

Adilson Roberto Locali Pereira

Resilient, sustainable, and nutritious:
pigeon pea protein fractions as high-potential food ingredients

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Tese apresentada como parte dos requisitos para obtenção do título de Doutor em Engenharia e Ciência de Alimentos, junto ao Programa de Pós-Graduação em Alimentos, Nutrição e Engenharia de Alimentos, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista “Júlio de Mesquita Filho”, Câmpus de São José do Rio Preto.

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Resumo

O feijão guandu (*Cajanus cajan*) é uma leguminosa nativa de países emergentes da África e da Ásia, apresentando alta resistência a ambientes secos. Seu alto teor de proteínas, que varia de 18 a 28%, a torna uma leguminosa potencial para o desenvolvimento de novos produtos vegetais a partir de fontes alternativas ainda pouco exploradas. Contudo, a produção de feijão guandu está limitada aos pequenos produtores. Uma forma de expandir a produção de feijão guandu em larga escala é compreender melhor a sua composição proteica e a sua funcionalidade nos sistemas alimentares. Neste trabalho frações proteicas do feijão guandu foram obtidas, caracterizadas e avaliadas quanto às suas propriedades funcionais. A globulina 7S foi a proteína presente em maior abundância nas frações proteicas do feijão guandu. Esta tese propôs um protocolo para a purificação da globulina 7S do feijão guandu em escala preparativa, visando a recuperação de rendimento suficiente para a caracterização das propriedades físico-químicas e funcionais das frações proteicas. Dados experimentais obtidos em laboratório para diferentes parâmetros físico-químicos mostraram boa concordância com valores calculados a partir de estudos preditivos utilizando a sequência e composição de aminoácidos do feijão guandu. Além do foco em proteínas, esta tese também apresenta uma extensa caracterização da fração lipídica e de carboidratos de ingredientes à base de feijão guandu, além da investigação de compostos minoritários, como os inibidores de tripsina. Para avaliar a funcionalidade, foram avaliadas as propriedades espumantes das frações proteicas. As frações proteicas do feijão guandu apresentaram propriedades espumantes semelhantes aos agentes espumantes de origem animal, como isolado proteico de soro de leite e albumina sérica bovina. Os resultados obtidos nesta tese mostram que as frações proteicas do feijão guandu apresentam alto potencial para aplicação como ingredientes alimentícios na indústria.

Palavras-chave: Feijão guandu. Globulinas. Produtos plant-based. Fracionamento de proteínas.

Abstract

Pigeon pea (*Cajanus cajan*) is a pulse native to emerging countries in Africa and Asia, presenting high resistance to dry environments. Its high protein content, which varies from 18 to 28%, makes it a potential pulse crop for the development of new plant-based products from alternative sources that are still little explored. However, pigeon pea production is limited to small producers. One way to expand pigeon pea production to a large scale is to better understand its proteins composition and, its functionality in food systems. In this work, pigeon pea protein fractions were obtained, characterized and evaluated for their functional properties. The 7S globulin was the protein present in greatest abundance in pigeon pea protein fractions. This thesis proposed a protocol for the purification of pigeon pea 7S globulin on a preparative scale, aiming the recovery of a sufficient yield for the characterization of physico-chemical and functional properties of the protein fractions. Experimental data obtained in the laboratory for different physicochemical parameters showed good agreement with values calculated from predictive studies using the sequence and amino acids composition of pigeon pea. In addition to the focus on proteins, this thesis also presents an extensive characterization of the lipid and carbohydrate fraction of pigeon pea-based ingredients, in addition to the investigation of minor compounds such as trypsin inhibitors. To evaluate functionality, the foaming properties of the protein fractions was evaluated. Pigeon pea protein fractions showed foaming properties similar to foaming agents of animal origin, such as whey protein isolate and bovine serum albumin. The results obtained in this thesis show that pigeon pea protein fractions have high-potential for application as food ingredients in the industry.

Keywords: Pigeon pea. Globulins. Plant-based products. Protein fractionation.

Résumé

Le pois d'Angole (*Cajanus cajan*) est une légumineuse originaire des pays émergents d'Afrique et d'Asie, présentant une grande résistance aux environnements secs. Sa teneur élevée en protéines, variant de 18 à 28 %, en fait une légumineuse prometteuse pour le développement de nouveaux produits d'origine végétale à partir de sources alternatives encore peu explorées. Cependant, la production de pois d'Angole est limitée à de petits producteurs. Une manière d'augmenter la production de pois d'Angole est de mieux comprendre sa composition protéique et, ses propriétés techno-fonctionnelles dans les matrices alimentaires. Dans ce travail, des fractions protéiques variées de pois d'Angole ont été obtenues, caractérisées et évaluées pour leurs propriétés fonctionnelles. La globuline 7S est la protéine la plus abondante dans les fractions protéiques du pois d'Angole. Cette thèse a développé un protocole pour la purification de la globuline 7S du pois d'Angole à une échelle préparative, visant à obtenir un rendement suffisant pour la caractérisation des propriétés physico-chimiques et fonctionnelles des fractions protéiques. Les données expérimentales obtenues en laboratoire pour différents paramètres physico-chimiques ont montré une bonne concordance avec les valeurs calculées à partir d'études prédictives utilisant la séquence et la composition en acides aminés du pois d'Angole. En plus de se concentrer sur les protéines, cette thèse présente également une caractérisation approfondie de la fraction lipidique et glucidique des ingrédients à base de pois d'Angole, en plus de l'étude de composés minoritaires tels que les inhibiteurs de trypsine. Enfin, les propriétés moussantes des fractions protéiques ont été évaluées. Les fractions protéiques du pois d'Angole ont montré des propriétés moussantes similaires à celles de protéines d'origine animale, tels que l'isolat de protéines de lactosérum et l'albumine de sérum bovin. Les résultats obtenus dans cette thèse montrent que les fractions protéiques du pois d'Angole ont un fort potentiel d'application en tant qu'ingrédients alimentaires dans l'industrie.

Mots clés: Pois d'Angole. Globulines. Produits à base de plantes. Fractionnement des protéines.

Table of Contents

Introduction	16
Chapter 1. Pigeon pea, an emerging source of plant-based proteins.....	22
1. Introduction	23
2. Pigeon pea: a brief historical perspective	24
3. Nutritional and antinutritional profile.....	29
4. Seed processing and functional properties of pigeon pea flour	30
4.1 Non-thermal processes.....	30
4.2 Thermal processes.....	33
5. Pigeon pea proteins	34
5.1 Protein composition derived from genome sequencing	34
5.2 Protein composition derived from differential solubility and chromatography	35
5.3 Amino acid profile and protein secondary structure	38
6. Methods for pigeon pea protein isolation	41
6.1 Extraction process and protein recovery	41
6.2 Possible applications of co-products from the protein extraction process	51
7. Functional properties.....	51
7.1 Protein solubility, surface hydrophobicity and zeta potential	52
7.2 Water and oil holding capacity	59
7.3 Foam properties.....	59
7.4 Emulsifying properties	60
7.5 Gel forming properties.....	61
8. Bioactive properties and digestibility	62
8.1 Bioactive peptides.....	62
8.2 Digestibility	62
8.3 Antioxidant activity	64
9. Application of pigeon pea in new food products	65
10. Perspectives for future work.....	66
Acknowledgments	67
References	68
Chapter 2. Objectives, experimental approach and methods of the study.....	76
Part I. Objectives and experimental approach	76

Part II. Source of raw material and production of the pigeon pea protein fractions.....	77
1. Source of raw materials	77
2. Pre-treatment of pigeon pea seeds and production of flour	77
3. Production of pigeon pea protein fractions	77
3.1 Precipitation-based extraction	77
3.2 Mild protein extraction	78
3.3 Purified fractions of pigeon pea 7S globulin	78
4. Sample coding	79
References	80

Chapter 3. Pre-treatment effects on the composition and functionalities of pigeon pea seed ingredients	81
1.Introduction	82
2. Materials and methods.....	84
2.1 Materials.....	84
2.2 Seed pre-treatments and flour production	84
2.3 Protein extraction	85
2.4 Composition of flours and protein concentrates	85
2.4.1 Cell wall polysaccharide composition, raffinose family oligosaccharides, and starch content	85
2.4.2 Total lipids, neutral lipids, phospholipids, and tocopherol content: extraction, identification, and quantification	86
2.4.3 Protein content: quantification and identification	87
2.4.4 Trypsin inhibitor activity (TIA)	87
2.4.5 Total soluble phenolic compounds (TSPC)	88
2.4.6 Moisture and ash contents	88
2.5. Characterization of endogenous fluorophores	88
2.6 Protein conformation and functional properties of pigeon pea protein concentrates ..	89
2.6.1 Intrinsic fluorescence spectroscopy.....	89
2.6.2 Protein solubility and electrostatic charge	89
2.6.3 Water and oil holding capacities (WHC and OHC)	89
2.6.4 Emulsifying properties	90
2.6.5 Foaming properties.....	90
2.6.6 Gelling properties	91
2.7 Statistical analysis	91
3. Results and discussion	91

3.1 Flour morphology and size distribution	91
3.2 Pigeon pea ingredients composition.....	93
3.2.1 Carbohydrate composition	93
3.2.2 Lipid composition.....	97
3.2.3 Protein composition.....	100
3.2.4 Minor compounds: trypsin inhibitor activity (TIA), total soluble phenolic compounds (TSPC), and ash content	101
3.3 Endogenous fluorophores in flours and protein concentrates.....	103
3.4 Protein concentrates obtained from soaked and soaked-boiled pigeon pea seeds: conformation and functionality	106
3.4.1 Protein conformation.....	106
3.4.2 Solubility and electrostatic charge	106
3.4.3 Functional properties.....	109
4. Conclusions.....	111
Acknowledgments	112
References	113
Supplementary material	121

Chapter 4. Preparative-scale purification and physicochemical characterization of pigeon pea 7S globulin	126
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Part I. Preparative chromatography for purification of 7S globulin from pigeon pea seeds	127
1. Introduction.....	127
2. Materials and methods.....	129
2.1 Materials.....	129
2.2 Protein composition of pigeon pea flour by SE-HPLC.....	129
2.3 Extractability of pigeon pea proteins	129
2.4 Laboratory-scale purification of pigeon pea 7S globulin	130
2.4.1 Removal of small molecules from the protein extract	130
2.4.2 Optimization of fractionation conditions by anion exchange and size exclusion chromatography	130
2.5 Preparative scale purification of pigeon pea 7S globulin	131
2.6 Analytical characterization of the purified fractions	132
2.6.1 Process yield and protein recovery	133
2.6.2 Composition.....	133

2.6.3 Proteomic analysis	133
2.6.4 Polyacrylamide gel electrophoresis in sodium dodecylsulfate (SDS-PAGE)	133
2.6.5 UV-visible spectroscopy	134
2.6.6 Dynamic light scattering.....	134
2.6.7 Intrinsic fluorescence measurements.....	134
2.7 Determination of foaming properties	134
2.8 Statistical analysis	135
3. Results and discussion	136
3.1 Protein composition of pigeon pea flour	136
3.2 Extractability of pigeon pea proteins	141
3.2 Laboratory-scale purification	143
3.2.1. Desalting vs UF.....	143
3.2.2 Separation by anion exchange and size exclusion chromatography.....	145
3.3 Preparative scale purification.....	146
3.4 Analytical characterization of the pigeon pea 7S globulin	149
3.4.1 Yield and purity.....	149
3.4.2 Protein composition.....	150
3.4.3 Minor components	154
3.5 Foaming properties.....	156
4. Conclusion	158
Acknowledgments	158
References	159
Supplementary material	163
 Part II. Physicochemical characterization of purified pigeon pea 7S globulin	 172
1.Introduction	172
2.Materials and Methods	174
2.1 Materials.....	174
2.2 In silico analyses.....	174
2.3 Pigeon pea protein extraction and purification.....	174
2.4 Amino acid profile.....	175
2.5 Determination of the specific extinction coefficient	175
2.6 Refractive index increment (dn/dc).....	175
2.7 Protein solubility	175
2.8 Electrophoretic mobility	176
2.9 Dynamic light scattering (DLS)	176

2.10 Statistical analysis	176
3. Results and discussion	177
3.1 Amino acid composition, N-to-P factor and chemical score	177
3.2 UV-visible spectra and refractometric parameters	179
3.3 Effect of pH on protein solubility and surface charge	182
3.4 Effect of pH on the size distribution of proteins.....	184
4. Conclusion	186
Acknowledgments	186
References	187

Chapter 5. Air-water interfacial and foaming properties of crude and purified globulin from pigeon pea seeds	189
1.Introduction	190
2.Materials and methods.....	192
2.1 Materials.....	192
2.2 Crude protein extraction.....	192
2.3 7S globulin purification	192
2.4 Composition.....	193
2.5 Fluorescence measurements	193
2.6 Protein dispersions	193
2.7 SDS-PAGE	193
2.8 Particle size distribution.....	194
2.9 Surface tension and surface dilatational properties.....	194
2.10 Foaming properties.....	194
2.11 Statistical analysis	195
3. Results and discussion	196
3.1 Characterization of crude protein extracts and purified fractions	196
3.1.1 Composition.....	196
3.1.2 Fluorescence measurements	196
3.1.3 Protein composition and size distribution.....	198
3.2 Air-water interfacial properties.....	200
3.2.1 Adsorption behavior	200
3.2.2 Surface dilatational rheology	201
3.2.3 Lissajous plots.....	204
3.3 Foaming properties.....	206
3.3.1 Foamability	206

3.3.2 Foam stability.....	207
3.3.2.1 Appearance of foams	207
3.3.2.3 Foam stability and liquid drainage	208
4. Conclusion	210
Acknowledgments	210
References	211
Supplementary material	215
 Chapter 6. Discussion, Conclusions and Perspectives.....	217
Part I. General discussion.....	218
1. Pigeon pea protein fractions obtained by different fractionation processes	218
2. Composition of the pigeon pea protein fractions	220
2.1 Possible glycosylation of pigeon pea proteins.....	220
2.2 Accumulation of lipids in protein fractions	221
2.3 Modifications in endogenous fluorophores	223
2.4 Reduction in trypsin inhibitors activity (TIA)	225
3. Impact of seeds pre-treatment on the composition and nutritional quality of pigeon pea-based ingredients	226
4. Functional properties of pigeon pea protein fractions.....	228
4.1 Solubility of different protein fractions	228
4.2 Effect of seed pre-treatments on functionality	228
4.3 Foaming properties.....	229
5. Protein composition of pigeon pea.....	230
6. Possible mechanisms of pigeon pea 7S globulin proteolysis.....	231
Part II. General conclusions and perspectives.....	233
References	235
 Appendix	237

Introduction

The world population is expected to reach around 9.8 billion people by 2050. Rethinking food production for a growing number of people while reducing negative impacts on the environment is a key issue to be considered by producers, researchers and government agencies in the coming decades (CLARK *et al.*, 2019). One of the strategies for more sustainable diets involves reducing excessive consumption of proteins of animal origin and increasing the intake of vegetable protein sources (ROSENFELD, 2018). The 2030 Agenda for Sustainable Development, launched by the Food and Agriculture Organization of the United Nations (FAO), includes encouraging projects that promote the sustainable and large-scale production of pulses for human consumption that contribute to reducing greenhouse gas emissions, increased soil fertility and better use of agricultural areas (FAO, 2016).

Private companies have shown accelerated investment in alternative protein sources to recent years, with amounts reaching around 5 billion dollars in 2021 (GFI, 2022). Over the past two decades, several new brands of plant-based meat or dairy products have been founded, the majority in the United States (34%) and the United Kingdom (8%) (GFI, 2023). Among the main ingredients used in the formulation of new plant-based products are soy, pea and wheat proteins, species widely studied to produce plant-based protein concentrations and isolates (AIMUTIS, 2022). However, it is estimated that the production of soybeans and peas, for example, will not be sufficient to meet the demand for plant-based proteins expected in the coming years (FOSCHIA *et al.*, 2017, RAHAMAN *et al.*, 2016), so it is necessary to investigate new protein sources. Among leguminous seeds, there are several species that are still underutilized – called *orphan crops* or *minor crops* – that have great potential for agronomic development, nutritional requirements and food security (SAMAL *et al.*, 2023). Despite being species well known in their native countries for their socioeconomic impact, these crops are considered underutilized because they are still neglected by policymakers, mainstream agricultural researchers, plant breeders, extension services, technology providers and consumers in general (POPOOLA *et al.*, 2020, VERBEECKE *et al.*, 2023). Furthermore, they comprise species whose grain supply networks are non-existent or not yet well developed (ALI & BHATTACHARJEE, 2023). Another important aspect is that these species are highly resistant to different environmental conditions, being able to withstand dry environments and extreme temperatures (SAMAL *et al.*, 2023).

Pigeon pea (*Cajanus cajan* L. Millsp.) is a highly drought-resistant pulse native to emerging countries in Asia and Africa (TAPAL *et al.*, 2019). World production of pigeon pea is around 5.5 million tons, corresponding to around 6% of world pulse production. The FAO's *The Global Economy of Pulses* includes pigeon peas among the most important pulses in terms of amount of consumption and global production (FAO, 2019). India is the largest consumer and producer of pigeon pea, accounting for around 90% of global

production of this pulse (TAPAL *et al.*, 2019). It's high nutritional value, with protein content ranging from 18-28% of its seed composition (SUN *et al.*, 2020), makes pigeon pea a potential source to produce protein-rich ingredients. In most part of the world, however, pigeon pea are still little used in human consumption. When it was introduced to America, it was used to feed pigeons on the island of Barbados, in the Caribbean Sea, where the name in English came from (VAN DE MAESEN, 1990). In Brazil, for many years the species was mainly used to feed livestock, so that only in the last two decades has it been the focus of studies to develop cultivars more suitable for human consumption (GODOY *et al.*, 2005, MIANO *et al.*, 2020). Its production, however, remains restricted to small producers. In fact, pigeon pea is one of the pulses whose production is almost entirely restricted to small-scale farmers. One of the ways to boost its large-scale production is to overcome possible difficulties in processing the seeds and a better understanding of their protein composition.

Some difficulties in processing pulses such as pigeon pea involve the hard-to-cook phenomenon and the presence of antinutritional factors. Antinutritional factors such as trypsin inhibitors, phytates, tannins, saponins, among others, can hinder the action of digestive enzymes on proteins, compromising their digestibility and absorption by the body (SAHNI *et al.*, 2020, SUN *et al.*, 2020). Pre-treatments such as soaking and boiling are among the primary technological methods to reduce the effects of antinutritional factors in pulses (CODA *et al.*, 2015, ZHOU *et al.*, 2023). Although such pre-treatments have already showed efficient in reducing anti-nutritional compounds in pigeon pea (DEVINDRA *et al.*, 2012, ONWUKA, 2006), it can affect protein structure and the functionality of ingredients based on pre-treated seeds, being a topic that still need further investigation.

The protein composition of pigeon pea is also a topic that needs further studies. Globulins account for around 60% of pigeon pea proteins, with 7S β -conglycinin being its main protein (SINGH & JAMBUNATHAN, 1982). Pigeon pea seeds also contain 11S glycinin, 2S albumin and other proteins in a smaller proportion (KRISHNAN *et al.*, 2017). Studies have investigated the production of pigeon pea protein concentrates and isolates, with the evaluation of their functional properties (ADENEKAN *et al.*, 2018, FERNÁNDEZ SOSA *et al.*, 2021, 2023, MWASARU *et al.*, 1999a, MWASARU *et al.*, 1999b). The alkaline solubilization method followed by isoelectric precipitation is the most used extraction method to obtain protein concentrates and isolates. Such methods enable the recovery of protein extracts rich in globulins, but which also contain other contaminants such as: other protein groups, sugars, phenolic compounds, phospholipids, among others (GRAVEL & DOYEN, 2023). To understand the role of pigeon pea 7S globulin in such complex mixtures, purification by chromatographic techniques can be employed. Krishna & Bhatia (1985) were the first to propose the isolation and purification on a laboratory scale of 7S β -conglycinin from pigeon pea. This study allowed to identify that pigeon pea 7S β -conglycinin is a glycoprotein and has a subunit around 70 kDa and two subunits around 57 kDa, with a molecular weight of 180 kDa in its native state. This pioneering

study presented fundamentals about the composition of the main protein in pigeon pea. However, to also evaluate the functionality of these proteins it is necessary to obtain them in sufficient quantities by migrating the laboratory-scale purification process to a preparative-scale process.

Therefore, the objective of this thesis was to extract, purify, characterize and evaluate the functional properties of different protein fractions from pigeon pea. Firstly, the impact of soaking and boiling pre-treatments on the functionality of pigeon pea protein concentrates obtained by alkaline solubilization followed by isoelectric precipitation was investigated. Then, pigeon pea 7S globulin was purified on a preparative scale using a mild extraction process and characterized for its physicochemical properties. The data of the investigated parameters were compared with theoretical values obtained by predictive studies using pigeon pea sequences and their amino acid composition. Finally, the air/water interfacial and foaming properties of purified 7S globulin and crude globulin extract were investigated. To investigate the use of pigeon pea proteins as possible alternative sources to proteins of animal origin, in studies of foaming properties, whey protein isolate and bovine serum albumin were used as controls.

The manuscript will be divided into five chapters:

Chapter 1 consists of a review of the state of the art regarding the use of pigeon pea seeds to produce food ingredients with a focus on seed pre-treatments and protein extraction methods.

Chapters 2, 3 and 4 present the experimental results. Chapter 2 focuses on the impact of seed pre-treatments on the composition and functionality of pigeon pea-based ingredients. Chapter 3 is focused on the purification and physicochemical characterization of pigeon pea 7S globulin. Chapter 4 presents the behavior at the air/water interface and foaming properties of pigeon pea protein fractions.

Finally, chapter 5 presents a general discussion of the results obtained in this thesis. Also, a general conclusion and perspectives for future work are presented.

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Chapter 1. Pigeon pea, an emerging source of plant-based proteins

Preamble

Making use of neglected pulse crops is a way to promote biodiversity and combat malnutrition in emerging countries, as well as a strategy to provide alternative and resilient sources of plant-based proteins. Pigeon pea (*Cajanus cajan*) is a pulse native to tropical and subtropical regions, such as Asia, India, and South America. Despite its great nutritional potential, pigeon pea is an underutilized crop, and its production is restricted to smallholder cultivators. Pigeon pea exploration for food application is a means to boost the cultivation and valorization of this crop, in addition to contributing to meet the worldwide demand for high quality plant-based proteins obtained from sustainable crops. This review synthesizes the main research findings involving pigeon pea exploration for food applications, from the processing of its seeds and flours to the extraction and modification of its proteins, highlighting its potential as a food ingredient.

Main questions:

- What are the gaps in relation to research on the use of pigeon pea in the production of food ingredients?
- What is known about the seed composition of pigeon pea?
- What pre-treatments have been used to improve the nutritional quality of pigeon pea seeds? What are its impacts on techno-functional properties?
- What methods were used to fractionate pigeon pea proteins?

Scientific articles published in the last decades in the Food Science and Technology field on the production of food ingredients obtained from pigeon pea seeds were evaluated. Key findings were summarized, and gaps were discussed.

Main results:

- Seed pre-treatments impact the functionality of pigeon pea fractions;
- The amino acid profile of pigeon pea is similar to soybean and chickpea;
- The 7S globulin is the main proteins found in pigeon pea;
- Pigeon pea proteins show high homology with soybean proteins;
- Rheological behavior and interfacial properties of proteins need further study.

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Pigeon pea, an emerging source of plant-based proteins

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1. Introduction

Pigeon pea is a pulse native to emerging countries in Asia, Africa, and South America (Sun et al., 2020). FAO's The Global Economy of Pulses (FAO, 2019) includes pigeon pea among the most important pulses in terms of global production and consumption quantities, along with common bean, chickpea, dry pea, lentil, cowpea, mung bean, and urd bean. The world production of pigeon pea is about 5.5 million tons, contributing to 5.8 % of total pulse production across the world. India is both the largest consumer and producer of pigeon pea, accounting for 90 % of global production (Sarkar et al., 2020; Tapal et al., 2019). Myanmar, Malaw, and Nepal are some of the major exporters of pigeon pea, with a limited domestic demand. Pigeon pea is also cultivated in small quantities in some countries in Latin America and the Caribbean (FAO, 2019).

Pigeon pea is considered one of the most drought-tolerant pulses, due to a deep root system, and a germplasm with a higher osmotic adjustment in relation to other pulses (FAO, 2019; Kumar et al., 2023). This allows pigeon pea plants to perform better, even with low water potential, by moderating photosynthetic functions, stomatal conductance, and delaying leaf senescence (Basu, 2016; Kumar et al., 2023). Nevertheless, despite these convenient agronomic characteristics, its production is almost entirely restricted to small growers. In addition, its average yields vary significantly depending on agronomic practices, density of planting and seed variety (Adenekan et al., 2018; Fuller et al., 2019), since it is primarily grown as an intercrop.

A key factor in boosting large-scale production of pigeon pea seeds is the development of new cultivars. While most pulses are naturally only self-pollinators,

pigeon pea shows a certain degree of cross-pollination, carried out by honey bees. This opens route to develop hybrids with improved agronomic features (FAO, 2019). One of the key breeding targets is the development of short-duration cultivars that can mature in about 130-145 days, instead of 250-280 days of long-duration cultivars. This would reduce the time of field occupancy and minimize the risks of frost damage (Singh et al., 2018). The development of new drought-tolerant cultivars of pigeon pea has been carried out by the International Center for Agricultural Research in the Dry Areas (ICARDA), in Lebanon, and the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), in India^{4,9}. Cultivars more suitable for human consumption, with shorter cooking times and better technological properties, have also been the focus of studies by the Brazilian Company of Farming Research (EMBRAPA)(Abranches et al., 2021; Miano et al., 2020).

Beyond agronomic challenges, a better understanding and expansion of the possible uses of pigeon pea for food applications is essential to reduce the gap between small-scale farmers and large-scale production (FAO, 2019). Investigations into the potential of pigeon pea-derived ingredients have gained prominence in recent literature, driven by the growing demand for alternative sources of plant-based proteins. The studies cover aspects from seed pre-treatments to improving nutritional quality and evaluating functional properties of flours (Acevedo et al., 2017; Adenekan et al., 2018; Chinma et al., 2022; Sharma et al., 2019; Sun et al., 2020) to fractionation processes to produce protein concentrates and isolates (Bravo et al., 2022; Sosa et al., 2021, 2023; Olagunju et al., 2018a, 2018b, 2021; Xu et al., 2021).

However, to the best of our knowledge, no comprehensive review has covered the main findings regarding the production and properties of pigeon pea-based protein-rich ingredients in the past decade. Highlighting such advances is important to enhance the use of this pulse, in addition to understanding which aspects still need more attention in the coming years. This review summarizes the main research outcomes related to pigeon pea, focusing on seed processing and protein extraction, as well as on the functional properties of interest to the food industry. This review also intends to disclose the potential of this nutritionally valuable pulse as an approach to encourage its further exploration as a food ingredient in future years.

2. Pigeon pea: a brief historical perspective

Pigeon pea (*Cajanus cajan* (L.) Millsp.), a pulse belonging to the *Fabaceae* family, grows in semi-arid, tropical, and subtropical regions of the world. The English name for this pulse derived from Barbados island, in the Caribbean Sea, where the seeds were used to feed pigeons (Van de Maesen, 1990). Depending on the region, pigeon pea can also be referred to as *Congo pea* and *Red gram*. The origin of this pulse is somewhat contradictory. While many believe the species is native to Africa, there is evidence supported by modern genetic data that its origin is from East India (Fuller et al., 2019). It

is believed that the culture was introduced in South American countries in the 17th century, brought by the slave route from Africa (Sarkar et al., 2020).

Cajanus cajan is a perennial tree (Figure 1) that can measure up to two meters in height with a growth time of 6 to 9 months. The seeds are about 2-mm thick, and their color can vary from black to cream (Adenekan et al., 2018; Fuller et al., 2019; Tiwari et al., 2008). Its cotyledons are made up of several plant cells where the starch granules are dispersed in a protein matrix (Figure 2). Pigeon pea is a crop that adapts well to hot climates and low humidity, contributing to soil fertility and to sustainable agriculture in regions that are already facing the first consequences of global climate change (Acevedo et al., 2013, 2017; Mizubuti et al., 2000; Sarkar et al., 2020).

The world production of pigeon pea reached about 5.5 million tons in 2021 (FAO, 2022) (Fig 3a). Comparatively, the world production of dried beans is on average 24 million tons, chickpea production is about 13 million tons, dry pea production is about 11 million tons, cowpea production is about 7 million tons, lentil production is about 5 million tons and faba bean production is about 4 million tons (FAO, 2019).

India is the country that mostly consumes and cultivates pigeon pea, accounting for about 90% of its world production (Fig 3b). Since 2010, however, pigeon pea production has been gradually increasing in Africa, the Caribbean and South and Central America. In India, this pulse is used mainly in the production of typical soups made from legume seeds, called *dhal* (Acevedo et al., 2017; Tapal et al., 2019). In the Caribbean and Southeast Asia, grains are harvested while still unripe and consumed as fresh vegetables (Fuller et al., 2019). In South American countries, such as Brazil and Argentina, pigeon pea for pulse consumption represents only small to medium-sized productions to serve as subsistence agriculture (Acevedo et al., 2013; Mizubuti et al., 2000). An alternative reason that drives pigeon pea cultivation in Brazil is its use as an intercrop for helping nitrogen fixation in the soil, in addition to the use of leaves and branches as green manure or as fodder for animal feed (Magrini et al., 2019).

Despite the easy adaptation to different soils, pigeon pea production has remained stagnant in the last five decades (Fuller et al., 2019). Bringing knowledge about the nutritive potential of pigeon pea to the population in general is another measure that can be taken to make the best use of this pulse (Onimawo & Akpojobwo, 2006; Oshodi et al., 1993). The number of scientific articles related to pigeon pea grew about 12.5 times in the period between 2015 and 2020, according to data from the Web of Science platform (Fig 3c). In the same period, all articles published in the Food Science and Technology category grew by 50% and publications referring to peas and soybeans increased by 65%. The number of publications involving pigeon pea, however, is still small, but shows potential for further expansion in future years.

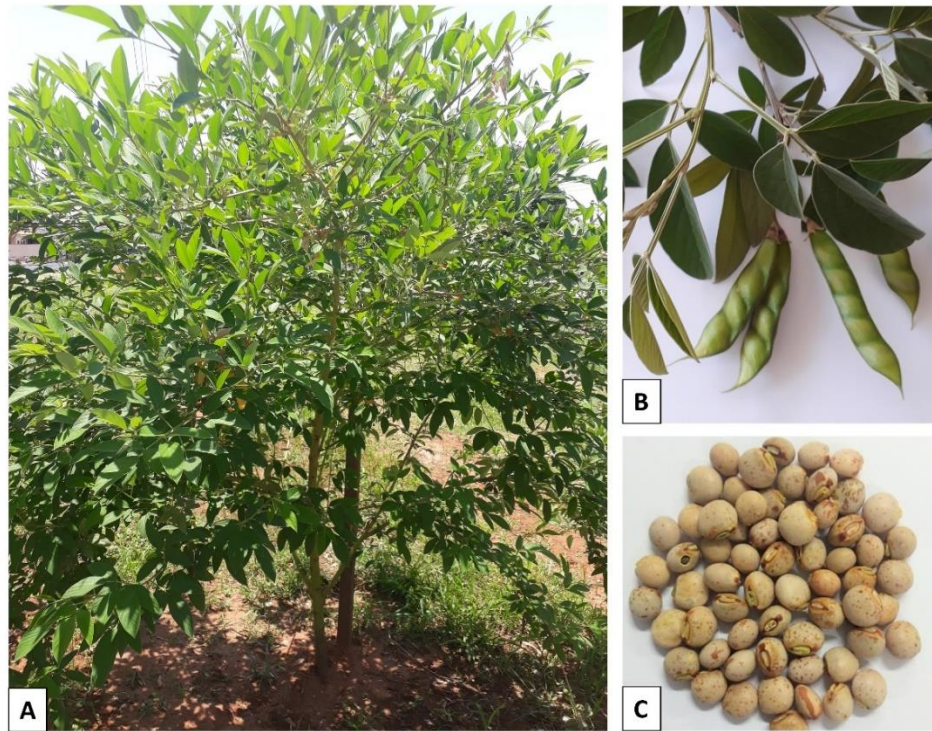


Figure 1.1. Pigeon pea (*Cajanus cajan* (L.) Millsp.) tree (a), green pods (b) and dry seeds (c).

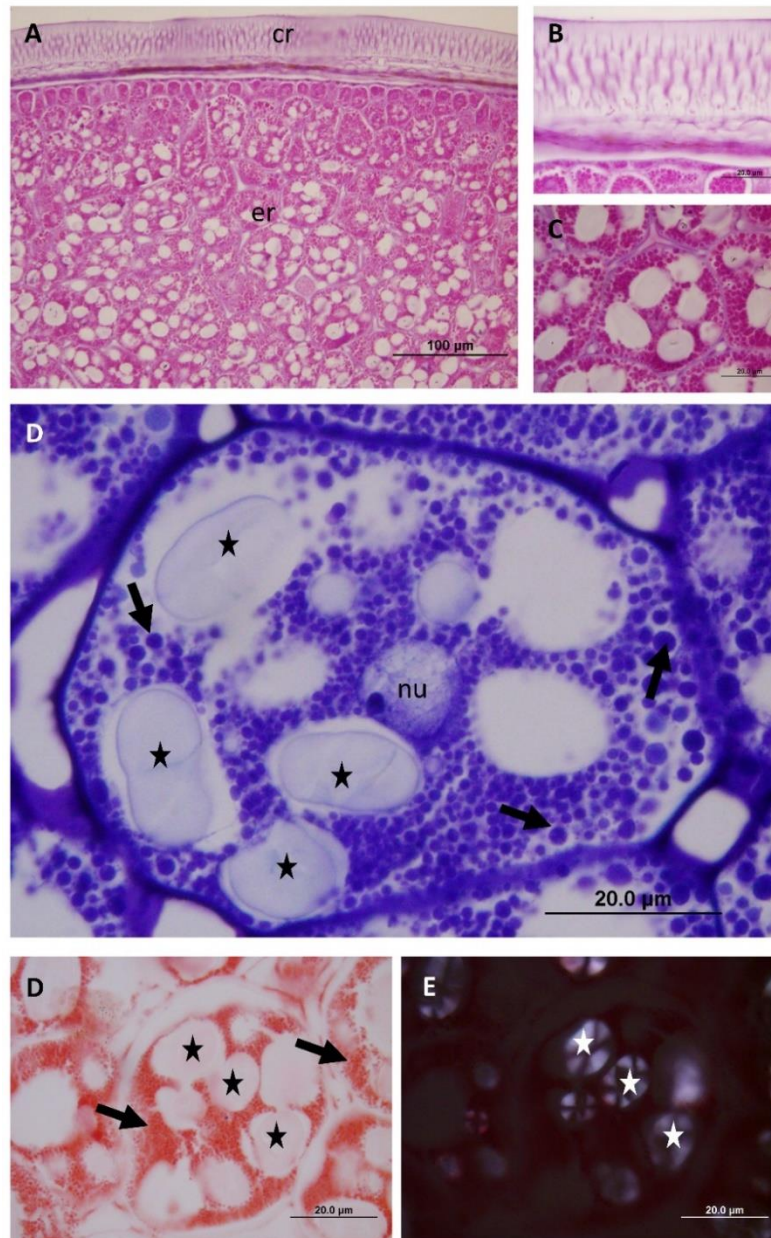


Figure 1.2: Histological sections of pigeon pea (*Cajanus cajan*) seed, obtained from formalin-fixed seeds embedded in paraffin and sectioned at 5-µm sections using rotative microtome. (A) General view of seed stained by haematoxylin-eosin. Coat region (cr) and endosperm region (er). (B) Detail in seed coat region that in the mature seed is dry and usually consists of dead cells. (C) Detail in the endosperm region occupying most of the seed volume. (D) Endosperm cell stained by toluidine blue at pH 4.0 for identification of anionic groups of the granular proteins in the cytoplasm (arrows); The nucleus is indicated by (nu) and starch granules are chromophobes and are indicated by stars. (E) Endosperm cell stained by Xylidine Ponceau at pH 2.5 for identification of cationic groups of total proteins. The protein granules are identified in red (arrows) and the starch is chromophobes too by this stain (stars). (F) The same region of the figure E visualized under polarized light for identification of starch granules with characteristic birefringence (stars).

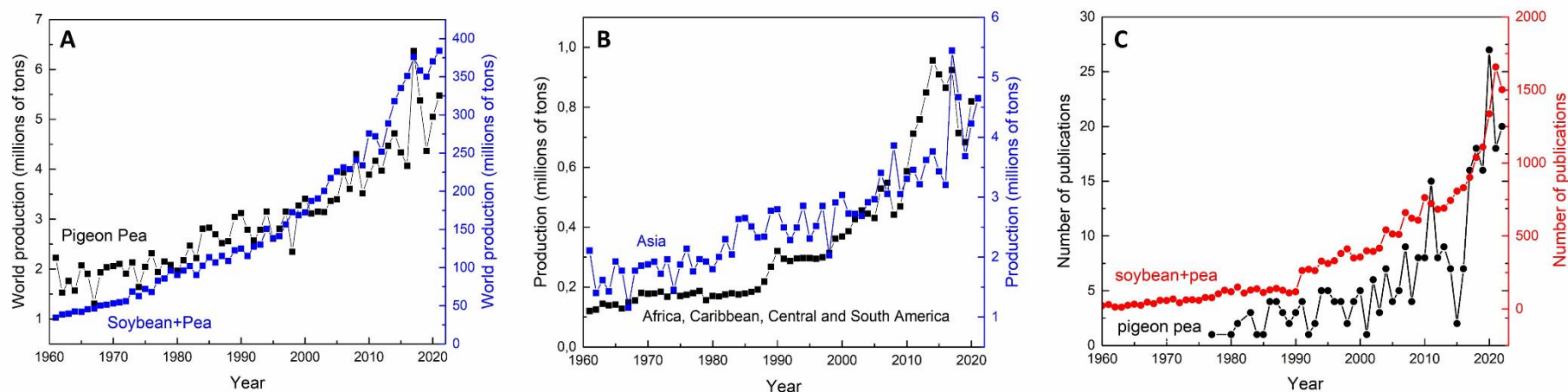


Figure 1.3. Dashboard with production data, scientific publications and nutritional aspects of pigeon pea (*Cajanus cajan*). **(a)** World production and **(b)** production in Asia, Africa, Caribbean, Central and South America of pigeon pea seeds compared to soybean and pea (1960-2022). Source: FAOSTAT. **(c)** Evolution of scientific publications on pigeon pea in the 'Food Science and Technology' field over the years compared to soybean and pea. Source: Web of Science data base.

3. Nutritional and antinutritional profile

Proteins represent 18 to 28 wt.% (dry basis) of the composition of pigeon pea seeds (Sun et al., 2020). A high protein content was also reported in the leaves (19.4 wt.%, dry basis) (Yang et al., 2020). The amino acid profile of seed proteins is similar to that of soybean, with about 43 wt.% of the total amino acids being essential amino acids. The presence of valine, leucine, isoleucine, glutamic acid, phenylalanine, and lysine stands out. The contents of these last three amino acids were found to be higher in pigeon pea than in chickpeas; however, it is low in methionine and cysteine, two sulfur-rich amino acids (Adenekan et al., 2018; Devindra et al., 2012; Fernández Sosa et al., 2021; Oshodi et al., 1993).

Carbohydrates correspond to the largest fraction of pigeon pea seeds, constituting about 60 wt.% (dry basis) of its total composition. Miano et al. (2020) found that the soluble fiber content ranged from 4.2 to 7.9 wt.%, whereas the insoluble fiber content was between 19.0 and 26.4 wt.%. The largest portion of the total carbohydrates in pigeon pea is starch: about 50%, 23.2% referring to the amylose fraction (Acevedo et al., 2013).

Lipids account for a small fraction of the pigeon pea composition (approximately 1.5-5.0 wt.%, dry basis), as in other similar pulses. The fatty acid profile is similar to that of soybean, with the predominant fatty acids being linoleic acid and palmitic acid, which represent about 54.8 and 21.4 wt.% of the total lipid composition, respectively (Oshodi et al., 1993).

The ash content in pigeon pea is about 3.5 wt.% (dry basis) of the seed composition. Oshodi, Olaofe, & Hall (1993) quantified the composition of minerals in pigeon pea seeds and obtained significant values of potassium (1308 mg/100g), magnesium (110 mg/100 g) and calcium (81.4 mg/100 g). On the other hand, the authors observed a low sodium content (9.9 mg/100 g). In a study by Yang et al. (2020), calcium (581 mg/100 g), magnesium (138.8 mg/100 g), iron (51.5 mg/100 g), sodium (32.5 mg/100 g), manganese (6.8 mg/100 g), copper (1.4 mg/100 g), and zinc (0.7 mg/100 g) were identified. Pigeon peas are richer in calcium than common beans and chickpeas (Awasthi et al., 2022; Vásquez et al., 2021).

In addition to these nutrients, pigeon pea seeds also present phenolic compounds (23.2 mg gallic acid equivalent/g), flavonoids (15.1 mg quercetin equivalent/g), tannins (0.4 g galic acid/100 g), tocopherols (1.1 – 9.3 mg/100 g), and vitamin B1 (0.4 mg/ 100 g) and B2 (0.3 mg/100 g) (Ferreira et al., 2004; Sharma et al., 2019; Torres et al., 2007). Some polyphenols and flavonoids have been identified in *Cajanus cajan* leaves (Prabhakar et al., 2023), but studies focusing on the identification of these compounds in the seeds are scarce in the literature. Nix et al. (2015) observed the presence of phytoalexins, cajanol, cajanin and two isoprenylated flavones in pigeon pea soaked-seeds.

Although rich in several macro- and micronutrients, the species also has antinutritional factors, as in most pulses. Antinutritional factors are compounds that

interfere with nutrient absorption by the human body, and may, for instance, inhibit the action of digestive enzymes and impair the bioavailability of proteins. Some polyphenols, for example, can form insoluble complexes with proteins, making their absorption difficult (Adenekan et al., 2018). Other antinutritional factors found in pigeon pea include saponins, protease inhibitors, phytic acid, phytolectins and oligosaccharides (Sun et al., 2020). Ene-Obong (1995) determined levels of trypsin inhibitors of up to 14.4 mg/g of flour, whereas tannins had values of 0.97 mg of catechin equivalent/g of flour, and phytates and phytic acid had values equal to 8.48 mg/g of flour. Some pre-treatments in seeds can help reducing their levels of antinutritional compounds. Soaking for 12 h followed by cooking for 60 min was shown to eliminate tannins and reduce the trypsin inhibitor compounds in pigeon pea by 77% (Onwuka, 2006). Germination, e.g., was shown to be efficient in reducing some antinutritional factors in pigeon pea by action of activated proteolytic enzymes (Sharma et al., 2019). The protein extraction process may also help reducing the concentration of antinutritional factors in the produced fractions. Adenekan et al. (2018) reported that the protein extraction process from pigeon pea, using different solvents (water, methanol, acetone, and ammonium sulfate), resulted in a drastic reduction in tannin and phytate levels. Trypsin inhibitor and cyanogenic glycoside were not detected in the protein isolate. All isolation methods used showed to be effective in significantly reducing the concentrations of the antinutritional factors evaluated.

Despite its high nutritional value, there is still a need to better characterize the composition of pigeon pea, especially regarding the composition of carbohydrates, lipids, and proteins. Currently, studies only report proximal compositions, but information about their composition profile is still scarce and needs to be better characterized with the aid of analytical chromatography techniques, for example. The same occurs for its minor compounds, which, in addition to identification, also need clarification regarding their impacts on the functionality and digestibility of the seeds.

4. Seed processing and functional properties of pigeon pea flour

Pulse seeds are often subjected to pre-treatment before processing, either in the food industry or in-home cooking. The main objective is to enhance their nutritional properties by reducing the antinutritional compounds and improving sensory aspects. Seed treatment can involve non-thermal (soaking, germination, ultrasound, fermentation, etc.) or thermal (cooking, roasting, microwave, etc.) processes (Acevedo et al., 2013; Lee et al., 2015; Onimawo & Akpojovwo, 2006).

4.1 Non-thermal processes

A simple non-thermal process that is employed is the soaking of the seeds. Hydration makes the seeds softer, in addition to promoting the activation of cell wall enzymes responsible for increasing the solubility of some compounds in the husk and reducing cooking time (Silva et al., 1981).

Kate et al. (2020) studied the dynamics of mass transfer in pigeon pea seeds during soaking. The authors noted that the beginning of the process is characterized by filling the empty cotyledon cavities with water and possible leaching of solids. In a second stage of the process, there is a loss of solids (about 5% of the total weight). Such solids are mainly made up of seed husks and loosely delimited solids on the surface of the cotyledons.

Miano et al. (2020) evaluated different pigeon pea cultivars during hydration kinetics in a water bath (25 °C / 660 min). The seed hydration percentage ranged from 57.2 to 99.7 wt.% (amount of hydrated seeds / total amount of seeds). The authors observed that some seeds did not hydrate after the process, which was attributed to heterogeneity between grains at harvest (presence of mature and unripe seeds) and variations of cultivars in relation to the permeability of the husks.

In a study conducted by Vásquez et al. (2021), the influence of ultrasound application and the use of NaHCO₃ solutions on the hydration kinetics of pigeon pea was evaluated. Whereas the application of ultrasound accelerated hydration kinetics, the addition of NaHCO₃ inhibited grain hydration by approximately 23 %. The decrease in the hydration rate due to salt addition (2 wt.%) was attributed to the high pH (8.4), since hydration in alkaline medium can cause changes in the composition of the shell and the cotyledon of seeds, modifying cell diffusion mechanisms.

Dehulling is a non-thermal process commonly used after soaking. Pigeon pea is usually dehulled to improve digestibility and reduce antinutritional compounds that may be present in the husks. Some compounds are responsible for binding the bark to the cotyledon, such as the disaccharide galactomannan and the glycol protein bonds, which makes the dehulling process more difficult at the industrial level. It is known that pigeon pea is difficult to peel due to the presence of gums and mucilage between the husk and the cotyledon. Such compounds form a network of cellulosic microfibrils embedded in a non-starch carbohydrate matrix, and a mixture of enzymes (xylanase, cellulase and pectinase) proved to be efficient in dissolving the gums and mucilage present between the husk and cotyledon of pigeon pea, facilitating dehulling. The enzyme mix increased the dehulling efficiency, reduced the amount of energy needed to cook the seeds and increased the protein content (Dabhi et al., 2019). Enzymatic pre-treatment has also been used for the same purpose in other species, such as basmati rice and horse grain (Arora et al., 2007; Sreerama et al., 2008).

In addition to this, the germination of seeds such as soybeans, chickpeas, lentils, and beans has been investigated and revealed to cause phytochemical changes in these seeds that impact their technological characteristics. Increase in ascorbic acid, phenolic compounds, antioxidant activity and tocopherols are some of the changes observed in germinated seeds. Some enzymes such as α - and β -amylase and proteases can be activated during the germination process, causing the breakdown of carbohydrates and proteins into simpler forms. This leads to better digestibility of these nutrients, in addition

to affecting its functionality, due to changes in their structures (Acevedo et al., 2017; Sharma et al., 2019).

Oloyo (2004) studied the effects of germination of pigeon pea. Seeds were left to germinate for 5 days, and the nutritional and functional properties of the generated flours were evaluated. Regarding the proximate composition, the authors noticed a decrease in protein (21.9 to 15.3 wt.%) and carbohydrate (62.6 to 59.5 wt.%) contents during germination. On the other hand, the contents of lipids, fibers and ash increased throughout germination. The authors attributed the decrease in protein and carbohydrate contents to the consumption of these macronutrients during the seed germination process. Antinutritional compounds such as oxalate and phytic acid decreased, whereas the levels of tannins, phenolic compounds and trypsin inhibitor compounds increased.

Sharma et al. (2019) observed that longer seed germination times and higher temperatures increased the levels of phenolic compounds in pigeon pea. The flavonoid contents in pigeon pea increased by more than 70 % in seeds germinated at 35 °C for 48 h, compared to non-germinated seeds. The increase in the levels of phenolic compounds was also reflected in the antioxidant activity of the flours, which was higher than that of the non-germinated seeds.

In the study of Chinma et al. (2022), germination of pigeon pea seeds for 72h also reduced the levels of phytic acid, tannins, and trypsin inhibitors by 62, 64 and 61 %, respectively, compared to untreated seeds. On the other hand, there was a 76 % increase in the level of total phenolic compounds, which may increase the antioxidant activity. In addition to increasing protein content by 18 %, seed germination resulted in a decrease in lipid content by 29 %, which was attributed to the consumption of fatty acids during germination for energy production. However, these results oppose those of Oloyo et al. (2004). The duration of the germination process seems to be a critical factor modulating the levels of such macronutrients.

Acevedo et al. (2017) investigated the effect of germination for 5 days on the seeds' microstructure and on the functional properties of pigeon pea flour. The authors observed that seed germination modified the cotyledon protein matrix, but preserved the starch granule shape, whereas treatments such as soaking and boiling affected both seed microstructures. Regarding the functional properties, germination allowed a subtle increase in the water holding capacity of the flour.

Germination is one of the non-thermal processes that alters the composition of pigeon pea macronutrients the most, especially when carried out over long periods. Among the main advantages observed, the reduction of antinutritional compounds helps to improve seed digestibility. However, protein levels can decrease and the impact of this process on the functionality of ingredients produced with germinated seeds still needs to be further clarified.

Finally, pigeon pea fermentation was investigated by Lee et al. (2015). The authors used *Bacillus subtilis* strains in seed fermentation, producing the fibrinolytic

enzyme nattokinase. The results suggest that pigeon pea fermented with *Bacillus subtilis* can bring benefits to cardiovascular health, helping to prevent hypertension, due to increased antioxidant activity and action of the nattokinase enzyme compared to non-fermented seeds. Fermentation also increased the contents in flavonoids and total phenolic compounds.

4.2 Thermal processes

Processes that involve heat can cause changes in the conformation of proteins and gelatinization of starch granules in the pulses, affecting their functional properties. Starch and proteins are the macronutrients that contribute to change the functional and textural properties of pulse flours after heating. In aqueous suspensions and at high temperatures, starch granules swell and open due to breakage of the amylopectin double helix, while amylose leaches through the swollen granules, leading to gelatinization (Acevedo et al., 2013). The gelation properties of legume proteins can be attributed to the globulin fraction present in these seeds (Onimawo & Akpojovwo, 2006). The thermal properties of pigeon pea have been investigated using differential scanning calorimetry (DSC) to determine the starch gelatinization temperature and protein denaturation temperature, in addition to rheological analyses to determine the pasting temperature of flours.

The gelatinization temperature of pigeon pea starch is reported to be between 82.0 and 83.6 °C, whereas the protein denaturation temperature is around 96 °C (Acevedo et al., 2013; Maninder et al., 2007). Sosa et al. (2021) determined a denaturation temperature of 90.4 °C for the globulin fraction, but failed to detect the denaturation temperature for the albumin fraction using DSC analysis, due to the high flexibility of the tertiary conformation of the corresponding polypeptides. The denaturation temperature of pigeon pea protein isolates also showed to be dependent on pH, ranging from around 92 °C for pH 2.1 to 103 °C for pH 8.3 (Sosa et al., 2023).

Another important thermal property for processing pulses is the pasting temperature, defined as the minimum temperature necessary to cook the flours (Acevedo et al., 2017). When this temperature is reached, changes in the structure of starch granules begin, leading to their gelatinization and increased viscosity (Sharma et al., 2019). The pasting temperature for pigeon pea is reported between 81.6 °C and 87.5 °C (10 to 20 wt.% solids), and the use of different pre-treatments may affect these values (Acevedo et al., 2013; Maninder et al., 2007; Sharma et al., 2019; Tiwari et al., 2008).

Cooking is a recurrent process applied to pulses in the industry, as well as, of course, in home cooking. Miano et al. (2020) observed that cooking pigeon pea (previously hydrated) for 20 min at 98 °C was sufficient to make the grains soft (minimum penetration force in a firmness test). A similar value was determined by Tiwari et al. (2008). The heat treatment resulting from cooking showed to affect the functional properties of pigeon pea flour. Soaking (6 h) followed by prolonged cooking of the seeds (60 min) reduced the pasting temperature from 81.6 °C to 74.1 °C, in addition to

increasing the water holding capacity and delaying the creaming process of emulsions stabilized by flour (Acevedo et al., 2017). Steam cooking of pigeon pea was evaluated by Tiwari et al. (2008) and proved to be an efficient pre-treatment for seed dehulling. Onimawo & Akpojobwo (2006) submitted pigeon pea seeds to a roasting process (80-100 °C/1 h) and evaluated the functional properties and antinutritional compounds of the resulting flours. The flours produced after roasting showed moisture content of 5.2- 4.5%. Authors determined higher values of water holding capacity and oil holding capacity for seeds that underwent roasting and they attribute this to the heat dissociation of proteins, swelling of crude fiber and gelatinization of starch. However, a decrease in the emulsifying and foaming properties was observed. This result may be related to the denaturation of pigeon pea proteins at high temperatures. Regarding the antinutritional compounds, the roasting process at 80 °C reduced the cyanide contents in the flours by approximately 28%, whereas a further reduction (approximately 66%) was attained by roasting at 100 °C. The phytic acid content was also reduced by roasting by approximately 20%.

Seed treatment by microwaves led to gelatinization of starch granules, due to high temperatures and the presence of water (Sun et al., 2020). Acevedo et al. (2017) applied microwaves to pigeon pea seeds submerged in water (seed-to-distilled water ratio of 1:10 g/mL) at different power levels (50, 70 and 100%). The authors observed that microwave treatment decreased protein solubility over a broad range of pH values, compared to untreated flour. This decrease in solubility was attributed by the authors to increased surface hydrophobicity due to changes in the secondary structure of proteins caused by heating, in order to expose the hydrophobic amino acids and result in the formation of disulfide bridges. It was also observed that the treatment helped to delay the creaming process of emulsions stabilized by pigeon pea flour.

5. Pigeon pea proteins

The protein composition of pigeon pea has been evaluated to some extent by different research groups, addressing aspects such as protein quantification, identification, amino acid profile and protein secondary structures. The outcomes are summarized in this section, to identify similarities with other pulses, specificities, and aspects that still need to be clarified regarding pigeon pea proteins.

5.1 Protein composition derived from genome sequencing

Pigeon pea genome sequencing (Varshney et al., 2012) provides a first insight into seed protein composition. The protein sequences resulting from the sequencing are available in the public deposit UniProt Knowledgebase (UniProtKB). A total of 28 sequences of full-length proteins are retrieved using the search query *C. cajan* and protein type (albumin, globulin, glutelin, prolamin) or *C. cajan* storage (October 2021). This includes 6 albumins, 8 7S-globulins, 3 11S-globulins, 7 glutelins and 4 oleosins sequences as reported in Table 1. No prolamin sequence was found. The UniProtKB Align software was used to compare the similarity of protein primary sequence within each

protein group (unpublished data). The albumin, 7S-globulin and oleosin display very low sequence homology, below 6%. The 11S-globulin and glutelin display higher homology (34-40%). This suggests that a single genotype presents a large diversity of proteins. Each protein type is encoded by a family of genes implying a polymorphism, similarly to most seed storage proteins (Krishnan, 2001).

From the protein sequences, several physicochemical properties can be estimated using the protParam feature of ExPASy webserver. The molecular weight of the albumin fraction ranges from 9.7 to 15 kg mol⁻¹, with isoelectric point (pI) comprised between 4.6 and 7.6 (Table 1). The molecular weight of the 7S-globulin fraction ranges from 21 to 45 kg.mol⁻¹. Through SDS-PAGE and peptide mass fingerprinting analyses, Sousa et al. (2020) identified the following groups of proteins in this range of molecular weight: vicilin, phaseolin α -type, phaseolin β -type. This suggests a homology between pigeon pea 7S-globulins and pea/kidney bean 7S globulins. However, the average theoretical isoelectric point (pI) of pigeon pea 7S-globulins is around 7.5, which is much larger than that of pea/kidney bean 7S globulins (pI 4-6) (Helmick et al., 2021). This may lead to specific physical chemical and functional properties of pigeon pea 7S-globulins. The 11S-globulin fraction identified in pigeon pea genome is expected to have molecular weight around 50-53 kg mol⁻¹ and pI around 6. This is similar to pea legumin A, identified by peptidomic analysis (Helmick et al., 2021; Sousa et al., 2020). Concerning the glutelin sequences, the molecular weight is about 38-39 kg mol⁻¹ and the pI around 5.2 – 5.8. In UniprotKB, they are annotated to belong to the 11S globulin family, similarly to rice glutelin (Wen, 1985). To our knowledge, no detailed characterization of pigeon pea glutelins has been undertaken to confirm their presence in pigeon pea seeds/flour and their structural similarity to 11S globulins and rice glutelin. A last group of proteins has been identified through the genome sequencing: oleosins. They are expected to be around 10-16 kg mol⁻¹ and to have basic pI (9.6 - 11.7). Oleosins are localized on the surface of oil bodies (oleosomes) and are abundant in oil seeds (Tzen et al., 1990). Since the lipid content of pigeon pea is low, the amount of oleosin is expected to be small.

5.2 Protein composition derived from differential solubility and chromatography

The protein composition of seed coat, embryo, cotyledons, whole seed and flour of pigeon pea was determined by successive solubilization following an Osborne-like procedure (Singh et al., 1990; Singh & Jambunathan, 1982). Four groups of proteins were identified: albumins, soluble in water, globulins, soluble in 0.5 M sodium chloride solution in 0.01 M phosphate buffer (pH 7.0), glutelins, soluble in 0.1 M sodium hydroxide, and prolamin, soluble in 70% ethanol/water mixture. Globulins were the main proteins found in pigeon pea flour (60-66%), followed by glutelins (19-25%), albumins (7-9%), and prolamins (2-5%) (Singh et al., 1990). The protein composition was found to depend on the pigeon pea variety and on the investigated seed compartment. Cotyledon and embryo were found richer in globulins than seed coat (Singh et al., 1990). Differential solubility gives an overall picture of flour/seed protein composition. However, it is

unreliable for a quantitative estimation of each protein group since substantial level of albumins may be found in globulin fraction. SDS-PAGE has been used to describe it qualitatively. Most prominent bands of pigeon pea proteins were located at 47 and 64 kDa, corresponding to two 7S globulin subunits (Boachie et al., 2019). Smaller bands were observed at 21, 35 and 36 kDa, in the region of the 11S globulins.

Liquid chromatography techniques such as size exclusion and ion-exchange chromatography have been used to purify and better characterize pigeon pea proteins at lab scale. Krishna, Mitra and Bhatia (1977) purified globulins by solubilizing the proteins in phosphate buffer solution (pH 7.2) followed by protein precipitation (pH 4.7) in a $(\text{NH}_4)_2\text{SO}_4$ saturated solution. Fractionation of purified globulins by size exclusion chromatography resulted in fractions α , β and γ , the first two corresponding to 11S-legumin, present in small quantities in pigeon pea, and 7S-vicilin, the main pigeon pea protein, respectively.

Vicilin from pigeon pea seeds was also purified from the precipitated globulins fraction by a zonal isoelectric precipitation procedure in a Sephadex G-50 column followed by further purification in a DEAE-Sephacel column (Krishna & Bhatia, 1985). While globulin proteins had subunits ranging from 72 to 20 kDa, purified vicilin had two subunits at 72 and 57 kDa. According to the authors, pigeon pea vicilin differs from those of *Vicia* and *Pisum* species by the absence of low molecular weight subunits. However, a similar pattern is found in vicilins of *Phaseolus vulgaris* and *Glycine max*, which suggests that *Cajanus cajan* is closer to common bean and soybean on this matter.

Table 1.1. Pigeon pea proteins retrieved from UniprotKB (October 2021) database and the corresponding number of amino acids, molecular weight and isoelectric point (pI) computed using the ProtParam feature of ExPASy webserver.

Protein type	Uniprot code	Protein name	Number of amino acids	Molecular weight (g mol ⁻¹)	pI
Albumin	A0A151QN35	Albumin-1	101	11003	5.6
	A0A151QQ22	2S albumin	124	14459	5.0
	A0A151QQZ7	Albumin-1	102	11097	7.6
	A0A151QR00	Albumin-1	102	11085	6.8
	A0A151R635	Albumin-1	92	9720	4.5
	A0A151R6F6	Albumin-1	101	10980	6.7
Globulin	A0A151SLE6	Basic 7S globulin	259	27260	4.7
	A0A151STZ0	Basic 7S globulin 2	397	42564	9.0
	A0A151TW66	Basic 7S globulin	192	21288	6.7
	A0A151U0J2	Basic 7S globulin	379	40412	5.9
	A0A151U0M6	Basic 7S globulin 2	414	44845	8.4
	A0A151U0Q4	Basic 7S globulin	409	43794	8.7
	A0A151U0R9	Basic 7S globulin	364	38814	8.7
	A0A151U5Z8	Basic 7S globulin	413	43424	8.1
	A0A151RNW3	Glycinin	473	53413	5.1
	A0A151TUL0	Glycinin G3	446	50177	5.8
Glutelin	A0A151TUN4	Glycinin G3	465	52503	5.8
	A0A151RZS9	Glutelin type-A 1	356	38308	5.7
	A0A151SUU8	Glutelin type-A 1	356	38191	5.4
	A0A151T731	Glutelin type-A 1	356	38161	5.2
	A0A151TM61	Glutelin type-A 1	356	38509	5.6
	A0A151UBW6	Glutelin type-A 2	361	39259	5.2
	A0A151UBZ3	Glutelin type-A 2	358	38974	5.5
	A0A151UC62	Glutelin type-A 2	358	38764	5.8
Oleosin	A0A151SJ77	Oleosin 5	156	16676	9.6
	A0A151SLJ5	Oleosin 16 kDa	152	16349	9.6
	A0A151T0X9	Oleosin 18.5 kDa	135	14631	9.8
	A0A151TT63	Oleosin 5	104	10712	11.7

In another work, γ -proteins, rich in sulfur amino acids, were purified from pigeon pea globulins (Krishna & Bhatia, 1986). The γ -proteins presented two subunits (32 and 20 kDa) linked by disulfide bonds. Amino acid analysis showed that this protein has 3 to 4 times more sulfur amino acids in its composition, when compared to legumin and vicilin.

More recently, Sosa et al. (Sosa et al., 2021) extracted an albumin and a globulin fraction from pigeon pea with a sequential extraction procedure in water and in Tris-HCl buffer, respectively, and subsequent purification of the fractions by size exclusion chromatography. The authors characterized the fractions by fluorescence spectroscopy, differential scanning calorimetry, and surface hydrophobicity measurements and found that the structure of the globulins was less flexible and more compact compared to that of the albumin fraction.

In a study by Bravo et al. (2022), the main subunit of 7S-vicilin from pigeon pea (≈ 50 kDa) was purified and proteolyzed to investigate the antibacterial, antihypertensive and antioxidant properties of the resulting bioactive peptides. Despite not presenting antibacterial properties against the microorganisms evaluated (*Escherichia coli*, *Candida albicans*, and *Staphylococcus aureus*), pigeon pea peptides showed high antioxidant activity and antihypertensive activity comparable to Captopril, a well-known and potent angiotensin-converting inhibitor enzyme (ACE). The results suggest that bioactive peptides from pigeon pea have potential for application in dietary supplements and even in alternative medicine.

It has been established that pigeon pea seeds contain several protein groups based on Osborne's definition. Globulins, in particular 7S-globulin, are the major type of storage proteins. The protein composition was found to vary depending on the variety and on the seed compartment. However, to the best of our knowledge, no attempt using analytical chromatography has been undertaken to quantify each protein groups, as done for pea proteins for example (Baniel et al., 1998). This would provide a better description of the variability of pigeon pea protein composition that is probably a major driver of the nutritional and the functional quality of pigeon pea. Still, the purification and characterization of the 7S-vicilin has brought fundamental knowledge on the structural properties of the major pigeon pea protein. Furthermore, large-scale purification of pigeon pea proteins, based on mild process such as preparative chromatography and achieving reasonable yield, would allow for investigating the functional properties of pigeon pea proteins.

5.3 Amino acid profile and protein secondary structure

The amino acid profile of pigeon pea reveals that the species has a substantial amount of essential amino acids in its composition (Table 2), and it has been reported to be very close to that of chickpeas (Devindra et al., 2012). The levels of amino acids such as valine, isoleucine, phenylalanine, leucine and lysine in pigeon pea are above those recommended for infant feeding according to Oshodi et al. (1993). The authors point out

that the same is true for adult individuals. Mwasaru et al. (2000) found that the amino acids present in pigeon pea are mostly hydrophilic (approximately 60 wt.%), whereas the hydrophobic residues represent about 35% of the total amino acids, and the cyclic and sulfur residues represent approximately 5 and 1.5%, respectively.

No significant difference was observed in the amino acid profile of protein isolates obtained by the isoelectric precipitation method when compared to pigeon pea flour (native protein), which indicates that the extraction process had little influence on the protein composition (Adenekan et al., 2018). A similar result was observed by Olagunju et al. (2018b) in the production of pigeon pea protein hydrolysates using different proteases. Although some specific amino acids, such as glutamic acid, valine, and tryptophan had higher levels in the hydrolysates compared to the non-hydrolyzed protein isolate, the amino acid profile of the proteins was similar for both materials.

Olagunju et al. (2018b) observed that hydrolysis with thermoase increased the content of branched-chain amino acids (BCAAs) by 7%, as well as that of most hydrophobic amino acids (alanine, glycine, leucine, valine, proline, isoleucine, and phenylalanine); this result was attributed to the specificity of thermoase to cleave proteins from peptide bonds constituted by hydrophobic amino acids, thus increasing the content of associated amino acids and, consequently, increasing the hydrophobicity of the peptides, which might improve the antioxidant activity of protein extracts in lipid-containing foods.

Regarding the protein secondary structure, Sun et al. (2020) observed the predominance of β -sheet structures (47%) in pigeon pea proteins; random coil, α -helix, curved, and β -antiparallel structures constitute 16, 14, 13, and 10% of the overall structure, respectively. The authors also determined a total of sulfhydryl groups and disulfide bonds of 22.6 and 7.9 $\mu\text{mol g}^{-1}$ for pigeon pea proteins. The proportion of β -sheet and β -strand can be related to protein digestibility, and a decrease in the proportion of β -sheet and/or an increase in the random coil contributes positively to an increase in digestibility. There are indications that the pigeon pea globulin fraction might be more digestible than the albumin fraction, due to the lower proportion of β -strands found in its structure.

Chapter 1. Pigeon pea, and emerging source of plant-based proteins

Table 1.2. Amino acid profile of pigeon pea proteins.

Amino acids	Composition (g 100 g ⁻¹ of pigeon pea protein)								
Essential amino acids									
Histidine	3.09	4.41- 4.78	3.61	2.66	3.98- 6.24	1.81- 1.91	3.29	3.41	3.60
Isoleucine	3.74	3.48- 3.82	3.92	5.16	3.46- 3.68	6.30- 6.50	3.54	4.13	3.90
Leucine	6.97	7.61- 8.88	6.79	13.79	6.88- 8.37	9.27- 11.05	7.22	8.57	7.20
Lysine	5.96	7.05- 7.66	7.40	6.05	7.05- 7.56	5.00- 6.54	6.38	5.86	6.80
Methionine	0.89	0.84- 1.77	0.70	1.32	0.75- 0.97	1.98- 2.17	1.09	0.32	1.00
Phenylanine	6.78	7.42- 8.87	3.54	17.64	6.80- 8.58	9.46- 10.50	10.10	9.71	9.70
Theronine	2.87	3.27- 4.01	1.36	3.02	3.27- 3.93	7.16- 8.40	3.45	2.64	3.80
Tryptophan	0.61	0.20- 0.78	0.09	0.53	0.20- 0.32	2.18- 3.91	1.15	-	-
Valine	4.01	3.46- 4.18	6.71	14.32	3.46- 3.87	11.00- 12.01	4.02	5.73	4.40
Non-essential amino acids									
Alanine	3.61	4.00- 4.24	15.47	5.46	3.82- 4.04	2.56- 3.70	4.31	6.28	4.60
Arginine	5.59	6.91- 7.24	2.79	10.06	6.91- 7.57	3.18- 4.40	6.05	3.23	6.30
Asparagine	-	-	-	2.65	-	3.77- 4.23	-	-	-
Aspartic acid	8.44	10.77- 11.55	1.26	0.43	9.53- 11.59	3.11- 3.61	8.78	10.56	10.40
Cystine	0.66	0.43- 1.01	-	-	0.43- 0.60	4.06- 5.12	1.01	-	1.20
Glutamic acid	15.03	18.04- 20.45	6.48	8.10	19.90- 22.50	1.67- 1.95	20.34	24.71	19.00
Glycine	2.96	3.12- 3.78	1.60	1.22	3.12- 3.63	10.32- 11.14	3.56	3.95	3.80
Proline	3.87	4.83- 5.64	0.72	-	4.83- 5.06	2.44- 3.00	4.79	5.41	4.30
Serine	3.77	4.59- 6.33	2.20	2.33	5.60- 6.27	4.13- 5.29	4.84	4.77	5.00
Tyrosine	2.49	2.47- 2.79	1.86	4.41	2.60- 2.77	6.95- 7.12	2.70	0.44	3.00
Reference	(Xu et al., 2021)	(Olagunju et al., 2021)	(Yang et al., 2020)	(Tapal et al., 2019)	(Olagunju et al., 2018b)	(Adenekan et al., 2018)	(Ene-Obong, 1995)	(Oshodi et al., 1993)	(Devindra et al., 2012)

6. Methods for pigeon pea protein isolation

According to Tapal et al. (2019), there is no commercial production of pigeon pea protein fractions, not even in countries where it is grown in large quantities, such as India. In parallel, pigeon pea has received increasing attention from researchers in recent years (FAO, 2019), which may indicate the beginning of the reversal of this scenario.

Several studies have focused on the production of pigeon pea protein isolates and concentrates, in addition to protein hydrolysates (Akintayo et al., 1999; Akoja et al., 2017; Mwasaru, Muhammad, Bakar, & Che Man, 1999; Mwasaru, Muhammad, Bakar, & Man, 1999; Olagunju et al., 2018b, 2018a; Tapal et al., 2019). Protein hydrolysates are widespread in the food industry, as they enable the reduction of antinutritional factors, as well as increase protein digestibility and release of bioactive peptides (Olagunju et al., 2020; Putra et al., 2021; Tapal et al., 2019; Xu et al., 2021). In the next sections, the main points regarding the extraction of pigeon pea proteins are presented.

Pulse proteins can be extracted using chemical, physical, or biotechnological methods. The most commonly used chemical method is the aqueous fractionation process that can be divided into three stages: (i) flour defatting using solvents such as petroleum ether, n-hexane, or n-pentane to remove lipids and other lipophilic compounds that can interfere in the protein extraction; (ii) extraction of proteins using salts, water, alcohols, or buffer solutions; and (iii) precipitation of proteins using ammonium sulfate, ethanol, methanol, acetone, or acids (hydrochloric acid, citric acid, etc.) (Kumar et al., 2021).

Among the physical methods, the main processes are assisted by technologies that improve protein recovery. Some examples are: pulsed electric field-assisted extraction, microwave-assisted extraction, high pressure-assisted extraction, and ultrasound-assisted extraction.

Among the biotechnological methods, the enzyme-assisted extraction and the bacteria-assisted extraction are the most prevalent. Enzyme-assisted extraction is an approach that allows the recovery of high-quality plant proteins, due to the action of proteases that promote greater release of proteins from the polysaccharide matrix present in the seeds.

6.1 Extraction process and protein recovery

Pigeon pea proteins have mostly been extracted using the conventional chemical process of aqueous fractionation by alkaline solubilization followed by isoelectric precipitation (Figure 4 and Table 3) (Mwasaru et al., 2000; Olagunju et al., 2020). This method consists of solubilizing proteins at alkaline pH (pH 8.0 – 12.0) and then precipitating the protein fractions at a pH close to their isoelectric point (pH 4.0 – 5.0). Precipitated proteins can be separated by centrifugation, ultracentrifugation, and filtration. Parameters such as solubilization pH, solvent used, temperature, ionic strength, extraction time, and solids:solvent ratio can affect protein extractability and process yield (Mwasaru et al., 2000). The optimization of pigeon pea protein extraction, carried out by

Mwasaru et al. (2000), showed that the optimal process conditions were at pH 8.5, with a solids:solvent ratio ranging from 1:5 to 1:25, which resulted in extraction yields around 75%. In the work by Sosa et al. (2021), the use of a higher alkalization pH (11.0) allowed greater protein recovery (67.5 %) compared to lower pHs (yield between 49.9 and 51.2%). The authors also evaluated the extraction of albumin and globulin fractions from pigeon pea and obtained extraction yields of 30.6 % for albumin, and 6.6 % for globulin. No extraction method could be considered the best to meet all the criteria evaluated in the work, but authors emphasized that the isoelectric precipitation method with pH variation for protein extraction is a simple, cheap and fast method to obtain protein ingredients. Mwasaru et al. (1999) also evaluated the production of pigeon pea protein isolates at different solubilization pHs. Protein recovery ranged from 35.1 to 58.1 %, whereas protein content in the isolates ranged from 78.1 to 83.4 wt.%. The authors also evaluated the micellization method in protein extraction, using 0.25 M NaCl solution at pH 6.5. They achieved a higher protein recovery (40.2 %), with the extract showing 82.8 wt.% protein as well as a higher solubility as compared to the isoelectric precipitation method (Mwasaru, Muhammad, Bakar, & Man, 1999). Besides, ultrasound-assisted extraction was investigated by Sun et al. (2020) and resulted in a 26 % increase in the extractability of pigeon pea proteins compared to untreated seeds.

In addition to the process of alkaline extraction followed by isoelectric precipitation, some works had also used acid extraction followed by isoelectric precipitation (Tapal et al., 2019) and salt-induced extraction followed by isoelectric precipitation (Mwasaru, Muhammad, Bakar, & Man, 1999) (Figure 5). These methods allow the isolation of globulin-rich fractions from pigeon pea, mainly. Albumins can also be extracted in these processes and be present in low quantities in protein concentrates or isolates (Sosa et al., 2021). Tapal et al. (2019) used pH 2.0 to solubilize the proteins, followed by isoelectric precipitation at pH 4.5. The protein content determined in the isolate was 85 % and the extraction yield was 37 %.

Other solvents such as methanol, acetone, and ammonium sulfate were shown to be good precipitants of proteins from pigeon pea, allowing extraction of pigeon pea protein isolates containing about 91 % of proteins (Adenekan et al., 2018). On the other hand, the work by Xu et al. (2021) showed that protein hydrolysis impaired the recovery of proteins. While the protein isolate produced by isoelectric precipitation had 87 wt.% of proteins, the hydrolysates produced with the enzymes alcalase and bromelain had 60 and 20 wt.%, respectively. The authors attributed the low protein content identified in hydrolysates to the conversion of proteins into small peptides and free amino acids, which makes them more difficult to quantify by the Bradford assay.

Beyond chemical extraction processes, the use of enzymes in protein extraction processes has been investigated for peas, pinto beans, mung beans, and soybeans to improve the protein recovery (Kumar et al., 2021). Furthermore, bacteria-assisted processes were evaluated by Emkani et al. (2021) for extracting an albumin-rich fraction and a globulin-rich fraction from pea through a process assisted by lactic fermentation.

The process increased the protein content of the albumin fraction as compared to the traditional process of protein precipitation using HCl or lactic acid. Nevertheless, the protein content in the globulin fraction was reduced using the process assisted by lactic acid fermentation. Such biotechnological routes, even though not commonly explored yet, may deserve attempts on pigeon pea protein fractionation.

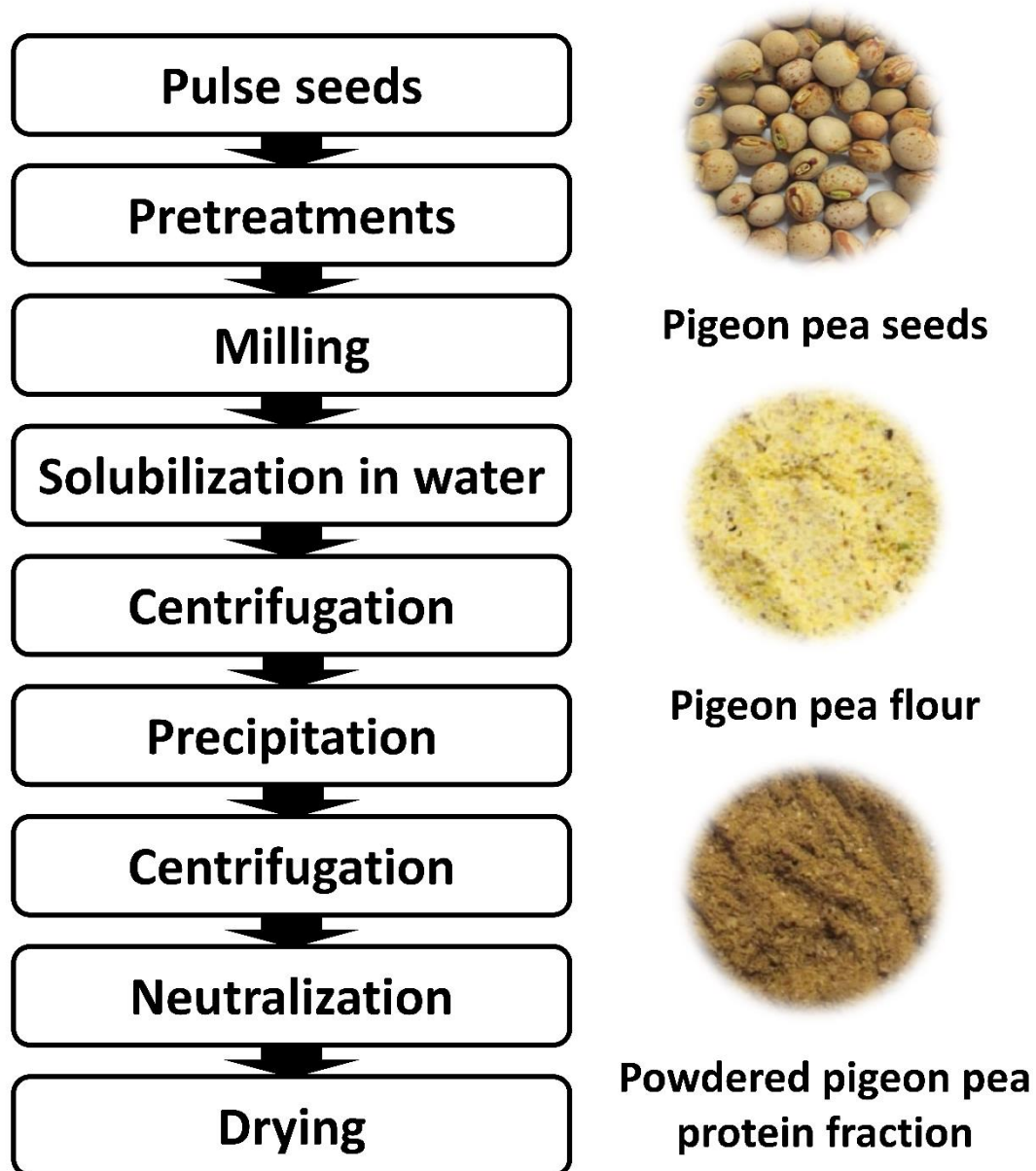


Figure 1.4. Conventional aqueous fractionation process to produce protein fractions from pigeon pea seeds.

Chapter 1. Pigeon pea, and emerging source of plant-based proteins

Table 1.3. Pigeon pea protein extraction process.

Reference	Extraction method	Flour treatment	Flour:solvent ratio	Solubilization pH	Precipitation pH	Centrifugation conditions	Fraction	Protein content (%)	Properties evaluated
(Xu et al., 2021)	Isoelectric precipitation method followed by enzymatic hydrolysis with alcalase and bromelain (1 %, w w ⁻¹ , enzyme/substrate ratio)	Defatting and milling	1:10	10.0	5.0	8,000 g (4 °C/30 min)	Protein isolated, alcalase hydrolysed protein (DH = 10.65 %) and bromelain hydrolysed protein (DH = 4.82 %)	87	Molecular weight profile (SDS-PAGE and SEC); Functional properties (solubility, surface hydrophobicity, WHC, OHC); Amino acid profile; Antioxidant activity (DPPH, NO scavenging and ORAC); Anti-inflammatory activity.
(Boachie et al., 2019)	Isoelectric precipitation method followed by enzymatic hydrolysis with pepsin, papain and thermolysin (1:100, w w ⁻¹ , enzyme/substrate ratio)	Milling	1:10		4.0	7,000 g (4 °C/30 min)	Pepsin hydrolysed protein, papain hydrolysed protein and thermolysin hydrolysed protein		Molecular weight profile (SDS-PAGE); Surface hydrophobicity; Amino acid profile; Particle characterization (particle size, zeta potential and polydispersity index); DPP-4 inhibition assay.

Table 1.3. continued

Reference	Extraction method	Flour treatment	Flour:solvent ratio	Solubilization pH	Precipitation pH	Centrifugation conditions	Fraction	Protein content (%)	Properties evaluated
(Olagunju et al., 2021)	Isoelectric precipitation method followed by enzymatic hydrolysis with thermoase. An enzyme / substrate ratio of 1: 100 is used	Soaking in boiling water, dehulling and milling	1:15	9.0	4.5	11,000 g (20 °C/ 30 min)	Protein concentrated, thermoase hydrolysed protein (DH = 14 %) and peptide fractions (< 1 kDa, 1-3 kDa, 3-5 kDa, 5-10 kDa e > 10 kDa).	75.96- 80.40	Amino acid profile; Antioxidant activity (DPPH and FRAP); Inhibition of linoleic acid oxidation; Inhibition of ACE activity; Inhibition of renin activity; Inhibition of carbohydrate-hydrolyzing enzymes (α -amylase and α -glucosidase inhibition).
(Putra et al., 2021)	Peptide extraction by ultrasonic-assisted method (40 kHz, 100 % power, 10 min) followed by enzymatic hydrolysis by pepsin (2,000 U mL ⁻¹) and pancreatin (100 U mL ⁻¹) at 37 °C. Peptide fractionation was performed by subjecting the hydrolysate to dialysis.	Soaking, boiling, dehulling, fermentation and milling	1:10			7,000 g (15 min)	Peptide fraction (<1 kDa , 1–3.5 kDa, 3.5–14 kDa, >14 kDa)		In vitro digestion simulation; Peptide fractionation; Peptide absorption; Angiotensin-I-converting enzyme inhibitory activity assay
(Sosa et al., 2021)	Isoelectric precipitation method. Albumin and globulin fractions were extracted according to their solubility in different solvents	Milling and defatting in hexane		8.0- 11.0			Protein isolated and albumin and globulin fractions	90.84	Molecular weight profile (SDS-PAGE); DSC; SEC; Fluorescence spectroscopy; Surface hydrophobicity; Protein denaturation with urea; FTIR; Protein solubility.

Table 1.3. continued

Reference	Extraction method	Flour treatment	Flour:solvent ratio	Solubilization pH	Precipitation pH	Centrifugation conditions	Fraction	Protein content (%)	Properties evaluated
(Sun et al., 2020)	Isoelectric precipitation method	Milling, soaking, ultrasound and microwave treatments	1:10		4.0	18,000 g (4 °C/45 min)	Protein isolated		In vitro simulated gastrointestinal digestion; Molecular weight profile (SDS-PAGE); Protein solubility; Free sulfhydryl group and disulfide bond determination; Surface hydrophobicity; Particle characterization (particle size, zeta potential and polydispersity index); Fourier transform infrared (FTIR).
(Tapal et al., 2019)	Isoelectric precipitation method followed by hydrolysis using the enzyme pepsin (concentration ranging from 2 to 10%, w w ⁻¹) for 2h at 37°C.	Defatting and milling	1:10		4.5	8,000 rpm (30 min)	Pepsin hydrolysed protein (DH = 24-38 %).	74	In vitro digestibility; Molecular weight profile; Antioxidant activity (DPPH and Reduction power); Encapsulation of curcumin with pigeon pea protein isolate (SEM; particle size and curcumin concentration in microcapsules).

Table 1.3. continued

Reference	Extraction method	Flour treatment	Flour:solvent ratio	Solubilization pH	Precipitation pH	Centrifugation conditions	Fraction	Protein content (%)	Properties evaluated
(Olagunju et al., 2018a)	Isoelectric precipitation method followed by enzymatic hydrolysis. For enzymatic hydrolysis, an enzyme/substrate ratio of 1: 100 is used. Three treatments were carried out: hydrolysis with alcalase (pH 8.0, 50 °C), pancreatin (pH 7.5, 37 °C) and pepsin + pancreatin (pH 2.0 for pepsin followed by 7.5 for pancreatin, 37 °C). The peptides were fractionated by ultrafiltration.					9,000 g (4 °C/ 30 min)	Protein isolated, alcalase hydrolysed protein (DH = 24.22 %), pancreatin hydrolysed protein (DH = 27.17 %), pepsin-pancreatin hydrolysed protein (DH = 18.53 %) and peptide fractions (< 1 kDa, 1-3 kDa, 3-5 kDa, 5-10 kDa e > 10 kDa).	23.85- 56.82 %	Molecular weight profile (SDS-PAGE); Antioxidant activity (DPPH, SRSA; HRSA; ABTS; ORAC: FRAP), Inhibition of linoleic acid oxidation).
(Olagunju et al., 2018b)	Isoelectric precipitation method followed by enzymatic hydrolysis with pancreatin (pH 8.0, 37°C) and pepsin-pancreatin (pH 2.0, 37°C) for 4 h. Also, a sequential hydrolysis with pepsin followed by pancreatin for 2 h each was done. The enzyme/substrate ratio was 1:20 (w w ⁻¹).		1:20	9.0	4.5	9,000 g (4 °C/ 30 min)	Protein isolated, pepsin hydrolyzed protein, pancreatin hydrolyzed protein and pepsin-pancreatin hydrolyzed protein.		Amino acid profile; Antioxidant activity (DPPH; HRSA; ABTS; FRAP), Inhibition of linoleic acid oxidation); ACE inhibitory activity; Renin inhibitory activity; Antihypertensive activity.

Chapter 1. Pigeon pea, and emerging source of plant-based proteins

Table 1.3. continued

Reference	Extraction method	Flour treatment	Flour:solvent ratio	Solubilization pH	Precipitation pH	Centrifugation conditions	Fraction	Protein content (%)	Properties evaluated
(Adenekan et al., 2018)	Methanol precipitation method, water extraction method, ammonium sulfate extraction method and acetone precipitation method	Dehulling and milling		11		6,000 rpm (20 °C/30 min)	Protein isolated	91.35- 91.83	Antinutritional factors (phytic acid content, trypsin inhibitor and cyanogenic glucoside content); Functional properties (WHC, OHC, emulsion capacity and stability, foaming capacity and stability, protein solubility and bulk density); Amino acid profile; Nutritional properties (NB, BV, NPU, TPD, PER).
(Akoja et al., 2017)	Enzymatic hydrolysis method using papain enzyme (2 %) at 55 °C	Soaking in water for 5 h, dehulling, milling and defatting in hexane	1:1	8.0		3,000 rpm (4 °C/10 min)	Papain hydrolysed protein		Production and characterization of maize-pigeon pea hydrolysate flours blends (bulk density, WHC, OHC, swelling capacity, dispersibility, emulsification capacity, foaming capacity and stability, pasting properties); Sensory evaluation of snacks produced with maize-pigeon pea hydrolysate flours blends.
(Mwasaru et al., 2000)	Isoelectric precipitation method			8.5			Protein isolated	83.4	Protein solubility; Emulsifying properties; Whipping properties; Gelation properties.
(Akintayo et al., 1999)	Isoelectric precipitation method	Dehulling and milling	1:10	8.5	4.5	8,000 rpm (30 min)	Protein concentrated	72	Foaming properties; Gelation properties.

Table 1.3. continued

Reference	Extraction method	Flour treatment	Flour:solvent ratio	Solubilization pH	Precipitation pH	Centrifugation conditions	Fraction	Protein content (%)	Properties evaluated
(Mwasaru, Muhamad, Bakar, & Man, 1999)	Isoelectric precipitation method and micellization technique. The isoelectric precipitation method was evaluated to vary the extraction pH and the method of micellization was performed with the use of 0.25 M NaCl solution (pH 6.5).	Milling	1:10	8.5, 9.5, 10.5, 11.5 and 12.5	4.5	5,000 g (15 min)	Protein isolated	78.1 a 83.4	Hydrophobicity; Molecular weight profile (SDS-PAGE); Isoelectric focusing; Color; DSC.
(Mwasaru, Muhamad, Bakar, & Che Man, 1999)	Isoelectric precipitation method and micellization technique. The isoelectric precipitation method was evaluated to vary the extraction pH and the method of micellization was performed with the use of 0.25 M NaCl solution (pH 6.5).	Milling	1:10	8.5, 9.5, 10.5, 11.5 and 12.5	4.5	5,000 g (15 min)	Protein isolated	78.1 a 83.4	Functional properties (protein solubility, WHC, OHC, emulsifying properties, whipping properties and gelation properties).
(Devindra et al., 2012)	Isoelectric precipitation method	Milling and defatting in hexane				12,000 g (15 min)	Albumin and globulin fractions	88.9- 99.4	Amino acid composition of different seed components.

Abbreviations. ABTS: 2,20-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; BV: biological value; ACE: angiotensin converting enzyme; DG: degree of hydrolysis; DPPH: 2,2-diphenyl-1-picrylhydrazyl; DPP-4: dipeptidyl peptidase-4; DSC: differential scanning calorimetry; FRAP: ferric reducing ability of plasma; FTIR: Fourier transform infrared spectra; HRSA: hydroxyl radical scavenging activity; NB: nitrogen balance; NPU: net protein utilization; OHC: oil holding capacity; ORAC: Oxygen radical absorbance capacity; PER: protein efficiency ratio; SDS-PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SEC: size exclusion chromatography; SEM: scanning electron microscopy; SRSA: superoxide radical scavenging activity; TPD: true protein digestibility; WHC: water holding capacity.

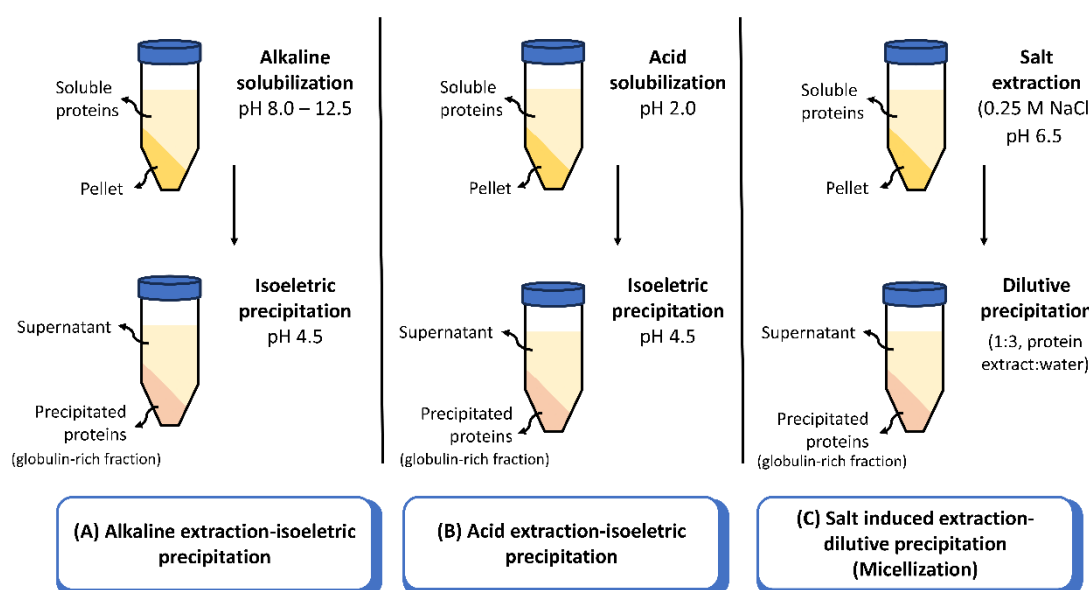


Figure 1.5. Extraction methods for isolation of pigeon pea proteins. **(a)** Alkaline extraction followed by isoelectric precipitation, **(b)** Acid extraction followed by isoelectric precipitation, and **(c)** salt-induced extraction followed by dilutive precipitation (micellization). Source: methods described by Mwasaru et al. (2000) and Tapal et al (2019).

The aqueous fractionation processes have some disadvantages, as they are time-consuming, require high-water consumption and involve possible losses in protein functionality due to exposure to very alkaline or acidic pHs (Kumar et al., 2021). A more sustainable approach that can be employed is the dry fractionation of pulse flours. Dry fractionation is a promising route to preserve protein functionality, as it only involves milling and air-classification or electrostatic sorting processes. This avoids the need for water solubilization and subsequent drying steps to obtain protein fractions, which represents a much more sustainable process (Schutyser et al., 2015). It could also be used as a preliminary step of aqueous fractionation to enrich the raw material in proteins (Yang et al., 2022). For pigeon pea proteins, however, only processes that used the wet route are reported in the literature. Dry fractionation could be an important process to be explored for pigeon pea in future years, mainly to understand the functionality of highly concentrated protein ingredients (70 – 90 % proteins, using aqueous fractionation methods) in comparison with ingredients mildly concentrated in proteins (50 – 60 % proteins, by dry fractionation). With a milder fractionation in perspective, the need to better understand the profile of non-proteinaceous compounds in pigeon pea is reinforced, as suggested in section 3.

6.2 Possible applications of co-products from the protein extraction process

The pellet produced from the aqueous fractionation of plant proteins is rich in carbohydrates that are eliminated by centrifugation during the process. Although less studied, pigeon pea carbohydrates also have properties of interest to the food industry, which implies that the co-products of the protein extraction process are likely to produce food ingredients.

Olagunju et al. (2021) isolated native starch granules from pigeon pea seeds and modified it through acetylation. Acetylation enabled a greater water and oil holding capacity, a lower retrogradation tendency and a lower glycemic index compared to native pigeon pea starch, highlighting the potential of pigeon pea modified starch for applications as a thickening and stabilizing agent. Guleria & Yadav (2022) evaluated the rheological properties of native, cross-linked, oxidized and hydroxy propylated starches from pigeon pea. The chemical modifications resulted in changes to several properties of starches, reducing paste clarity, gel hardness, swelling power, and solubility. The cross-linked starch presented better thermal stability and, consequently, a more stable structure. Finally, Singh et al. (2023) evaluated cellulose nanocrystals from pigeon pea stem waste. Cellulose was extracted from the stems using the chlorite bleaching and NaOH treatment method, followed by production of cellulose nanocrystals by acid hydrolysis with HCl. The cellulose nanocrystals showed good thermal stability (>324.3 °C), being highlighted as an interesting material for application in the food packaging sector.

7. Functional properties

The functional properties of plant-based proteins can be affected by intrinsic and extrinsic factors. Among the intrinsic factors are the size and shape of the proteins, net charge distribution, amino acid sequence, structure (secondary, tertiary and quaternary), interaction with other components of the food matrix, hydrophobicity/hydrophilicity ratio, and others. The extrinsic factors include moisture, temperature, pH, ionic strength, chemical additives, enzymes, and mechanical processes (Kumar et al., 2022). In addition, pre-treatments applied to seeds and the selected protein extraction processes can affect the functional properties of the resulting flours and protein extracts (Table 4). Functional properties such as solubility, water holding capacity, oil holding capacity, surface hydrophobicity, emulsifying capacity, foaming capacity, among others, are defined as physicochemical properties that provide information on how proteins behave in food systems, either when applied as a processing aid or as a macro-constituent of the product (Adenekan et al., 2018).

This section presents several functional properties investigated for pigeon pea proteins. The functionality of pigeon pea proteins showed similarities to other plant-based proteins such as cowpea, dolichos bean, jack bean, and field pea (Acevedo et al., 2013, 2017; Maninder et al., 2007; Mwasaru et al., 2000; Mwasaru, Muhammad, Bakar, & Man, 1999). There is still a need for studies that compare the functionality of pigeon pea with other plant sources and that evaluate the ability of pigeon pea proteins to be

analogous to proteins obtained from animal sources (e.g., egg protein, whey protein, etc.).

7.1 Protein solubility, surface hydrophobicity and zeta potential

Protein solubility can be considered a good index to determine its potential for applications in food systems (Adenekan et al., 2018). As the pH of a medium is increased to more alkaline regions, there is an increase in the solubility of proteins, a phenomenon that is associated with the amphoteric character of these molecules. At alkaline pH, proteins are strongly negatively charged, showing a greater interaction with water and, consequently, leading to greater solubility. In parallel, modifications in the conformation of proteins by alkalinization can cause their hydrophobic groups to become buried within their structure (Mwasaru et al., 2000; Xu et al., 2021).

In most works on pigeon pea, as well as in studies involving other plant-based proteins, the protein solubility is usually determined by the preparation of a protein suspension, followed by centrifugation, and determination of the protein concentration in the supernatant. It is important to highlight that these methods can also quantify proteins that are not necessarily soluble, but are in the supernatant phase in the form of small aggregates which do not sediment under the applied centrifugation conditions.

For a better understanding of the mechanisms that involve protein solubility, surface hydrophobicity is a commonly investigated property. This structural property affects the bioavailability and nutritional quality of proteins, having a direct relationship not only with their solubility but also with other functional properties, as observed by Sosa et al. (2021). These authors investigated the extraction of protein isolates and of albumin and globulin fractions from pigeon pea and reported that the extraction pH and the nature of the protein fraction impact the solubility of proteins in aqueous media. While the albumin fraction and protein isolates extracted at different alkaline pHs (8, 9, 10, and 11) presented solubility in water ranging from 60 to 74%, the globulin fraction presented a lower solubility in such conditions, around 21%. However, in buffer solution (pH 8, $\mu = 0.3$ M) the solubility of globulin was higher than that of albumin and other protein isolates (82%). The authors attributed the decreased solubility of protein isolates in the buffer solution to the presence of NaCl and Na₂HPO₄ salts (*salting out* effect). Regarding the surface hydrophobicity, higher values were determined by the authors for protein isolates and the globulin fraction, whereas the albumin fraction had lower hydrophobicity.

Chapter 1. Pigeon pea, and emerging source of plant-based proteins

Table 1.4. Functional properties of pigeon pea flour and protein extracts.

Pigeon pea flour										
Reference	Treatment	Pasting Temperature (°C)	Water holding capacity (g g ⁻¹)	Oil holding capacity (g g ⁻¹)	Least gelation concentration (%)	Thermal properties (°C)	Emulsifying properties	Foam properties	Surface Hydrophobicity	Solubility (%)
(Sharma et al., 2019)	Ground pigeon pea seeds were used as a control (A). Two processes were evaluated: soaking (10h / 25 °C) (B) and germination (48h at 25 (C), 30 (D) and 35 (E) °C).	(A) 87.50; (B) 87.70; (C) 89.40; (D) 90.70; (E) 93.85								
(Adenekan et al., 2018)	Milled pigeon pea seeds.		1.25	1.31			Emulsifying capacity (%): 0.40 Emulsion Stability (%): 0.40	Foaming capacity (%): 18.20 Foaming stability (%): 25.30		12.1
(Acevedo et al., 2017)	The flours were produced with native seeds (without treatment) (A); germinated for 5 days (B); soaked (6h) and boiled in water for 20 (C), 40 (D) and 60 (E) minutes; and treated in microwaves at powers of 50 (F), 70 (G) and 100 (H)% (output power 800 Watts).	81.6 (A); 82.8 (B); 83.1 (C); 75.2 (D); 74.1 (E); 81.4 (F); 82.2 (G); 82.5 (H)	1.00 (A); 1.50 (B); 1.72 (C); 1.73 (D); 1.74 (E); 0.99 (F); 0.98 (G); 1.00 (H)	1.11 (A); 1.14 (B); 1.13 (C); 1.13 (D); 1.11 (E); 1.13 (F); 1.12 (G); 1.11 (H)						
(Acevedo et al., 2013)	Milled pigeon pea seeds.	81.63			6-8	83.6 (starch gelatinization); 96.0 (protein desnaturation)				
(Tiwari et al., 2008)	Dehulled and ground seeds were used as control (A). Different dryings were carried out as pre-treatments on the seeds in order to facilitate dehulling, which are: wet method (B); hydrothermal method (C) and dry method (D).	84.7 (A); 84.7 (B); 87.5 (C); 85.5 (D)	1.05 (A); 1.00 (B); 1.35 (C); 1.10 (D)	1.25 (A); 1.20 (B); 1.30 (C); 1.10 (D)	6 (A); 8 (B); 8 (C); 8 (D)		Emulsifying capacity (%): 40-80 Emulsion Stability (%): 30-60	Foaming capacity (%): 10-22 Foaming stability (%): < 20		

Chapter 1. Pigeon pea, and emerging source of plant-based proteins

Table 1.4. continued.

Pigeon pea flour										
Reference	Treatment	Pasting Temperature (°C)	Water holding capacity (g g ⁻¹)	Oil holding capacity (g g ⁻¹)	Least gelation concentration (%)	Thermal properties (°C)	Emulsifying properties	Foam properties	Surface Hydrophobicity	Solubility (%)
(Maninder et al., 2007)	Flours produced from seeds defatted from different Indian cultivars (AL-15 (A) and AL-201 (B)) were evaluated	83.4 (A); 83.5 (B)	1.37 (A); 1.39 (B)	0.98 (A); 0.96 (B)	12 (A); 14 (B)	81.8 (A); 82.2 (B)		Foaming Capacity (%): 34.5 (A); 37.3 (B) Foaming Stability (%): 80.0 (A); 80.0 (B)		
(Onimawo & Akpojobwo, 2006)	Ground seeds (without treatment) were used as control (A). Flours produced with roasted seeds in two different processes were evaluated: roasting at 80 ° C / 1h (B) and roasting at 100 ° C / 1h (C)		4.47 (A); 4.82 (B); 4.94 (C)	2.50 (A); 2.81 (B); 2.98 (C)	4 (A); 4 (B); 4 (C)		Emulsifying capacity (%): 80.0 (A); 75.0 (B); 50.0 (C) Emulsion Stability (%): 62.5 (A); 55.6 (B); 55.0 (C)	Foaming capacity (%): 600 (A); 325 (B); 300 (C) Foaming Stability (%): 410 (A); 295 (B); 235 (C)		

Chapter 1. Pigeon pea, and emerging source of plant-based proteins

Table 1.4. continued

Pigeon pea protein extracts										
Reference	Treatment	Pasting Temperature (°C)	Water holding capacity (g g ⁻¹)	Oil holding capacity (g g ⁻¹)	Least gelation concentration (%)	Thermal properties (°C)	Emulsifying properties	Foam properties	Surface Hydrophobicity	Solubility (%)
(Xu et al., 2021)	Protein isolate (A), protein hydrolysate produced by the enzyme alkalase (B) and protein hydrolysate produced by the enzyme bromelain (C) were evaluated.		3.90 (A); 4.00 (B); 7.60 (C)	3.20 (A); 3.00 (B); 6.00 (C)					100-120 (A); < 30 (B); < 30 (C)	68.7 at pH 12.0 (A)
(Sosa et al., 2021)	Albumin (A) and globulin (B) fractions of pigeon pea were evaluated. Protein isolates produced with different alkalization pH (8.0 (C), 9.0 (D), 10.0 (E), 11.0 (F)) were also evaluated.					90.4 (B); 94.7 (C); 95.9 (D); 96.7 (E); 96.5 (F)			1843 (A); 2600 (B); 2377 (C); 2138 (D); 2122 (E); 2622 (F)	71 (A); 21 (B); 70 (C); 69 (D); 74 (E); 60 (F)
(Adenekan et al., 2018)	Protein isolates produced by different methods were evaluated: aqueous extraction (A), methanol precipitation extraction (B), ammonium sulfate extraction (C) and acetone precipitation extraction (D).		1.55 (A); 1.60 (B); 1.51 (C); 1.50 (D)	1.40 (A); 1.45 (B); 1.45 (C); 1.40 (D)			Emulsifying capacity (%): 1.40 (A); 1.40 (B); 2.17 (C); 2.23 (D) Emulsion Stability (%): 91.20 (A); 91.47 (B); 89.10 (C); 90.43 (D)	Foaming capacity (%): 33.3 (A); 35.37 (B); 35.10 (C); 33.23 (D) Foaming stability (%): 54.57 (A); 53.83 (B); 55.73 (C); 52.33 (D)		97.03 (A); 97.13 (B); 90.70 (C); 92.63 (D)

Chapter 1. Pigeon pea, and emerging source of plant-based proteins

Table 1.4. continued

Pigeon pea protein extracts										
Reference	Treatment	Pasting Temperature (°C)	Water holding capacity (g g ⁻¹)	Oil holding capacity (g g ⁻¹)	Least gelation concentration (%)	Thermal properties (°C)	Emulsifying properties	Foam properties	Surface Hydrophobicity	Solubility (%)
(Mwasaru et al., 2000)	Pigeon pea protein isolate solutions containing 0 (A), 0.1 (B), 0.2 (C), 0.3 (D), 0.4 (E) and 0.5 (F) M of NaCl were evaluated.				14 (B); 14 (C); 10 (D); 6 (E); 6 (F)		Emulsifying capacity (%): 39.50 (A); 43.09 (B); 42.26 (C); 39.34 (D); 43.75 (E); 42.42 (F) Emulsion Stability (%): 44.98 (A); 47.86 (B); 50.67 (C); 57.36 (D); 51.47 (E); 56.35 (F)	Foaming capacity (%): 34.0 (A); 21.0 (B); 40.2 (C); 54.3 (D); 59.0 (E) 64.2 (F) Foaming stability (%): 77.8 (A); 29.3 (B); 21.7 (C); 24.7 (D); 32.1 (E); 35.6 (F)		96.9 (pH 12.0) (E)
(Akintayo et al., 1999)	The functional properties of pigeon pea protein concentrate were evaluated for the impact of different factors. Protein solutions containing 0.00 (A), 0.25 (B), 0.50 (C), 1.00 (D) % (w w ⁻¹) of NaCl were evaluated. The influence of ionic strength on protein solutions was evaluated under the following conditions: 0.0 (E), 0.5 (F), 1.0 (G). The influence of the protein extract concentration was evaluated in 2 (H), 4 (I), 6 (J), 8 (K) and 10 (L)% (w / w). The influence of pH on the protein extract was evaluated at 2 (M), 4 (N), 6 (O), 8 (P), 10 (Q)				6 (A); 2 (B); 12 (C); 14 (D); 4 (F); 8 (G); 4 (M); 10 (N); 6 (O); 12 (P);			Foaming capacity (%): 80 (A); 110 (B); 60 (C); 40 (D); 80 (E); 100 (F); 50 (G); 80 (H); 110 (I); 126 (J); 140 (K); 152 (L) /140 (M); 90 (N); 80 (O); 110 (P); 128 (Q) Foaming stability (%): 90.9 (A); 55.5 (B); 66.6 (C); 71.4 (D); 90.9 (E); 55.5 (F); 67.5 (G); 90.9 (H); 66.6 (I); 50.0 (J); 41.6 (K); 40.0 (L); 42.0 (M); 54.0 (N); 58.8 (O); 50.0 (P); 45.4 (Q)		

Chapter 1. Pigeon pea, and emerging source of plant-based proteins

Table 1.4. continued

Pigeon pea protein extracts										
Reference	Treatment	Pasting Temperature (°C)	Water holding capacity (g g ⁻¹)	Oil holding capacity (g g ⁻¹)	Least gelation concentration (%)	Thermal properties (°C)	Emulsifying properties	Foam properties	Surface Hydrophobicity	Solubility (%)
(Mwasaru, Muhammad, Bakar, & Man, 1999)	Protein isolates were obtained by micellization (A) and isoelectric precipitation using alkalization pHs of 8.5 (B), 9.5 (C), 10.5 (D), 11.5 (E) and 12.5 (F).		1.24 (A); 0.84 (B); 1.25 (C); 1.26 (D); 2.10 (E); 2.13 (F)	2.10 (A); 2.45 (B); 1.79 (C); 1.77 (D); 1.77 (E); 2.03 (F)	6 (A); 6 (B); 6 (C); 8 (D); 10 (E); 10 (F)		Emulsifying capacity (%): 30-50 Emulsion stability (%): 10-50	Foaming capacity (%): 15-40 Foaming stability (%): 65-80		79.22 (pH 12.0) (A)
(Mwasaru, Muhammad, Bakar, & Che Man, 1999)	Protein isolates were obtained by micellization (A) and isoelectric precipitation using alkalization pHs of 8.5 (B), 9.5 (C), 10.5 (D), 11.5 (E) and 12.5 (F).					93.78 (A); 92.00 (B); 92.70 (C); 91.22 (E)			3089 (A); 3053 (B); 3465 (C); 3479 (D); 1826 (E); 1673 (F)	

The ionic strength of the medium directly affects the solubility of proteins, as investigated by Mwasaru et al. (2000): the presence of NaCl decreased the solubility of pigeon pea protein isolate in acidic medium, whereas at the alkaline end, the solubility of proteins increased; protein suspensions in 0.5 and 0.2 M NaCl showed the lowest solubility for the acid and alkaline extremes, respectively, whereas the highest solubility was in a 0.4 M NaCl solution at pH 12.0 (96.9%). According to the authors, at low pH, carboxyl groups protonate and the protein acquires a globally positive charge, resulting in decreased Cl⁻ ion repulsion and hydrophobic interactions, leading to the formation of insoluble aggregates. On the other hand, at high pH values there is an increase in the negative charge on proteins, which combined with the *salting in* effect of NaCl, serves to dissociate protein aggregates and thus increase solubility. It was also observed that the presence of NaCl affected more the solubility of pigeon pea proteins than that of cowpea proteins.

The action of hydrolysis on protein solubility was assessed by Xu et al. (2021). The study showed a lower solubility of the hydrolysates compared to the protein isolate in the entire pH range evaluated, which the authors attributed to alterations in the protein profile. In fact, the action of proteases can result in hydrolysates with peptides of more heterogeneous protein profile (lower solubility). Regarding hydrophobicity, higher values were observed for isolates compared to hydrolysates; the low surface hydrophobicity of hydrolysates may be associated with the production of peptides, decreasing the hydrophobic structures on the surface of the hydrolysates.

The protein extraction method and the fractions obtained affect the surface hydrophobicity values. Mwasaru et al. (1999) reported that pigeon pea proteins extracted by isoelectric precipitation showed greater hydrophobicity than those extracted by micellization. The solvents chosen for the extraction processes, in turn, also play an important role in the protein solubility. Adenekan et al. (2018) demonstrated that, while aqueous extraction and methanol extraction led to about 97% solubility, ammonium sulfate and acetone extractions resulted in slightly lower solubilities (90.7- 92.6 %).

Finally, protein denaturation at high temperatures (> 90 °C), as in microwave processing of flour, has been reported to result in decreased water solubility of pigeon pea proteins. The microwave treatment also resulted in a reduction in the particle size of protein isolates (166 nm), compared to protein isolates produced from flour without pretreatment (457 nm). However, the polydispersity index showed no differences, indicating a heterogeneous distribution of particle sizes (> 0.3). The zeta potential ranged from -32.7 to -35.2 mV. The authors used protein samples dispersed in Milli-Q water (1 mg/mL) in the assays (Sun et al., 2020).

The thermolysin enzyme produced smaller particles in the pigeon pea protein hydrolysate (264.6 nm), as compared to papain (400 nm) and pepsin (578.2 nm) enzymes. The polydispersity of the particles ranged from 0.4 to 0.6, and the zeta potential of the hydrolysates was around -15 mV, indicating a low electrostatic stability and potential tendency of the hydrolysates to aggregate (Boachie et al., 2019).

7.2 Water and oil holding capacity

The water and oil holding capacities (WHC and OHC, respectively) of pulse-based ingredients are of great importance for the incorporation of these ingredients in meat, dairy, confectionery and bakery products. Whereas WHC is essential for hydration processes and ingredient incorporation in water-continuous matrices, OHC helps in texture and better retention of aromas and flavors (Acevedo et al., 2017).

The presence of $-\text{COOH}$, $-\text{NH}_2$ and $-\text{OH}$ groups in the structure of proteins is largely responsible for the WHC of proteins. The unfolding of the exposed quaternary structure increases the protein's affinity for water molecules which could contribute to the greater WHC. OHC has been associated with lipid-protein interactions resulting from non-covalent bonds, electrostatic bonds, and hydrogen bonds (Acevedo et al., 2017; Xu et al., 2021).

Both the flour and the protein extracts of pigeon pea have good WHC and OHC properties. Flour is rich in carbohydrates and fiber, hydrophilic compounds that can easily interact with water and contribute to the WHC value (Acevedo et al., 2013). However, WHC and OHC of pigeon pea protein isolates have been shown to be greater than those of their flours (Acevedo et al., 2013, 2017; Adenekan et al., 2018; Tiwari et al., 2008; Xu et al., 2021).

In flours, one way to increase these capacities is the addition of some pretreatment steps. It has been shown that soaking, cooking, germination and roasting of pigeon pea contribute to the increase in WHC and/or OHC of their flours. On the other hand, treatments based on the use of microwaves were not able to increase these indices (Acevedo et al., 2013; Onimawo & Akpojovwo, 2006; Tiwari et al., 2008).

In the case of protein extracts, Mwasaru et al. (1999) highlighted that extraction at a more alkaline pH (pH 12.5) led to an increase in WHC compared to extracts obtained under lower alkalization conditions (pH 8.5). The inverse was observed by the authors for OHC, with higher values being obtained in less alkaline regions.

Protein hydrolysis can also contribute to produce ingredients with greater WHC and/or OHC. Xu et al. (2021) showed that the WHC of a pigeon pea protein hydrolysate produced with bromelain almost doubled, compared to the non-hydrolyzed protein isolate. The same behavior was observed for OHC. The authors attributed these results to changes in protein structures, causing greater or lesser exposure of polar groups (e.g., $-\text{COOH}$, NH_2 , $-\text{OH}$).

7.3 Foam properties

A foam is formed when air is injected into a liquid and trapped in the form of bubbles. What defines a good foaming ability of a given material is the presence of surface-active molecules, such as proteins that can reduce surface tension at the air-water interface (Adenekan et al., 2018) and form viscoelastic interfacial films.

Surface hydrophobicity is positively correlated with foaming properties, as the initial anchoring of proteins at the air-water interface is facilitated by surface hydrophobic

patches. The ability of proteins to unfold at the interface is another important aspect of foam formation. Molecular flexibility can expose previously buried hydrophobic portions, which can contribute to a more rapid reduction in surface tension, increasing foamability (Damodaran, 2006; Foegeding et al., 2006).

While whey and egg are proteins widely used in the production of foams in foods, soybean, peas, chickpea, and wheat are examples of plant-based proteins used as substitutes for animal proteins as foam-forming agents. Globulins are the most explored vegetable protein fraction regarding foaming capacity, because the conventional method of aqueous fractionation of vegetable proteins results in a globulin rich-fraction (more soluble in high pH conditions). However, studies have shown that globulins have poor interfacial and foam-stabilizing properties when compared to animal-derived proteins, due to their highly ordered structure and limited ability to unfold and adsorb at the interface (Adenekan et al., 2018). Conversely, the albumin fraction (generally present in the pellet from the aqueous fractionation process) has a foaming activity superior to that of globular proteins (Murray, 2020; J. Yang & Sagis, 2021).

Several studies evaluated the foaming properties of pigeon pea flours and derived protein extracts. However, to date, no studies have further investigated the impact of the interfacial properties of these proteins on the foaming capacity.

Mwasaru et al. (1999), in a study with pigeon pea protein isolates, showed that foam volume expansion decreases with increasing pH. The authors also highlighted that the use of saline solutions made it possible to increase the formed foam volume, as it improves protein solubility. On the other hand, the presence of salts impaired the rheological properties of the formed films, decreasing the foam stability over time. Akintayo et al. (1999) observed that the foaming capacity of pigeon pea protein concentrates increased when low concentrations (0.25% , $w\ v^{-1}$) of NaCl were added. The foaming capacity had also a direct relationship with the increase in the concentration of protein concentrate, with better foam volumes being observed at concentrations equal to 10% ($w\ v^{-1}$). Both the foaming capacity of pigeon pea protein isolates and the resulting foam stability were superior compared to those obtained with pigeon pea flour (Adenekan et al., 2018). Although it forms a rather small volume of foam, which is characteristic of pulses' flour, pigeon pea flour generated foams with good stability ($> 80\%$) (Ene-Obong, 1995).

7.4 Emulsifying properties

Pigeon pea flours and protein extracts were shown to have the potential to stabilize oil-in-water (O/W) emulsions (Adenekan et al., 2018; Mwasaru et al., 2000; Mwasaru, Muhammad, Bakar, & Che Man, 1999; Onimawo & Akpojovwo, 2006; Tiwari et al., 2008). Adenekan et al. (2018) reported that pigeon pea protein extracts showed better results in the stabilization of emulsions than pigeon pea flours (obtained by milling the seeds). The authors obtained emulsion stability values that reached 90%, this

percentage being the measure of the emulsion layer that remained stable after heating (85 °C/15 min).

Mwasaru et al. (1999) observed that the pH of protein extraction significantly affected their emulsifying properties. While the emulsifying capacity increased slightly as the extraction pH increased from 8.5 to 11.5, extraction conditions with higher alkalinity (pH 12.0) decreased the emulsifying capacity of pigeon pea protein isolates. The authors explained that the emulsification functionality depends on the botanical source, in addition to the protein solubility and conformational stability.

The addition of NaCl was also shown to influence the emulsifying properties of protein isolates. In a work conducted by Mwasaru et al. (2000), the addition of 0.1 and 0.4 M of NaCl contributed positively to increase the emulsifying capacity, due to the better solubility conditions of the proteins.

Roasting pigeon pea seeds at temperatures of 80 and 100 °C was shown to have a negative impact on emulsifying properties, according to Onimawo & Akpojovwo (2006). The authors observed reductions in emulsifying capacity and emulsion stability compared to flour that was not produced from roasted seeds. The decrease in emulsifying properties may be related to the denaturation of proteins at high temperature.

7.5 Gel forming properties

Gelation is a functional property that is linked to the formation of a percolating network that provides a solid-like matrix with the retention of water, lipids, sugars and flavor in foods, in addition to affecting their final consistency. Pigeon pea flour was able to form self-supporting gel (Acevedo et al., 2013), highlighting its potential for application in foods that require a firm gel consistency. Part of this is due to the presence of starch in the flour, which contributes to gel formation.

Acidic pH conditions showed to be more beneficial for the formation of gels, compared to alkaline pHs, as observed by Akintayo et al. (1999) and Mwasaru et al. (Mwasaru, Muhammad, Bakar, & Che Man, 1999). These authors also observed that gelation was improved under conditions of moderate ionic strength, even causing a reduction from 14 to 6% in the least concentration for gel formation.

According to Tapal et al. (2019), the production of protein hydrolysates from pigeon pea can also affect the least concentration for gel formation. The authors determined that too high levels of enzyme (8-10 wt.%) did not result in gel formation. Conversely, the addition of enzyme up to a concentration of 6 wt.% was ideal to obtain firm gels.

Sosa et al. (2023) evaluated the gelling properties of pigeon pea protein isolates extracted by alkaline solubilization (pH 8.0) followed by isoelectric precipitation. The gelling properties of pigeon pea protein isolate were dependent on pH and ionic strength, with gels obtained at a pH close to the isoelectric point of proteins (pH = 3.9) showing a denser network and higher hardness, in texture profile analysis (TPA), and greater water retention capacity, compared to more alkaline pHs. The authors also observed pH-

dependent changes in the color of the gels, varying from light brown at pH = 2.1 to dark brown at pH = 8.3, which is an important aspect for consumer acceptance.

8. Bioactive properties and digestibility

8.1 Bioactive peptides

Bioactive peptides are nutraceutical agents that have health benefits, acting in disease prevention. Reactive or hydrophobic groups of proteins are found within their structure, that is, the sequence of reactive peptides remains inactive within their primary structure. The release of these peptides through enzymatic protein hydrolysis is possible and has shown to be promising for pigeon pea (Olagunju et al., 2018a).

By means of bioinformatics analysis, Boachie et al. (2019) evaluated forty pigeon pea proteins and showed that almost 50% of the amino acids were associated with the inhibition of DPP-4, an enzyme linked with the treatment of diabetes mellitus (type 2). The evaluation using bioinformatics projected the pepsin enzyme as the best protease to trigger the release of bioactive peptides with DPP-4 inhibitory functions. However, *in vitro* assays conducted by the authors showed that the thermolysin enzyme released the most active DPP-4 inhibitors. Differences between the bioinformatics simulation and the *in vitro* assays were attributed by the authors to the nature and behavior of the protein under laboratory conditions (acid pH for pepsin catalysis and the structural conformation of proteins) which are unincorporated parameters to existing bioinformatics tools for enzymatic hydrolysis.

Olagunju et al. (2021) also evaluated the bioactive activity of pigeon pea peptides in controlling postprandial blood glucose levels. The results showed that the hydrolysate and peptide fractions (obtained through membrane ultrafiltration) may have potential as a natural inhibitor of α -glucosidase, the enzyme responsible for hydrolyzing starch and increasing the level of glucose in the bloodstream.

Olagunju et al. (2018b) evaluated the anti-hypertensive effect of pigeon pea protein isolates and protein hydrolysates through an assay with hypertensive rats. The hydrolysates were instantaneous in reducing blood pressure, confirming the effectiveness of the enzymatic hydrolysis process. Pepsin hydrolysate had the fastest action in lowering blood pressure with a maximum lowering effect of -30.91 mmHg after 2 h of oral administration. The authors found similar blood pressure lowering effect reported for mung bean hydrolysate prepared with alcalase and for hemp seed and yellow pea pentapeptides (Aluko et al., 2015; Girgih et al., 2014; Li et al., 2006).

8.2 Digestibility

Food digestibility can be studied using *in vitro* and *in vivo* methods. The *in vitro* strategy consists of subjecting foods to conditions that simulate the digestion process (e.g., treatment with mixed digestive enzymes, pH changes, etc.). The *in vivo* approach allows for understanding the real effects that nutrient digestion has on a living body, usually using rats or pigs. Protein digestibility can provide insights into the stability of

proteins during digestion and how they withstand the digestive process. *In vitro* assays have some advantages over *in vivo* assays, such as greater reproducibility, greater speed and lower cost. However, such methods may provide an overestimated result for the actual nutritional value, as it disregards biologically unavailable amino acids. In recent years, an international group of researchers developed the INFOGEST protocol (<http://www.cost-INFOGEST.eu/>) with the aim of standardizing *in vitro* digestion methods and enabling a better comparison between results (Gomes et al., 2020; Ogawa et al., 2018).

Protein Digestibility Corrected Amino Acid Score (PDCAAS) is an index used to assess the nutritional quality of proteins and more reliably estimate the protein value of foods for human consumption. The maximum value of PDCAAS is 1.0, which means that after the digestion process, one unit of protein provides 100% of the essential amino acids required for human consumption. However, studies have shown that PDCAAS generally underestimates the value of high-quality proteins and overestimates the value of other proteins. In addition, the presence of antinutritional factors may make the PDCAAS measurement inappropriate for predicting plant-based proteins quality (Gomes et al., 2020).

Another method used to assess food digestibility is the Digestible Indispensable Amino Acid Score (DIAAS). Performed in the small intestine (ileum) of pigs, it is shown to be a more adequate and accurate estimate for humans, avoiding the limitations of the PDCAAS procedure (evaluated in rats). However, the applicability of DIAAS for proteins of plant sources is limited by some aspects, such as: low representation of plant-based foods within the scoring structure; failure to translate differences in nitrogen-to-protein conversion factors between plant-based and animal-based foods; focus on isolated nutrients rather than the food matrix; inadequate recognition of increased digestibility of heat-treated and processed plant-based foods (Craddock, 2021).

Some studies were carried out to evaluate pigeon pea digestibility, mainly using *in vitro* methods or assays conducted in rats. Although the findings of these works may have inherent limitations of the used methods, they provide an idea of how pretreatments can affect pigeon pea digestibility. In a study conducted by Sousa et al. (2020) on the *in vitro* digestion of some pulse flours and isolated proteins, quantification of the amount of free amino acid groups after digestion showed that pigeon pea fractions were the evaluated ingredients that released the greatest amounts of equivalent glutamic acid, compared to sorghum, peanuts, black beans and wheat fractions. Sun et al. (2020) evaluated the digestibility of pigeon pea flours submitted to different treatments. Gastrointestinal digestion was conducted by submitting the flour samples to simulated digestive fluids containing electrolytes, enzymes (α -amylase, pepsin and pancreatin), CaCl_2 , and water. The flour processed by microwaves showed greater digestibility (71.6%) when compared to the control flour (54.4%). According to the authors, the loss of secondary protein structures (due to microwave treatment) may explain the improvement in digestibility.

Tapal et al. (2019) determined the digestibility of pigeon pea protein isolates using pepsin and pancreatin enzymes. The degree of hydrolysis achieved in the *in vitro* digestibility assay was about 50%. It was observed that the method used had an impact mainly on the degradation of proteins with molecular mass between 45 and 66 kDa. The authors highlighted that the peptides produced by hydrolysis have bioactive potential against oxidative stress and hypertension, according to bioinformatics analysis using the BIOPEP database.

Sharma et al. (2019) determined pigeon pea digestibility using α -amylase (for starch digestion), and pepsin and pancreatin (for protein digestion). Both starch and protein digestion were improved by seed germination. Germination at 35 °C for 48 h resulted in a starch digestibility of 39.1% and a protein digestibility of about 98.3%, while non-germinated seeds had a result close to 20 and 70%, respectively.

Membrane filtration improved the digestibility of peptide fractions extracted from pigeon pea, which ranged from 90.6 to 93.6% (Olagunju et al., 2021). Size uniformity of the peptides, made possible by filtration, can contribute to better action of the proteases in relation to the hydrolysate that did not undergo ultrafiltration and had peptides of various sizes.

Adenekan et al. (2018) evaluated the *in vivo* digestibility of pigeon pea protein isolates using rats. The authors observed values of nitrogen balance, biological value, net protein utilization, true protein digestibility and protein efficiency ratio equal to 0.66 g, 90.0%, 93.0%, 93.1 and 1.4, respectively.

8.3 Antioxidant activity

In a study conducted by Tekale et al. (2016), 37 compounds including some short peptides were identified in *Cajanus Cajan* seeds. According to the authors, pigeon pea is a source of important bioactives that contribute to its antioxidant and iron chelating activity.

Yang et al. (2020) determined IC₅₀ (antioxidant concentration to achieve 50% inhibition) values of 2536 and 1250 $\mu\text{g mL}^{-1}$ for aqueous extracts of pigeon pea seeds, by the DPPH (2,2-diphenyl-1-picrylhydrazyl) and NO radical elimination methods, respectively. The authors determined a positive correlation between the levels of phenolic and flavonoid compounds and the values found in the antioxidant activity assays.

Sharma et al. (2019) reported that germination increased the antioxidant capacity of pigeon pea. Antioxidant activity was determined by the DPPH, metal chelating activity and reducing power methods, with values of 47.9, 87.7 and 197.3% for seeds germinated at 35 °C for 48 h, while non-germinated seeds presented values of 21.6, 42.0 and 98.3%, respectively.

Xu et al. (2021) evaluated the antioxidant activity of pigeon pea protein isolate and hydrolysates. The protein isolate showed greater antioxidant activity (evaluated by the DPPH and NO radical scavenging methods) than the hydrolysates. Nevertheless, a

different result was observed in a study conducted by Tapal et al. (2019), in which the protein hydrolysate showed better antioxidant capacity by the DPPH method than the protein isolate. Similar behavior was observed for the reducing power assay. According to the authors, the antioxidant activity of hydrolysates depends on the composition of peptides generated after hydrolysis. The antioxidant activity of protein hydrolysates was also superior to that of protein isolates in a study conducted by Olagunju et al. (2020), using the DPPH, ABTS, FRAP and hydroxyl radical scavenging methods.

Olagunju et al. (2018b) evaluated the antioxidant activity of pigeon pea hydrolysates and peptides separated by ultrafiltration through different methods, such as DPPH, ABTS, FRAP, superoxide radical scavenging activity, ORAC and inhibition of linoleic acid oxidation. In general, low-molecular mass peptides, especially the <1 kDa fraction, exhibited superior antioxidant properties when compared to higher molecular weight fractions. The hydrolysates produced from the enzyme alcalase and pancreatin hydrolysates were better than the hydrolysates produced by the combination of pepsin and pancreatin.

9. Application of pigeon pea in new food products

There is a wide range of applications for plant-based proteins in the development of food products, including use in food supplementation, emulsifying, foaming, and gelling agents, hydrogels for pharmaceutical applications and use in edible coatings (Kumar et al., 2022). Some functional properties have already been investigated for pigeon pea flours and protein extracts (as presented in the previous section). Yet, the use of pigeon pea seeds and their protein-rich ingredients in new formulations with sensory acceptance assessment is still little or non-existent in the literature.

Torres et al. (2007) produced flours from germinated pigeon pea seeds (4 days/20 °C) and investigated their application in pasta formulations. A formulation of 100 % semolina was used as control and formulations added with 5, 8 and 10 % (w/w) of germinated pigeon pea flour were evaluated for their nutritional composition, cooking time and sensory acceptance. The protein content of the germinated flour was 29 %. Pigeon pea flour supplementation resulted in shorter cooking times (20 – 30 % shorter than the formulation with 100 % semolina) and better protein, fat, dietary fiber and mineral content. No difference was observed by consumers in the overall acceptance of pastas produced only with semolina and pastas produced with pigeon pea flour supplementation.

A protein binder (protein content = 32 %, w/w) from pigeon pea was used in the formulation of beef sausages by Mongi & Gomezulu (2022), with concentrations ranging from 0 to 6 % (weight of protein binder/weight of beef meat). The descriptive analysis showed that the 6 % concentration led to greater acceptance compared to the other concentrations tested, showing that the increase in the amount of pigeon pea proteins in the formulations increased the sensory profile and consumer acceptance. However, control sausages made with chemical phosphate binder were the most

accepted by consumers. Studies that also use protein binders from other plant sources as controls are encouraged to better understand the performance of pigeon pea proteins in this type of application.

The application of pigeon pea protein hydrolysate in a maize-based snack was investigated by Akoja et al. (2017). The hydrolysate was produced with the addition of the enzyme papain (2 %, w/w), but the protein content of the hydrolysate was not reported by the authors. Maize flour was added with pigeon pea protein hydrolysate at concentrations of 5, 10, 15 and 20 % to produce snacks, with the 5 % concentration being the one that showed the best overall acceptance by consumers, together with the control formulation (without addition of protein hydrolysate).

A study conducted by Anchang & Okafor (2022) evaluated the effect of different formulations of breakfast cereals using mango, pigeon pea and sorghum flour on the composition of antinutritional factors. Twenty-eight samples were evaluated from the combined mixture-process linear multiplication model used in the study. The authors evaluated the levels of some antinutritional compounds such as lectins, phytates and tannins. Increasing the concentration of pigeon pea flour in breakfast cereals resulted in an increase in the lectin content. Increasing the concentrations of pigeon pea flour and sorghum flour was also accompanied by an increase in the tannin content. The protein content of the formulations was not determined by the authors.

It is noted that only flours or ingredients slightly more concentrated in proteins were used in new product formulations. The use of pigeon pea protein concentrates and isolates in product development is scarce and should be investigated as it may allow for better performance, when a certain amount of protein is required, in addition to the fractionation process potentially removing compounds that have negative effects on consumer acceptance, such as tannins, for example. In general, pigeon pea applications appear promising for the development of cereal-based products and meat-like products. However, the possible advantages of using pigeon pea ingredients over other plant sources remain to be clarified.

10. Perspectives for future work

In recent years, there has been a growing interest in the exploration of pigeon pea proteins, mainly to produce concentrates, isolates, and hydrolysates. The works raised in this review showed promising results, using mostly isoelectric precipitation extraction methods to obtain protein extracts from pigeon pea that can be used as food ingredients. The studies carried out range from improvements in the processing of pigeon pea seeds, to the investigation of their technological properties to produce functional foods. It is possible to obtain protein extracts of pigeon pea in a sustainable way, using simple methods that do not use organic solvents in the extraction. The investigation of its proteins is a way of promoting biodiversity and boosting the expansion of this pulse cultivation in future years.

Some gaps were observed in the evaluated literature that are relevant to guide future studies on pigeon pea:

(I) Minor compounds (phenolic compounds, flavonoids, antinutritional compounds, etc.) must be identified and quantified into more depth (using, e.g., chromatographic techniques), and their implications for the nutritional and functional value of pigeon pea ingredients should also be evaluated;

(II) The protein composition of pigeon pea should be characterized into detail. Protein purification has been little explored in the literature, yet is important for a better understanding of how each protein fraction contributes to the overall functionality of pigeon pea protein ingredients. Such studies are still relevant to assist in the selection of cultivars with the desired protein profile;

(III) Alternative protein extraction methods must be evaluated, mainly aiming at better process yield and protein recovery. Studies that use processes assisted by emerging technologies and dry fractionation of proteins, in particular, are encouraged;

(IV) The rheological behavior and interfacial properties of pigeon pea protein should be further investigated to provide mechanical underpinning of certain functional properties (emulsifying, gelling), and support for the development of new products;

(V) The digestibility of pigeon pea proteins has been little evaluated and needs to be further clarified. Some studies have investigated the digestibility of protein isolates, concentrates or hydrolysates, but there is no knowledge of the digestibility of these proteins in more complex food systems. The way in which pigeon pea proteins associate with other components, such as carbohydrates and lipids, must also be clarified;

(VI) Sensory analyses must be conducted to evaluate the acceptance of seeds and products based on pigeon pea flour and protein extracts. Comparison with foods based on animal proteins or vegetable proteins most used currently (soy, peas, etc.) is of potential interest.

Clarifying these points should be the focus of future studies on pigeon pea proteins to better understand their functional properties, improve extraction yield, assess nutritional value, and enhance consumer acceptance. These efforts aim to harness pigeon pea proteins as sustainable and nutritious alternatives to address global protein demand.

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Chapter 2. Objectives, experimental approach and methods of the study

Part I. Objectives and experimental approach

. Several studies in the literature have investigated the effects of pre-treatments on seeds to improve the nutritional quality of pigeon pea seeds, aiming at better digestibility (SHARMA et al., 2019; ADENEKAN et al., 2018). In parallel, protein concentrates and isolates have been obtained from its seeds, using the conventional aqueous fractionation route with protein precipitation step (SUN et al., 2020; SOSA et al., 2021). The main advances regarding the processing of pigeon pea seeds were elucidated in Chapter 1, as well as gaps that still need to be better investigated, as its protein composition.

The general objective of this thesis is to obtain, characterize and evaluate the functional properties of different protein fractions from pigeon pea. To achieve this, the study was divided into three parts:

- The first part consists of evaluating the effect of seed pre-treatments, soaking and boiling, on the functional properties of pigeon pea protein concentrates obtained from pre-treated seeds (Chapter 3). An extensive characterization of the composition of proteins, carbohydrates and lipids present in the protein fractions was performed. The reduction of antinutritional compounds was investigated by determining the trypsin inhibitors activity, raffinose family oligosaccharides and total soluble phenolic compounds. The techno-functional properties (emulsifying, foaming, gelling) of protein concentrates were discussed considering the changes observed in the ingredients composition;
- The second part consists of the development and implementation of a protocol for preparative-scale purification of 7S globulin from pigeon pea (Chapter 4). The procedure for protein purification was optimized on a laboratory scale and expanded to a preparative scale, with stages of protein extract desalting and separation by anion exchange and size exclusion chromatography. The physicochemical properties of the purified protein fractions were evaluated;
- The third part consists of the evaluation of air-water interfacial and foaming properties of pigeon pea protein fractions (Chapter 5). In this section, the 7S globulin purified in Chapter 4 was used as a reference. Crude protein extracts were obtained from two types of pigeon pea seeds. The protein fractions obtained had their foaming properties compared to proteins of animal origin such as whey protein isolate, bovine serum albumin and lysozyme.

Part II. Source of raw material and production of the pigeon pea protein fractions

The following section presents experimental details about the raw material used and the methods for producing the different protein fractions investigated in this thesis. In this section, only general explanations will be presented about the methodologies, with the aim of providing a more comprehensive view regarding the different protein extraction processes that were used. For more information, Chapters 3, 4 and 5 present the complete methods used for each part of the research.

1. Source of raw materials

Two types of pigeon pea seeds were used: commercial seeds (cv. unknown) obtained from a local supplier in São Paulo (Brazil); and pure line seeds (cv. g18-95) donated by the Brazilian Agricultural Research Corporation (Embrapa). Due to the higher availability of commercial seeds, it was used in the studies in Chapters 3 and 4. To evaluate the air-water interfacial properties and foaming properties, in Chapter 5, both types of seeds were used.

2. Pre-treatment of pigeon pea seeds and production of flour

For the studies conducted in Chapter 3, commercial seeds were subjected to soaking and soaking+boiling pre-treatments. In the soaking pre-treatment, the seeds were soaked in distilled water (1:10) for 24 h at room temperature. After pre-treatment, the water was removed, and the seeds were dried in an oven at 60°C overnight. In the boiling pre-treatment, soaked seeds were added and kept in boiling water (1:5) for 30 min. After boiling, the cooking water was discarded, and the seeds were dried in an oven at 60° overnight. Raw seeds (without pre-treatment) were also placed in an oven at 60°C overnight. After drying, the seeds were milled in a disk mill at 1730 rpm for 5 min to produce flour. Flours produced with raw, soaked and soaked-boiled seeds were called FC, FS and FB. Flour from pure line seeds (FE) was obtained in the same way, without any pre-treatment step.

3. Production of pigeon pea protein fractions

Different production methods were used in this thesis to obtain protein fractions from pigeon pea. In Chapter 3, the conventional method of protein extraction by alkaline solubilization followed by isoelectric precipitation was used. While for the studies carried out in Chapters 4 and 5, a mild protein extraction process at pH 8.0 was employed, followed by extract desalting. Purified fractions of 7S globulin were obtained by liquid chromatography techniques. The following sections briefly describe each method used.

3.1 Precipitation-based extraction

In this method, FC, FS and FB flours were used to produce pigeon pea protein concentrates. First, the flours were dispersed in distilled water (1:10) and the pH was

adjusted to 12.0. The suspension was stirred for 1h and centrifuged to collect the protein-rich supernatant. The supernatant had its pH adjusted to 4.5, to promote protein precipitation. A new centrifugation was performed to collect the precipitated proteins. The precipitated proteins were dispersed in distilled water and the pH was adjusted to 7.0. The protein concentrates were freeze-dried for 72h and the powders were recovered. Pigeon pea protein concentrates obtained from FC, FS and FB flours were named PPC-C, PPC-S and PPC-B.

3.2 Mild protein extraction

In this method, flour from commercial and pure line seeds were used. For protein extraction, 200 g of each flour were dispersed in 1 L of 50 mM Tris buffer solution pH 8.0. The suspension was stirred for 1 h in a mechanical stirrer at 225 rpm. After stirring, the suspension was centrifuged and the supernatant was collected. Another 1 L of 50 mM pH 8.0 Tris buffer solution was added to the pellet followed by agitation and centrifugation with collection of the supernatant. The two supernatants were pooled, centrifuged again in the same conditions, and filtered to remove small particles. To remove the salts and small molecules like polyphenols, the extracts were injected into a desalting column. The column was equilibrated with pH 8.0 sodium carbonate buffer. The injection was conducted at a flow rate of 50 mL/min. The desalted extracts were frozen and freeze-dried for 72 h. The crude extract from commercial seeds is referred to as CPE-C and the crude extract from pure line seeds as CPE-E.

3.3 Purified fractions of pigeon pea 7S globulin

In this method, only commercial seeds were used to obtain purified protein fractions. The proteins were previously extracted using the same procedure described in item 3.2. After obtaining the desalted extract, it was separated by anion exchange chromatography. The desalted extract was injected in column filled with Q-Sepharose Fast Flow resin at a flow rate of 13 mL/min. After separation in the anion exchange column, purification was conducted following two distinct routes: (i) process with dialysis and (ii) process without dialysis. In the process with dialysis, protein fractions were subjected to dialysis against ammonium carbonate buffer pH 8.0 (1 g/L) at 4 °C with four changes of buffer every 3 h. After dialysis, the samples were freeze-dried for 72 h, and the powders were recovered. The purified fractions were solubilized in 0.1 M phosphate buffer pH 7.0 and injected in a column filled with Cellufine GCL 2000 gel filtration resin equilibrated with 0.1 M phosphate buffer pH 7.0. Fractions were collected, pooled and subjected to dialysis and freeze-drying as previously described. In the process without dialysis, protein fractions were injected directly onto the Cellufine GCL 2000 gel filtration column equilibrated with ammonium carbonate buffer pH 8.0. Fractions were collected, pooled and freeze-dried. The purified fraction of 7S globulin obtained by the first process was recovered in its native state, being called N-7S. On the other hand, the purified fraction of 7S globulin obtained by the second process was recovered in a partially hydrolyzed state, being called H-7S.

4. Sample coding

To summarize the nomenclature used for the samples evaluated, Table 2.1 presents the codes with descriptions for each flour and protein fraction produced in this thesis.

Table 2.1 Coding of the evaluated samples.

Sample	Code
Flour produced from raw commercial seeds (cv. unknown)	FC
Flour produced from pure line seeds (cv. g18-95)	FE
Flour produced from soaked commercial seeds	FS
Flour produced from soaked-boiled commercial seeds	FB
Protein concentrate produced with FC using a precipitation-based extraction	PPC-C
Protein concentrate produced with FC using a precipitation-based extraction	PPC-S
Protein concentrate produced with FC using a precipitation-based extraction	PPC-B
Crude protein extraction with FC using a mild protein extraction	CPE-C
Crude protein extraction with FE using a mild protein extraction	CPE-E
Native 7S globulin from pigeon pea	7S
Partially hydrolyzed 7S globulin from pigeon pea	H-7S

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Chapter 3. Pre-treatment effects on the composition and functionalities of pigeon pea seed ingredients

Preamble

Soaking and boiling can improve the digestibility of seeds by reducing anti-nutritional factors and the hard-to-cook phenomenon. The literature review on pigeon pea showed that some studies have evaluated the effect of soaking and boiling pre-treatments on the functional properties of the resulting flours. However, there is a scarcity of studies that have evaluated the use of pre-treated seeds to produce protein concentrates or isolates. In addition to this, there is also a lack of a more in-depth characterization of the composition of these ingredients, in terms not only of proteins, but also of carbohydrates and lipids, that are potentially impacted by these pre-treatments. In this section, the production of pigeon pea protein concentrates using soaked and soaked-boiled seeds and its impacts on composition and functionality of pigeon pea based-ingredients are investigated. This study brings new insights into the effects of soaking and boiling-based pre-treatments on the composition of pulse protein ingredients, and in turn, on their techno-functionalities as exemplified with their emulsifying, foaming and gelling properties.

Main questions

- What are the impacts of soaking and boiling pre-treatments on the composition of pigeon pea flour and protein concentrates?
- How are antinutritional compounds in pigeon pea affected by pretreatments?
- How are the functional properties of protein concentrates affected by seeds pre-treatments?

An extensive characterization of the composition of macronutrients (proteins, lipids and carbohydrates) and minor compounds (trypsin inhibitors, total soluble phenolic compounds) was carried out. Emulsifying, foaming and gelling properties of pigeon pea protein concentrates obtained from pretreated seeds were investigated.

Main results

- Mannose residuals in protein concentrates suggests the presence of glycosylated proteins;
- Phospholipids were removed during fractionation process using soaked-boiled seeds;
- Trypsin inhibitors activity decreased by 95% in the protein concentrate of soaked-boiled seeds;
- Boiling increased emulsion stability, water holding capacity, and least gelation concentration of the protein concentrates.

The content of this chapter is under review in the journal Food Hydrocolloids.

Pre-treatment effects on the composition and functionalities of pigeon pea seed ingredients

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1.Introduction

Protein transition is a topic widely discussed by government organizations and researchers. Partial replacement of animal-based proteins by plant-based proteins in the diets is among the initiatives to reduce the ecological footprint that seek to ensure greater sustainability for the planet, in addition to achieving food and nutrition security. This transition can be driven by diversifying the protein sources with the inclusion of pulses that are still underused in human nutrition (Bühler, Dekkers, Bruins, & Van Der Goot, 2020; Diedericks, Venema, Mubaiwa, Jideani, & Van der Linden, 2020). Pigeon pea [*Cajanus cajan* (L.) Millsp.] is a non-conventional crop native to regions of Asia and Africa. This pulse has a high protein content (20-28%), and is rich in minerals such as iron and calcium, and in B-complex vitamins. The easy adaptation to warmer and drier environments makes pigeon pea a resilient pulse, which is of great interest for strategies to overcome climate change effects (Locali-Pereira et al., 2023; Sahni, Sharma, & Surasani, 2020; Devindra, Sreenivasa, Bhaskar, & Mulimani, 2011).

Pigeon pea production is currently restricted to some countries in Asia and Africa due to a lack of knowledge about its nutritional and technological potential and relevant aspects to make large-scale production around the world possible. The low digestibility of

proteins, presence of antinutritional factors, such as trypsin inhibitors, and the hard-to-cook phenomenon are some aspects that can hamper the consumption of this pulse (Carter & Manthey, 2019). Processing can enhance the use of pulses by reducing the levels of antinutritional factors and by modifying the structures of starch granules and protein bodies, which in turn will modulate their functional properties (Sun, Ohanenye, Ahmed, & Udenigwe, 2020; Sahni et al., 2020).

Soaking is a non-thermal treatment that consists of hydrating the seeds; it may be used to reduce the cooking time of pulses and to assist in the removal of hydrophilic antinutritional compounds including tannins, trypsin inhibitors, and raffinose oligosaccharides, that migrate to the aqueous medium by leaching (Carter & Manthey, 2019; Girigowda et al., 2005; Yasmin et al., 2008). Thermal treatments such as boiling are used to eliminate thermolabile antinutrients such as chymotrypsin inhibitors, and improve seed digestibility and softness (Onwuka, 2006; Hernández-Infante et al., 1998; Patterson et al., 2017; Wang et al., 2021), in addition to their obvious effect of mitigating microbial contamination issues. (Alfaro-Díaz et al., 2021).

Soaking in combination with boiling has already proved to effectively eliminate antinutritional factors and improve pigeon pea digestibility. For instance, soaking pigeon pea seeds for 12 h followed by boiling for 40 min was sufficient to reduce the levels of tannins, trypsin inhibitors, hemagglutinin and hydrogen cyanide by 80% (Onwuka, 2006). The combination of both treatments was also efficient at removing phytates and compounds responsible for flatulence (Devindra et al., 2011; Igbedioh, Olugbemi, & Akpapunam, 1994).

In spite of having a positive impact on the nutritional quality of pigeon pea, the effects of these pre-treatments on the molecular properties of pigeon pea proteins, on the processability of the flours, and on the functionality of the resulting ingredients still need to be clarified. In particular, proteins are responsible for several technological properties of pulse-based ingredients (e.g., emulsifying, foaming, gelling) and their conformation can be partially or fully altered by heat treatments (Espinosa, Guevara-Oquendo, Newkirk, & Yu, 2020). The consequences of these pre-treatments on the other pigeon pea macromolecules (lipids, carbohydrates) have received little attention so far, yet is an important factor when considering ingredient functionality.

The aim of this work was therefore to evaluate the impact of soaking and soaking + boiling pre-treatments on the composition, protein conformation and techno-functional properties of pigeon pea protein ingredients.

2. Materials and methods

2.1 Materials

Pigeon pea seeds were purchased from a local supplier (São Paulo, Brazil). Soybean oil was supplied by Cargill (Minas Gerais, Brazil). Sodium hydroxide, hydrochloric acid, sodium phosphate, bovine serum albumin (BSA), trypsin from bovine pancreas, trypsin inhibitor from *Glycine max*, N- α -benzoyl-DL-arginine-p-nitroanilide (BAPNA), and gallic acid standard were purchased from Sigma-Aldrich®. Folin-Ciocalteu reagent was provided by Êxodo (São Paulo, Brazil). Sodium carbonate was purchased from Synth (Sao Paulo, Brazil). Protein assay dye reagent and Coomassie blue were purchased from Bio-Rad (California, USA). Tris-glycine SDS running buffer was purchased from ThermoFisher (Massachusetts, USA). Chloroform, methanol, and n-hexane were purchased from Biosolve Chemicals (Dieuze, France). Standards of α , β , γ and δ -tocopherol were purchased from Calbiochem and the γ -tocotrienol standard was purchased from Cayman Chemical (Ann Arbor, USA). Sodium sulfate anhydrous and acetic acid were purchased from Carlo Erba Reagents (Val de Reuil, France). Sodium chloride was purchased from VWR International (Radnor, USA). Neutral lipid standards DG 1.2 (CAS number 2442-61-7), DG 1.3 (CAS number 2465-32-9), C18:1 (CAS number 112-80-1) and TG 18:1 (CAS number 122-32-7) were purchased from Sigma Aldrich (St Louis, USA) and MG 18:3 standard was purchased from Nu-Check Prep (Minnesota, USA). The phospholipid standards phosphatidylglycerol (CAS number 383907-64-0), phosphatidylethanolamine (CAS number 97281-40-8), phosphatidylinositol (CAS number 383907-36-6), phosphatidylserine (CAS number 383908-63-2), phosphatidic acid (CAS number 475995-54-1), phosphatidylcholine (CAS number 97281-44-2), sphingomyelin (CAS number 475662-40-9), and lysophosphatidylcholine (CAS number 97281-38-4) were purchased from Avanti (London, UK).

2.2 Seed pre-treatments and flour production

Pigeon pea seeds were manually checked to remove damaged seeds and then subjected to two different physical pre-treatments: soaking, and soaking + boiling. Soaking was performed in distilled water (1:5, w v⁻¹) for 24 h at 25 °C. The soaking liquid was discarded, and part of the soaked seeds was then boiled in distilled water (1:3, w v⁻¹) for 30 min, leading to soaked + boiled seeds. A control consisting of raw seeds (without pre-treatments) was also evaluated. All seeds, including the control, were dried in an oven at 60 °C for approximately 12 h. After drying, the seeds were ground in a disk mill (J.R. Araújo & Cia Ltda., Brazil) at 1730 rpm to produce the flours. Flours produced with raw, soaked-, and soaked-boiled seeds were named FC, FS, and FB, respectively.

The size distribution of the flours was evaluated in a laser diffraction analyzer (Mastersizer 3000, Malvern) in the dry state. It was also assessed using a QICPIC image analyzer (Sympatec, USA) dispersing the flour (50 mg) in 0.5%-ethanol in water solution (1 L), in which the particle-shape images were recorded as well. The flours' morphology

was analyzed in a JEOL JSM-7500 F scanning electron microscope (JEOL Ltd., Japan) at 400× magnification by fixing small amounts of powders in SEM stubs and gold coating under vacuum before analysis.

2.3 Protein extraction

Pigeon pea flour was dispersed in distilled water (1:10, w v⁻¹) and the pH was adjusted to 12.0 (using 3 M NaOH) to promote protein solubilization, following the method used by Sun et al. (2020), with modifications. The dispersion was mechanically stirred (MA261, Marconi, Brazil) (1 h / 750 rpm), and then centrifuged (10,000 g, 30 min, 4 °C). The supernatant was collected, and its pH adjusted to 4.5 (using a 3 M HCl) to induce protein precipitation. A new centrifugation was performed (10,000 g, 30 min, 4 °C) and the protein-rich precipitate was collected and redispersed in distilled water, being neutralized to pH 7.0. The pigeon pea protein concentrates (PPC) were frozen, and finally freeze-dried for 72 h. After freeze-drying, the powdered extracts were stored in desiccator containing K₂CO₃ saturated solution (RH = 43.2%) until analysis. The PPC produced with raw, soaked- and soaked-boiled seed flours were named PPC-C, PPC-S and PPC-B, respectively. The macroscopic aspect of the pre-treated seeds, flours, and protein extracts is shown in Supplementary Figure 1.

2.4 Composition of flours and protein concentrates

2.4.1 Cell wall polysaccharide composition, raffinose family oligosaccharides, and starch content

The total amount of cell wall polysaccharides and their sugar composition were measured by chromatographic and colorimetric methods as previously described, with minor modifications (Le Gall, Even, & Lahaye, 2016). Briefly, flours and protein concentrates were subjected to hot ethanolic extraction using an automated solvent extractor (ASETM350, Thermo ScientificTM DionexTM; about 500 and 200 mg respectively per cell, at 100 °C with 80% ethanol 2 mL.min⁻¹ flow mode during 15 min). The insoluble alcoholic fractions were hydrolyzed with sulfuric acid to obtain a mixture of neutral and uronic sugars. The resulting neutral sugars were converted into alditol acetates prior to GC analysis (TraceGOLDTM TG-225MS GC Column (30 × 0.32 mm ID); TRACE GC Ultra Thermo ScientificTM; at 205 °C using H₂ as the carrier gas). Sugar solutions were used as external standards and inositol solution was used as internal standard for all measurements. The resulting uronic acids were quantified by meta-hydroxydiphenyl (MHDP) colorimetric method. The sugars arising from the cell walls through this procedure are referred to as non-starch polysaccharides (NSP).

For the determination of raffinose family oligosaccharides (RFOs), approximately 15 mg of flour or protein concentrate were mixed in absolute ethanol containing an internal standard (IS, melezitiose), incubated (15 min at 75 °C), and evaporated under dry air at 40 °C to obtain RFO extract (Gangola et al., 2014). Then, the

resulting pellet was redispersed in ultrapure water, vortexed, and centrifuged to collect the supernatant. After filtration, the samples were injected into an HPAEC-PAD (ICS-6000 Thermo Scientific™, column PA1, flow rate 0.25 mL/min, 30 °C, elution gradient with 50 to 500 mM NaOH for 40 min). For quantification, external standards of saccharose, raffinose, stachyose and verbascose, mixed in solution with melezitiose (IS) were used.

Total starch content was determined after sample amylolysis using two commercial enzymes (α -amylase from *Bacillus licheniformis* and amyloglucosidase from *Aspergillus niger*; E-BLAAM and E-AMGDF, Megazyme) followed by HPAEC-PAD analyzes (ICS-6000 Thermo Scientific™) (Le Gall, Even, & Lahaye, 2016).

The total carbohydrate content was expressed by the sum of starch, non-starch polysaccharides (NSP), raffinose family oligosaccharides (RFO), and saccharose contents.

2.4.2 Total lipids, neutral lipids, phospholipids, and tocopherol content: extraction, identification, and quantification

Total lipids were extracted using a method described by Bligh & Dyer (1959) and Folch, Lees, & Sloane Stanley (1957), with adaptations. First, 1 g of sample was weighed and hydrated in 6 mL of 50 mM pH 8.0 phosphate buffer overnight at 4 °C under stirring. Then, 50 mL of chloroform/methanol solution (2:1 v v⁻¹) were added. The mixture was stirred and filtrated under vacuum to recover the liquid phase. The powder was then recovered and the procedure was repeated twice. The filtrate was transferred into a separating funnel and 35 mL of NaCl solution (0.73% w/v) were added, mixed, and let to decant overnight at 4 °C. The lower phase was recovered after filtration on anhydrous sodium sulfate and glass wool, and the solvent was evaporated under vacuum (R-100, Rotavapor, Büchi, France), and then under nitrogen (N-Evap 111, Organomation, USA). After evaporation, the round flasks containing the lipid extract were weighed, and the total lipid content was calculated based on the mass recovered. The lipid extract was then collected in chloroform and stored in an amber flask at -80 °C until further analyses.

For quantification and identification of neutral lipids and phospholipids, a U-HPLC (Ultimate 3000 RSLC, Thermo Scientific™, France) equipped with an evaporative light scattering detector (ELSD, Sedex 85) was used (Keuleyan et al., 2023); prior to injection, the total lipid concentration was adjusted to 0.5 and 2.0 mg of lipids/mL of solvent, respectively. The amount of each compound was calculated using a calibration curve made with commercial standards of DG 1.2, 18:1, DG 1.3, 18:1, C18:1, TG 18:1 and MG 18:3, for neutral lipids; and phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidic acid, phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine, for phospholipids.

To investigate the tocopherol content and composition, aliquots of dried lipid extracts were solubilized in hexane to reach a final concentration of 10 mg of lipids/mL and injected in U-HPLC (Ultimate 3000 RSLC, Thermo Scientific™, France) equipped with a RS-fluorescence detector (Thermo Scientific™, France) (Keuleyan et al., 2023). The

excitation and emission wavelengths of the fluorescence detector were 295 and 330 nm, respectively. An external calibration curve of α , β , γ and δ -tocopherol was used.

2.4.3 Protein content: quantification and identification

The total nitrogen content of the flours and protein concentrates was determined by the Dumas combustion analysis method (Elementar, Langensfeld, Germany) using a nitrogen-to-protein conversion factor of 5.7 (Alamanou & Doxastakis, 1997).

Protein identification was carried out by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing and reducing conditions with 2- β -mercaptoethanol (5%, v v⁻¹), following the method described by Lawrence & Besir (2009). Samples were dispersed in Laemmli buffer (2 mg of protein/mL buffer) and heated at 95 °C for 5 min. Then, 10 μ L aliquots of the protein dispersions and molecular weight marker standard were deposited onto gradient polyacrylamide gels (4-12%, Bolt™ Bis-Tris Plus, ThermoFisher), and subjected to a 100 V-current, immersed in Tris-glycine SDS running buffer (Novex™) with constant voltage of 150 V. After the run, the gel was placed in a sensitization solution (2% phosphoric acid, 50% ethanol, v v⁻¹) for 1 h under agitation. Then, the gel was rinsed (2% phosphoric acid solution, v v⁻¹) for 40 min under agitation, transferred to a sensitization solution (17% ethanol, 2% orthophosphoric acid and 15% ammonium sulfate, v v⁻¹), and stained with Coomassie Brilliant Blue after 20 min. After complete staining, the gel was rinsed with distilled water and scanned.

2.4.4 Trypsin inhibitor activity (TIA)

The TIA of flour and protein concentrates was determined using the AOCS method Ba 12a-2020, which determines the presence of tryptic inhibitors by a colorimetric reaction in the presence of trypsin and a substrate (N α -benzoyl-DL-arginine 4-nitroanilide hydrochloride, BAPNA) and which was optimized and described by Liu et al. (2021) with slight modifications. Inhibitors were extracted from 300 mg of sample in 15 mL of 10 mM NaOH, sonicated for 3 min, stirred for 3 h at 600 rpm and finally centrifuged at 20 000 g for 5 min at room temperature. Supernatants were diluted with distilled water so that 60 μ L caused 30-70 % trypsin inhibition (linearity range). Extractions were performed in triplicates. The TIA assay was conducted in 96-well microplates. First, 60 μ L of a dilute extract was mixing with 150 μ L of BAPNA (0.4 mg mL⁻¹) prewarmed at 37°C. Then, 60 μ L trypsin solution (20 μ g mL⁻¹) was added to start the colorimetric reaction. The microplate was immediately place in an oven at 37°C. Exactly 10 min later, 30 μ L 30% (v v⁻¹) acid acetic were added to stop the reaction. A blank sample of each extract was prepared by adding acetic acid before trypsin solution to prevent the reaction. Concurrently, the reaction was run in the absence of inhibitors using the same procedure but by replacing the sample extract with an equal amount of water. Absorbance was measured at 410 nm using a microplate reader (Varioskan Lux, Thermo Fisher, USA). The ΔA_{410} , percentage of trypsin inhibition (%TIn) and TIA calculations were performed using equations 1, 2 and 3 below (Liu, 2021):

$$\Delta A_{410} = (A_{410R} - A_{410RB}) - (A_{410S} - A_{410SB}) \quad (1)$$

$$\%TIn = [\Delta A_{410} / (A_{410R} - A_{410RB})] \times 100 \quad (2)$$

$$TIA = (\%TIn \times \text{the amount of trypsin } (\mu\text{g})) / \text{mg of sample (or mg of protein)} \quad (3)$$

where ΔA_{410} is the absorbance at 410 nm corrected for the difference between the absence and presence of sample extract, A_{410R} is the reference reading, A_{410RB} is the reference blank, A_{410S} is the sample reading, A_{410SB} is the sample blank, %TIn is the percentage of trypsin inhibition, and TIA is the trypsin inhibitor activity.

2.4.5 Total soluble phenolic compounds (TSPC)

The content in TSPC was determined by the Folin-Ciocalteu method, according to Mba, Kwofie, & Ngadi (2019), with modifications. About 1 g of sample was diluted in 20 mL of 70% methanol solution (v v⁻¹), stirred for 1 h, and centrifuged (4000 g, 10 min). After centrifugation, 400-μL aliquots of the supernatant were collected and diluted in 600 μL of distilled water. Then, 1 mL of Folin-Ciocalteu reagent, previously diluted in distilled water (1:4, v:v), 2 mL of 20% Na₂CO₃ solution (w/v), and 1 mL of distilled water were added. The mixture was kept in the dark for 30 min for complete reaction and the absorbance was read in a spectrophotometer at 750 nm. Gallic acid was used to construct the standard curve (0 to 50 μg.mL⁻¹) and TSPC was expressed as mg of gallic acid equivalent (GAE) g⁻¹.

2.4.6 Moisture and ash contents

Moisture and ash contents were determined gravimetrically by drying in an oven at 105 °C until constant weight, and by incinerating in a muffle oven at 550 °C, respectively (AOAC, 1990).

2.5. Characterization of endogenous fluorophores

The occurrence, fluorescence patterns and intensity of the fluorophores endogenously present in the flours and protein concentrates were evaluated by conducting three-dimensional fluorescence measurements (excitation-emission fluorescence matrices, 3D-EEFM) using a spectrofluorimeter (FP-8550, Jasco, Japan). Fluorescence measurements were performed at excitation wavelengths range from 240 to 480 nm, and emission wavelengths range from 280 to 520 nm. A constant slit of 1 nm and a bandwidth of 2.5 nm were defined for both excitation and emission. The samples were evaluated in their solid (powders) and liquid (dispersions) states, to assess the influence of a solvent on the behavior of the endogenous fluorophores. For dry state, samples were evaluated using a powder cell and performing the measurements in front-surface mode; and in the liquid state using a quartz cuvette and performing the

measurements in right-angle mode. The liquid dispersions were prepared by dispersing the powders in a 50 mM pH 7.0 phosphate buffer (20 mg/mL of buffer) and centrifuging (10000 *g*, 10 min, 20 °C). The supernatants were diluted to reach an absorbance at 280 nm equal to 0.075, which is suited to right-angle measurements.

2.6 Protein conformation and functional properties of pigeon pea protein concentrates

2.6.1 Intrinsic fluorescence spectroscopy

The intrinsic fluorescence emission spectra of pigeon pea proteins were obtained using an ISS PC1 steady-state spectrofluorimeter (Champaign, IL, USA) at 25 °C (Caruso et al., 2016). Protein samples were diluted in 50 mM pH 7.0 phosphate buffer at a concentration of 0.15 mg mL⁻¹. The excitation wavelength was set at 280 nm and the emission spectra were recorded from 300 to 520 nm. A constant slit of 2 nm was defined for both excitation and emission. A 10 × 10 mm quartz cuvette was used for measurements, which were conducted in right-angle mode.

2.6.2 Protein solubility and electrostatic charge

Protein solubility was evaluated at pHs ranging from 2.0 to 12.0 using the Bradford method (Bradford, 1976). First, 100 mg of sample were diluted in 10 mL of distilled water at different pHs. The mixtures were vortexed and centrifuged (1000 rpm, 25 min, 25 °C). A 100 µL aliquot of the supernatant was collected from each tube and diluted in 1900 µL of distilled water. In a microplate, 10 µL of each solution were mixed with 200 µL of Coomassie dye reagent (G-250, Bio-Rad) previously diluted in distilled water (1:4, v v⁻¹). The plate was incubated for 5 min and the absorbance was read at 595 nm. Protein concentration was determined by constructing a standard curve of BSA at concentrations ranging from 0 to 0.5 mg/mL. Protein solubility (%) was determined as the ratio between the protein content in the supernatant and the protein content in the initial mass of sample.

The zeta potential of protein dispersions at different pHs (2.0 to 12.0) was determined in a Zetasizer Nano Series equipment (Malvern, UK) (Sun et al., 2020). Protein dispersions were prepared in the same manner as described for the protein solubility analysis. Each sample was analyzed in triplicate at 25 °C and the refraction indices used were 1.33 (for water) and 1.45 (for protein dispersions). For each reading, eleven repetitions were performed by the equipment.

2.6.3 Water and oil holding capacities (WHC and OHC)

The water holding capacity (WHC) and oil holding capacity (OHC) of the protein concentrates were determined according to Zhao, Shen, Wu, Zhang, & Xu (2021), with modifications. About 1 g of sample was weighed in a polypropylene centrifuge tube and 20 mL of distilled water or soybean oil were added. The mixture was vortexed and allowed to stand for 30 min. Then, the tubes were centrifuged (4500 rpm, 10 min, 25 °C) and the

supernatants were discarded. The tubes were inverted to drain any remaining liquid. Then, the samples were weighed again to calculate WHC and OHC by Equation 4:

$$WHC \text{ or } OHC (g\ g^{-1}) = \frac{m_2 - m_1}{m_1} \quad (4)$$

in which m_2 is the mass of sample + water or oil after centrifugation and m_1 is the initial mass of sample.

2.6.4 Emulsifying properties

To determine the emulsifying activity index (EAI) and emulsion stability index (ESI), protein dispersions (0.5 %, w w⁻¹) were prepared and the pH was adjusted to 7.0 (using 0.1 M NaOH and HCl solution). Then, 1.5 mL of soybean oil were added to 4.5 mL of protein dispersion and homogenized (20000 rpm, 1 min) (T-25 Ultra-Turrax®, IKA, Germany). An aliquot (25 µL) of the produced emulsion was diluted in 5 mL of 0.1% SDS (sodium dodecyl sulfate) solution. The absorbance of the diluted emulsion was determined by spectrophotometry (UV-M51, BEL Engineering, Italy) at 500 nm. The EAI and ESI were calculated according to Equations 5 and 6 (Tontul, Kasimoglu, Asik, Atbakan, & Topuz, 2018).

$$EAI (m^2/g) = \frac{2 \times 2,303 \times A_0 \times DF}{C \times \varphi \times 10000} \times 100 \quad (5)$$

$$ESI (min) = \frac{A_0}{A_0 - A_{10}} \times 10 \quad (6)$$

in which A_0 is the absorbance of the diluted emulsion after production, DF is the dilution factor, C is the weight of protein per volume of emulsion (g/mL), φ is the volume fraction of the soybean oil in the emulsion and A_{10} is the absorbance of the diluted emulsion 10 min after production.

2.6.5 Foaming properties

The foaming capacity was determined by adding 1.5 g of protein concentrates to 50 mL of distilled water (pH adjusted to 7.0), followed by stirring (20,000 rpm, 2 min) (T-25 Ultra-Turrax®, IKA, Germany) (Vinayashree & Vasu, 2021). The foam produced was added to a graduated cylinder and the volume was measured (t = 0 min). For foam stability, the foam volume was measured after 120 min of storage. Foaming capacity and foam stability were calculated using Equations 7 and 8

$$Foaming\ capacity\ (\%) = \frac{V_0 - V_i}{V_i} \times 100 \quad (7)$$

$$Foam\ stability\ (\%) = \frac{V_{120} - V_i}{V_i} \times 100 \quad (8)$$

in which V_i , V_0 , V_{120} are the volumes before stirring, after stirring and 120 min after stirring, respectively.

2.6.6 Gelling properties

The least gelation concentration (LGC) was evaluated according to Sahni et al. (2020). Protein suspensions in distilled water (pH = 7.0) were prepared at different concentrations (2 to 20 % w w⁻¹). The suspensions were vortexed for 30 s, placed in a boiling water-bath for 1 h, cooled in running water, and stored at refrigeration temperature for 24 h. After this period, the tubes containing the suspensions were inverted. The LGC was determined as the lowest concentration necessary for the gel not to flow after the tube inversion.

2.7 Statistical analysis

Data were analyzed using the OriginPro 2016 and XLSTAT software. Variance analysis (ANOVA) followed by Tukey post hoc test was applied to determine the difference between samples at a 5% significance level. The experiments were carried out in three independent replicates. The factors were sample type (flours and protein concentrate) and pre-treatments (control, soaking and soaking + boiling). Data were expressed as mean $\pm$ standard deviation. Significant differences between samples are indicated with different uppercase or lowercase letters.

3. Results and discussion

3.1 Flour morphology and size distribution

Soaking is a widely used process, both in domestic cooking and on the industrial scale, to improve the digestibility of pulse seeds. This pre-treatment has the main objective of hydrating the seeds, and in particular biopolymers constituents such as proteins, starch and pectin. This, in turn, facilitates subsequent cooking and is thus very helpful in reducing the hard-to-cook phenomenon often reported in underused pulses. Boiling makes pre-soaked seeds softer and more palatable, which are important attributes for their consumption. Heating results in protein denaturation, starch gelatinization, and pectin solubilization, mechanisms responsible for seed softening (Chigwedere, Njoroge, Van Loey, & Hendrickx, 2019). The impact of these phenomena appears in the size distribution, particle shape and morphology of the flours (Figure 3.1 and Supplementary Table 3.1).

The flour produced with raw seeds (control, FC) presented a bimodal size distribution in the dry state (blue solid line), with a small population between 10 - 100 μm and a larger population with size $> 100 \mu\text{m}$ (Fig. 3.1a). The soaked seeds generated a flour (FS) with a broader distribution between 100 and 1000 μm and only a shoulder between 10-100 μm , whereas soaking + boiling (FB) caused the size distribution shifting to the right, resulting in a well-defined peak between 200 and 2000 μm and suggesting

the formation of aggregates as a consequence of boiling. The size distribution measured in the flour suspensions (black dotted lines) partly overlapped with the distribution in dry state, with predominantly bimodal distributions for FC and FS, and trimodal for FB. A decrease in the smaller size range was observed in the dry state measurement for FS (Fig. 3.1a). As suggested by the SEM images, the starch granules in FS show a tendency to form aggregates, since we cannot see as many individual granules as in FC. Soaking may induce a change in the matrix around the starch granules, leading to agglomeration, which in turn leads to a shift towards larger size. When the FS sample was evaluated in the liquid state, however, we can see this peak again, more pronounced, probably due to the solubilization of the matrix between the starch granules.

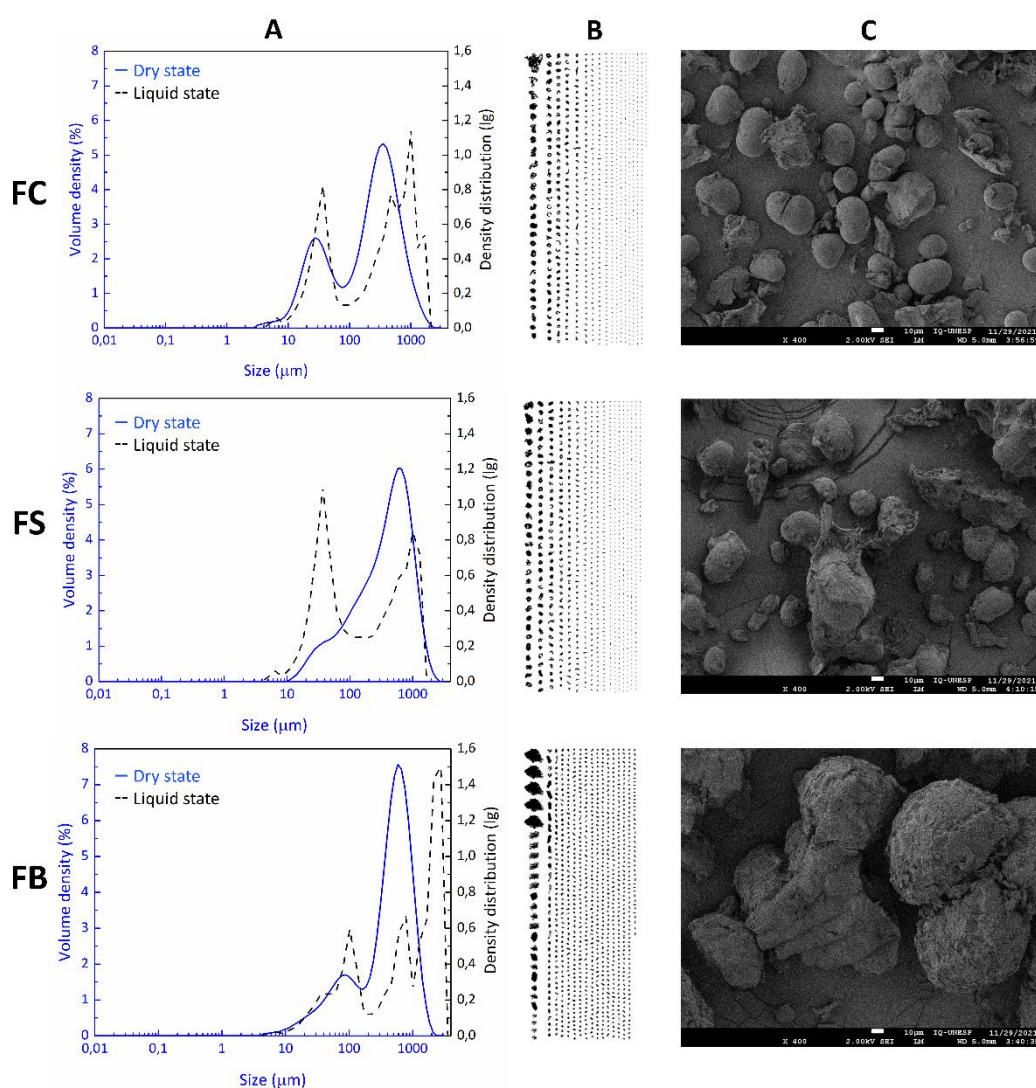


Figure 3.1. Size distribution (a), particle morphology (b) and scanning electron micrographics (SEM) (c) of pigeon pea flours. Size distribution measurements were carried out on samples in the dry state (solid blue line) and on aqueous dispersions containing 0.5% ethanol (dashed black line). FC: flour produced with raw seeds; FS: flour produced with soaked seeds; FB: flour produced with soaked+boiled seeds.

All the samples had a major population with size $> 100 \mu\text{m}$, of which the relative importance increases with applying pre-treatments. Starch granules generally have a size ranging from 1 to $110 \mu\text{m}$ and an oval or spherical shape (Hoover, 2001), like the particles that appear in great number in FC (Fig. 3.1c) and that probably correspond to the population observed in the size region between 1 and $100 \mu\text{m}$. The particle shape distribution and SEM images (Fig 3.1b and Fig 3.1c) of flours FS and FB corroborate these results. The particle shape distribution shows the incidence of a greater number of smaller and more uniform particles and few larger and uneven particles in FC and FS, whereas in FB there was a greater incidence of larger and uneven particles (Fig 3.1b.), which contributes to the shift of particle size distribution for sizes $> 1000 \mu\text{m}$.

The SEM images (Fig. 3.1c) shows the effect of the seeds pre-treatments in the flour morphology, such as the presence of native starch granules in non-treated flour (FC); starch granules with a more wrinkled surface due to hydration after soaking (FS); and larger structures formed after gelatinization of starch granules by boiling (FB) (Ma et al., 2021). Also, compact particles could be seen in FB, which may be directly related to the gelatinization of starch granules under high moisture and high temperature conditions. During thermal treatments, moisture content plays an important role in the morphological changes of starch granules, so that a higher moisture content can lead to a more compact starch structure (Zhang et al., 2022; Li et al., 2021). The starch content of the samples is further discussed in the following section, along with the composition of the pigeon pea ingredients.

3.2 Pigeon pea ingredients composition

As a preliminary characterization of the FTIR spectra of pigeon pea protein concentrates revealed the significant contribution of other macronutrients such as lipids ($3000\text{-}2800 \text{ cm}^{-1}$) and carbohydrates ($1200\text{-}900 \text{ cm}^{-1}$) (Andrade et al., 2019; Gholizadeh et al., 2014) (Supplementary Figure 3.2), the composition of these components in both flours and protein concentrates was characterized. In the following sections the impact of seed pre-treatments on major (polysaccharides, lipids, and proteins) and minor compounds (trypsin inhibitor activity, total soluble phenolic compounds, raffinose family oligosaccharides (RFOs), ashes) of pigeon pea ingredients are presented.

3.2.1 Carbohydrate composition

Carbohydrates are the largest constituents of pigeon pea flour, corresponding to approximately 60 wt. % of its total composition, regardless of the pre-treatments used (Table 3.1). Of this total content in flours, around 54-62% corresponds to starch, 27-29% corresponds to non-starch polysaccharides (NPS), around 7-11% correspond to raffinose family oligosaccharides (RFO), and around 4-6% correspond to sucrose (Table 3.2). Regarding the composition in non-starch polysaccharides (NSP), soaking+boiling pre-treatment decreased the levels of uronic acid and glucose residues from 2.8 and $8.4 \text{ g } 100 \text{ g}^{-1}$ to 1.8 and $7.9 \text{ g } 100 \text{ g}^{-1}$, respectively. This could be attributed to a solubilization of

some cell wall polysaccharides in the cooking water. The soaking+boiling pre-treatment also affects the soluble polysaccharide content: FB showed an increase in uronic acids suggesting an increased content of pectin as compared to FC and FS. Also, the combined pre-treatment seems to have completely solubilized the uronic acid content in flour. The increase in the soluble NSP content can be attributed to a modification of the structure of homogalacturonans during boiling, reducing the hard-to-cook phenomenon present in these seeds (Chigwedere et al., 2019, Mubaiwa et al., 2017).

The content of RFOs in pulses varies from 2 to 10 g 100 g⁻¹ (Guillon & Champ, 2002). In this work, the RFOs corresponded to around 7.3 g 100 g⁻¹ for FC, whereas in FS and FB this value was reduced by around 25 and 37%, respectively. Soaking leads to a reduction in RFOs levels probably through the leaching of raffinose, stachyose and verbascose into the soaking water. During boiling, RFOs can be reduced by heat-induced hydrolysis of oligosaccharides to disaccharides and monosaccharides (Onigbinde and Akinyele, 1983). Unlike other seeds such as chickpeas, lupin, and lima beans that present only traces of verbascose, pigeon pea is a species that presents high levels of this oligosaccharide, as discussed by Raja, Agasimani, Varadharajan, & Ram (2016). According to the authors, the reduction of these sugars through seed pre-treatments is beneficial for better digestibility of pigeon pea, as the consumption of RFOs is associated with flatulence problems.

Sucrose is the only disaccharide present in pigeon pea, except for some cultivars that may have small amounts of maltose (Agasimani, Varadharajan, & Ram, 2016). The sucrose content was 4 g 100 g⁻¹ for FC and decreased by about 48% with soaking, reaching 2.1 g 100 g⁻¹ for FS. However, the sucrose content in FB showed an increase of approximately 10% compared to FS, possibly resulting from the hydrolysis of oligosaccharides to sucrose.

The protein fractionation process resulted in a large decrease in carbohydrate content as compared to flours: 9-11 g 100 g⁻¹ for PPC-C and PPC-S and about 15 g 100 g⁻¹ for PPC-B (Table 3.1). Mannose and galactose residues were the most abundant NSP residues in the protein concentrates (about 1 g 100 g⁻¹ each), and their levels were not affected by pre-treatments ($p > 0.05$). Mannose residues had their content doubled in relation to the levels presented in flour counterparts, whereas the proteins were 3-fold concentrated in the fractionation process. The high mannose content in protein concentrates is surprising and appears not to be related to the pre-treatments used. The presence of mannose can be attributed to the potential existence of (galacto)(gluco)mannanes in protein concentrates but also to high mannose *N*-glycans of *N*-glycoproteins. It is known that soybean globulin, for example, is likely to be glycosylated, with mannose being one of the main sugars involved in this process (Thanh & Shibasaki, 1976). Soybean globulin has some homology with pigeon pea globulin, according to a study by Krishnan, Natarajan, Oehrle, Garrett, & Darwish (2017), so that the glycosylation of the main proteins in pigeon pea may occur in a similar way to that in soybean. However, more studies must be carried out to understand this possible phenomenon.

Table 3.1. Proximate composition, total soluble phenolic compounds (TSPC) and trypsin inhibitors activity (TIA) of pigeon pea flours and protein concentrates.

Sample	Proximate composition					Total amount of characterized compounds (g 100 g ⁻¹ , d.b)	TSPC	TIA	
	Moisture (g 100 g ⁻¹)	Protein (g 100 g ⁻¹ , d.b)	Lipid (g 100 g ⁻¹ , d.b)	Carbohydrate (g 100 g ⁻¹ , d.b)	Ash (g 100 g ⁻¹ , d.b)		mg GAE g ⁻¹	µg Tld mg ⁻¹ sample	µg Tld mg ⁻¹ protein
Flours									
FC	9.0 ± 0.0 ^b	19.5 ± 0.2 ^a	4.0 ± 0.2 ^a	64.6 ± 0.7 ^a	4.3 ± 0.0 ^a	92.4 ± 0.7 ^a	7.2 ± 0.0 ^a	8.3 ± 0.2 ^a	42.6 ± 0.8 ^a
FS	10.0 ± 0.3 ^a	21.5 ± 0.8 ^a	3.1 ± 0.1 ^b	61.1 ± 1.1 ^b	4.1 ± 0.0 ^b	90.3 ± 2.0 ^a	6.0 ± 0.1 ^b	5.7 ± 0.3 ^b	26.1 ± 1.1 ^b
FB	7.8 ± 0.1 ^c	20.2 ± 0.7 ^a	2.8 ± 0.0 ^c	64.5 ± 1.6 ^a	2.9 ± 0.0 ^c	90.4 ± 1.5 ^a	3.1 ± 0.0 ^c	1.2 ± 0.0 ^c	5.8 ± 0.2 ^c
Protein concentrates									
PPC-C	8.3 ± 0.0 ^c	63.7 ± 0.3 ^b	12.6 ± 0.4 ^a	11.1 ± 0.0 ^b	7.5 ± 0.0 ^a	94.9 ± 0.6 ^a	5.6 ± 0.1 ^a	11.5 ± 0.5 ^a	18.0 ± 0.8 ^a
PPC-S	10.1 ± 0.0 ^a	68.3 ± 0.5 ^a	9.3 ± 0.0 ^b	9.1 ± 0.4 ^c	7.2 ± 0.3 ^a	93.9 ± 0.9 ^a	5.7 ± 0.0 ^a	6.2 ± 0.3 ^b	9.0 ± 0.4 ^b
PPC-B	9.3 ± 0.0 ^b	62.2 ± 0.5 ^c	4.0 ± 0.1 ^c	15.4 ± 0.2 ^a	7.5 ± 0.0 ^a	89.1 ± 0.7 ^b	5.7 ± 0.1 ^a	0.6 ± 0.0 ^c	1.0 ± 0.0 ^c

Different letters in the same column (for flours and protein concentrates) indicate statistical difference ($p < 0.05$). Tld: trypsin inhibited; FC: flour produced with raw seeds; FS: flour produced with soaked seeds; FB: flour produced with soaked-boiled seeds; PPC-C: protein concentrate produced with FC; PPC-S: protein concentrate produced with FS; PPC-B: protein concentrate produced with FB.

Table 3.2. Carbohydrates composition in pigeon pea flours and protein concentrates.

	NSP composition									RFO composition				Sucrose	Starch
	Rha	Fuc	Ara	Xyl	Man	Gal	UA	Glc	Total	Raf	Sta	Ver	Total		
Total content															
FC	0.3 ± 0.0 ^a	0.0 ± 0.0 ^a	2.9 ± 0.1 ^a	2.6 ± 0.5 ^a	0.4 ± 0.0 ^a	1.3 ± 0.1 ^a	2.8 ± 0.1 ^a	8.4 ± 0.8 ^a	18.7 ± 0.5 ^a	1.0 ± 0.1 ^a	2.6 ± 0.1 ^a	3.7 ± 0.1 ^a	7.3 ± 0.2 ^a	4.0 ± 0.2 ^a	34.7 ± 0.4 ^b
FS	0.3 ± 0.0 ^a	0.1 ± 0.0 ^a	3.4 ± 0.3 ^a	2.9 ± 0.6 ^a	0.4 ± 0.0 ^a	1.5 ± 0.1 ^a	2.3 ± 0.1 ^b	7.1 ± 0.2 ^a	18.0 ± 1.2 ^a	0.5 ± 0.0 ^b	1.2 ± 0.0 ^c	3.8 ± 0.1 ^a	5.5 ± 0.1 ^b	2.1 ± 0.0 ^c	35.6 ± 0.2 ^b
FB	0.4 ± 0.0 ^a	0.0 ± 0.0 ^a	3.0 ± 0.2 ^a	2.2 ± 0.5 ^a	0.4 ± 0.0 ^a	1.6 ± 0.1 ^a	1.8 ± 0.1 ^c	7.9 ± 0.4 ^b	17.3 ± 0.6 ^b	0.5 ± 0.0 ^b	1.7 ± 0.0 ^b	2.4 ± 0.0 ^b	4.6 ± 0.1 ^c	2.3 ± 0.0 ^b	40.3 ± 1.9 ^a
Soluble content															
FC	n.d	n.d	0.1 ± 0.0 ^a	0.1 ± 0.0 ^a	0.1 ± 0.0 ^a	0.7 ± 0.0 ^b	1.5 ± 0.0 ^b	0.2 ± 0.0 ^a	2.7 ± 0.0 ^b	n.a	n.a	n.a	n.a	n.a	n.a
FS	n.d	n.d	0.1 ± 0.0 ^a	0.1 ± 0.0 ^a	0.1 ± 0.0 ^a	0.8 ± 0.1 ^b	1.4 ± 0.0 ^b	0.2 ± 0.0 ^a	2.6 ± 0.1 ^b	n.a	n.a	n.a	n.a	n.a	n.a
FB	n.d	n.d	0.1 ± 0.0 ^a	0.1 ± 0.0 ^a	0.1 ± 0.0 ^a	1.1 ± 0.0 ^a	2.1 ± 0.1 ^a	0.2 ± 0.0 ^a	3.7 ± 0.1 ^a	n.a	n.a	n.a	n.a	n.a	n.a
Total content															
PPC-C	n.d	n.d	0.6 ± 0.0 ^a	0.2 ± 0.0 ^a	1.0 ± 0.0 ^a	1.0 ± 0.1 ^a	0.8 ± 0.0 ^a	n.d	3.6 ± 0.1 ^a	0.2 ± 0.0 ^a	1.2 ± 0.1 ^a	1.4 ± 0.0 ^a	2.8 ± 0.1 ^a	1.8 ± 0.0 ^a	2.8 ± 0.1 ^a
PPC-S	n.d	n.d	0.4 ± 0.1 ^b	n.d	0.9 ± 0.1 ^a	0.8 ± 0.1 ^a	0.6 ± 0.0 ^b	n.d	2.8 ± 0.2 ^b	0.2 ± 0.0 ^a	0.9 ± 0.0 ^b	1.1 ± 0.0 ^b	2.1 ± 0.1 ^b	1.3 ± 0.0 ^b	2.8 ± 0.1 ^a
PPC-B	n.d	n.d	0.6 ± 0.0 ^a	0.1 ± 0.0 ^b	1.1 ± 0.1 ^a	0.8 ± 0.0 ^a	0.8 ± 0.0 ^a	n.d	3.4 ± 0.1 ^a	0.1 ± 0.0 ^a	0.7 ± 0.0 ^c	1.0 ± 0.0 ^b	1.7 ± 0.1 ^c	0.9 ± 0.0 ^c	9.4 ± 0.2 ^b

Different letters in the same column (for flours and protein concentrates) indicate statistical difference ($p < 0.05$). FC: flour produced with raw seeds; FS: flour produced with soaked seeds; FB: flour produced with soaked + boiled seeds; PPC-C: protein concentrate produced with FC; PPC-S: protein concentrate produced with FS; PPC-B: protein concentrate produced with FB. Rha, Fuc, Ara, Xyl, Man, Gal, UA, Glc, NSP, Suc, Raf, Sta, Ver, and RFO are rhamnose, fucose, arabinose, xylose, mannose, galactose, uronic acid, glucose, non-starch polysaccharides, sucrose, raffinose, stachyose, verbascose, and raffinose family oligosaccharides, respectively. Values below the detection limit are expressed as n.d and measures not performed are expressed as n.a.

Protein fractionation also affected the RFO composition of protein concentrates, which decreased even further compared to the original flours. PPC-C, PPC-S and PPC-B presented values of 2.8, 2.1, and 1.7 g 100 g⁻¹ of total RFOs, respectively, and all concentrates had a reduction of around 63% in relation to their flour counterparts. This significant reduction highlights the cumulative effect of seed pre-treatments with aqueous fractionation of proteins in improving the digestibility of pigeon pea-based ingredients. It has been reported that in processes to produce globulin-rich extracts, the protein precipitation step at acidic pH generates a side stream that is rich in water-soluble proteins, such as albumins, and sugars (Yang et al., 2022). It is possible that this step is essential to further reduce the levels of RFOs and sucrose in protein concentrates.

Finally, of the total carbohydrates in protein concentrates, around 25-31% correspond to the starch fraction for PPC-C and PPC-S (Table 3.2). For the protein concentrate produced with soaked-boiled seeds, however, a greater accumulation of starches was observed (corresponding to 61% of the total carbohydrates in PPC-B). This can be explained by the starch gelatinization that occurs during boiling, leading to greater release and solubilization of amylose and amylopectin (Watson & Johnson, 1965).

3.2.2 Lipid composition

The total lipid content in pigeon pea flour was reduced by soaking and boiling pre-treatments (Table 3.3). While FC presented a total lipid content of 4 g 100 g⁻¹, FS and FB showed values of 3.1 g 100 g⁻¹ and 2.8 g 100 g⁻¹, respectively.

Neutral lipids corresponded to 50% of the total lipids in FC, whereas FS and FB had 45 and 71% of neutral lipids in their composition, respectively. All flours showed a predominance of triglycerides (1-1.8 g 100 g⁻¹) and the same level of free fatty acids (0.2 g 100 g⁻¹). However, only samples subjected to pre-treatments showed detectable levels of monoglycerides, which is an indication of triglyceride hydrolysis during pre-treatments (Abdel-Rahman, El-Fishawy, El-Geddawy, Kurz, & El-Rify, 2007). The total phospholipid content was around twice higher in FB, compared to FC and FS. This may seem surprising, since it is conceptually not possible that additional lipids would be incorporated in the seeds (and resulting flour) upon soaking + boiling. A possible explanation is that this pre-treatment would improve the extractability of lipids, in particular polar lipids, which also implies that extraction might not have been complete for FC and FS, although conducted with the recommended Folch extraction. Comparable analyses are hardly available in literature; one of the only references found is the work of Liukkonen, Moontfoort & Laaksot (1992), who observed a moderate increase in the phospholipid content for flour of oat soaked-steamed-grains, compared to a reference with non-steamed grains.

Table 3.3. Neutral lipids, phospholipids and tocopherols content of pigeon pea flours and protein concentrates.

	Total lipids (g 100 g ⁻¹ , d.b)	Neutral lipids (g 100 g ⁻¹ , d.b)				Phospholipids (g 100 g ⁻¹ , d.b)				Tocopherols (µg g ⁻¹ lipids)	
		TG	MG	FFA	Total	PE	PI	PA	PC	Total	γ-tocopherol
Flours											
FC	4.0 ± 0.2 ^a	1.8 ± 0.1 ^a	n.i	0.2 ± 0.0 ^a	2.0 ± 0.1 ^a	n.d	0.3 ± 0.0 ^a	n.d	n.d	0.3 ± 0.0 ^b	877.0 ± 215.0 ^a
FS	3.1 ± 0.1 ^b	1.0 ± 0.0 ^b	0.2 ± 0.0 ^a	0.2 ± 0.0 ^a	1.4 ± 0.1 ^b	n.i	0.1 ± 0.0 ^b	0.1 ± 0.0	n.d	0.3 ± 0.0 ^b	304.0 ± 70.6 ^b
FB	2.8 ± 0.0 ^c	1.6 ± 0.1 ^a	0.2 ± 0.0 ^a	0.2 ± 0.0 ^a	2.0 ± 0.0 ^a	0.2 ± 0.0	n.i	n.i	0.4 ± 0.0	0.6 ± 0.0 ^a	218.6 ± 15.0 ^b
Protein concentrates											
PPC-C	12.6 ± 0.4 ^a	6.4 ± 0.4 ^a	n.i	n.d	6.4 ± 0.2 ^a	n.d	0.8 ± 0.0 ^a	n.i	0.9 ± 0.0 ^a	1.7 ± 0.1 ^a	1274.5 ± 142.6 ^a
PPC-S	9.3 ± 0.0 ^b	3.4 ± 0.3 ^b	0.6 ± 0.0 ^a	n.d	4.0 ± 0.3 ^b	n.d	0.7 ± 0.0 ^a	n.d	0.6 ± 0.0 ^b	1.3 ± 0.0 ^b	377.5 ± 22.5 ^b
PPC-B	4.0 ± 0.1 ^c	1.1 ± 0.1 ^c	0.2 ± 0.0 ^b	0.4 ± 0.0	1.7 ± 0.1 ^c	n.i	n.d	n.i	n.d	n.d	167.1 ± 3.5 ^c

Different letters in the same column (for flours and protein concentrates) indicate statistical difference ($p < 0.05$). FC: flour produced with raw seeds; FS: flour produced with soaked seeds; FB: flour produced with soaked-boiled seeds; PPC-C: protein concentrate produced with FC; PPC-S: protein concentrate produced with FS; PPC-B: protein concentrate produced with FB. TG: triglycerides; MG: monoglycerides; FFA: free fatty acids; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PC: phosphatidylcholine; PA: phosphatidic acid. Values below the detection limit are expressed as n.d and compounds not identified are expressed as n.i.

Regarding tocopherols, only γ -tocopherol was identified in the samples (Table 3.3). The high content of γ -tocopherol observed for FC ($877 \mu\text{g g}^{-1}$ lipids) shows that pigeon pea is a good source of tocopherol, as well as several Canadian and Indian pulses, as demonstrated by Padhi, Liu, Hernandez, Tsao, & Ramdath (2017) and Krishnaa, Prabhakara, & Aitzetmüllerb (1997). Soaking alone considerably decrease the γ -tocopherol contents to $304 \mu\text{g g}^{-1}$ lipids, indicating that processes that do not use heat can also result in significant losses of such lipophilic compounds. Tocopherol losses were even greater when pre-treatment was combined with boiling (around $218 \mu\text{g g}^{-1}$ lipids). The degradation of tocopherols during processing has been reported for various ingredients and food products, and is most likely due to the capacity of these molecules to act as chain-breaking antioxidants in the presence of free radicals, notably when heat treatments (e.g., steaming) are applied in the presence of air (Hidalgo et al., 2008; Ottaway et al., 2010; Balakrishnan & Schneider, 2023).

The protein fractionation process resulted in the accumulation of lipids, which presented values of 12.6, 9.3 and $4.0 \text{ g } 100 \text{ g}^{-1}$ for PPC-C, PPC-S and PPC-B, respectively (Table 3.3). These values shows that the lipids initially present in raw-seeds and soaked-seeds flours were 3-fold concentrated for PPC-C and PPC-S. The soaking + boiling pre-treatment, however, resulted in only a 1.4-fold concentration of lipids in PPC-B, compared to its flour counterpart. It is important to highlight that although pigeon pea is a pulse with a low lipid content (<5% of total lipids in seeds) the fractionation process resulted in a significant concentration of the endogenous lipids, which was recently reported in other pulse protein ingredients (Grasberger et al., 2024; Keuleyan et al., 2023). This points out to a possible genericity of this lipid accumulation effect, but the exact mechanisms involved still need to be unraveled. Our results therefore confirm that investigating the mechanisms behind protein-lipid interactions during the fractionation of plant-based proteins is of great interest.

The neutral lipid content ranged from 43 to 51% of the total lipid composition of the protein concentrates. All protein concentrates showed a predominance of triglycerides, whereas monoglycerides were identified only for PPC-C and PPC-S. However, the level of free fatty acids doubled for PPC-B, when compared to its flour counterpart. This increase is a primary response to the occurrence of lipolysis reactions, and the wet fractionation process can intensify this phenomenon due to the homogenization step (Liukkonen, Moontfoort & Laaksot, 1992).

Soaking + boiling followed by proteins fractionation resulted in a complete loss in the phospholipid fraction in PPC-B (values below the detection limit). PPC-C and PPC-S presented values of 1.7 and $1.3 \text{ g } 100 \text{ g}^{-1}$ of total phospholipids, respectively, agreeing with the values reported by Keuleyan et al. (2023) for lupin protein concentrate and isolate ($1.4\text{-}1.9 \text{ g } 100 \text{ g}^{-1}$) but lower than the values reported for pea protein concentrate and isolate ($2.3\text{-}5.4 \text{ g } 100 \text{ g}^{-1}$). Thus, compared to the starting flours, phospholipids accumulated in the concentrates for the control and soaked samples, but were fully eliminated when the seeds were initially subjected to soaking + boiling. The latter effect

could potentially be due to the physical release of phospholipid-rich structures, which would then be eliminated upon the processing to yield the concentrate. The exact mechanisms are still to be unraveled, and certainly deserve attention as polar lipids are important drivers for the sensory and functional attributes of plant protein ingredients. Among the different types of phospholipids, only phosphatidylinositol and phosphatidylcholine were identified in pigeon pea protein concentrates. Like in flours, γ -tocopherol contents were the highest for PPC-C (1274 $\mu\text{g g}^{-1}$ lipids), decreasing to around 378 and 167 $\mu\text{g g}^{-1}$ lipids for PPC-S and PPC-B, respectively. This confirms the effect of soaking and boiling on tocopherol chemical degradation, as mentioned earlier.

3.2.3 Protein composition

The protein content of flours was comprised between 19.5 and 21.5%, (Table 3.1), not being affected by the applied pre-treatments ($p > 0.05$). Corzo-Ríos et al. (2022) and Danhassan et al. (2018) also did not observe changes in the protein content of seeds subjected to similar processes.

The SDS-PAGE analysis (Figure 3.2) showed three main protein bands in all the samples: two between 37 and 50 kDa and one between 50 and 75 kDa. These bands may correspond to 7S-vicilin subunits, the main protein of pigeon pea seeds (about 60% of their composition) (Sun et al., 2020). FC presented more bands < 37 kDa than FS and FB, which could be seen mainly in reducing conditions. Seed pre-treatments can lead to the loss of low molecular weight proteins. In work carried out by Sun et al. (2020), protein bands with molecular weight < 25 kDa completely disappeared in pigeon pea protein extracts produced with microwave-cooked seeds.

Regarding the low molecular weight proteins that can be lost by soaking and boiling treatments, trypsin inhibitors are of special interest, since their removal can improve the digestibility of these seeds. Krishnan et al. (2017) identified Kunitz trypsin inhibitors (KTI) in pigeon pea seeds with a molecular weight of 21 kDa. A protein band close to 21 kDa can be observed in FC (orange triangle symbol, under reduced conditions), being less visible in the other samples. The possible loss of KTI during the pre-treatment could directly affect the trypsin inhibitor activity (TIA) of the samples and will be further discussed in the next section.

The presence of a protein band close to 18 kDa (yellow triangle symbol, under not-reduced conditions) in FC could suggest the presence of lectins in the samples. Pigeon pea lectins have two polypeptide chains joined only by non-covalent and non-disulfide bonds and their presence in the seeds was reported in study of Siddiqui, Hasan, and Salahuddin (1995).

The extraction process increased the protein content of powders from 62.2 to 68.3% (Table 3.1). In SDS-PAGE, the identified bands are in the region of 7S-vicilin subunits (Sun et al., 2020). Thus, the extraction process eliminated proteins with molecular weight > 75 kDa and reduced the amount of proteins with molecular weight < 37 kDa.

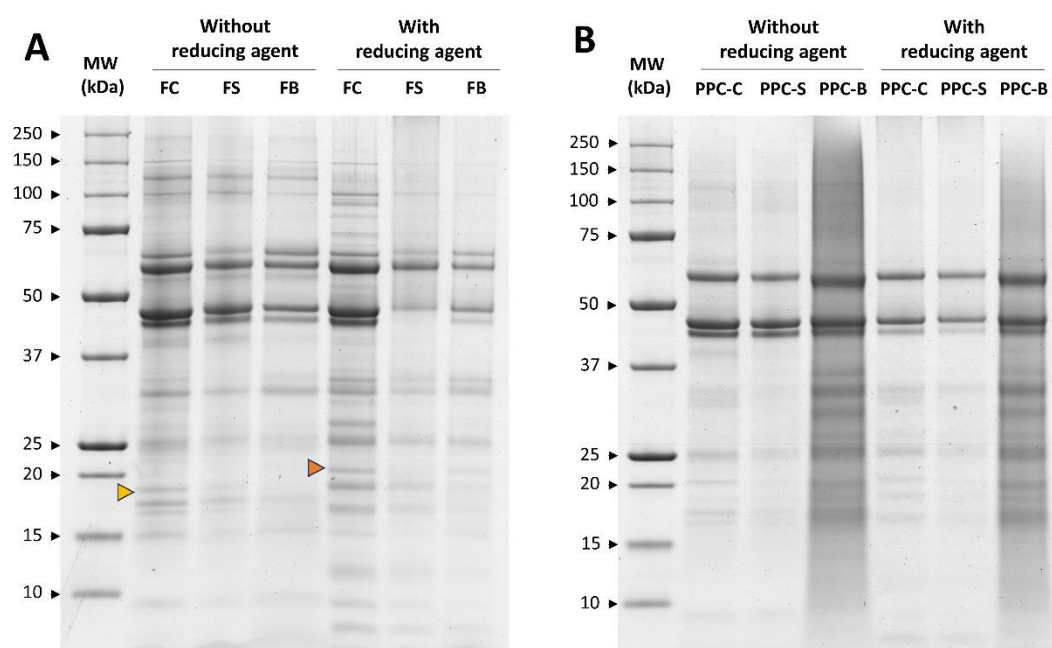


Figure 3.2. SDS-PAGE of pigeon pea flours **(a)** and protein concentrates **(b)** with or without the presence of the reducing agent 2-mercaptoethanol (10%, v v⁻¹). FC: flour produced with raw seeds; FS: flour produced with soaked seeds; FB: flour produced with soaked+boiled seeds; PPC-C: protein concentrate produced with FC; PPC-S: protein concentrate produced with FS; PPC-B: protein concentrate produced with FB.

3.2.4 Minor compounds: trypsin inhibitor activity (TIA), total soluble phenolic compounds (TSPC), and ash content

Trypsin inhibitors, phenolic compounds and minerals may be leached and/or degraded during soaking and boiling pre-treatments (Chigwerede et al., 2019), so we probed their content (Table 3.1).

The TIA was 8.3, 5.7 and 1.2 $\mu\text{g TId mg}^{-1}$ sample for FC, FS and FB, respectively. The soaking pre-treatment reduced TIA by 31%, whereas boiling treatment led to a reduction of about 86% for pigeon pea flour. As the protein content is similar for all samples, the same reduction percentages are observed if we consider the TIA results expressed in mg of protein (Table 3.1). The reduction of TIA is a good indicator of the efficiency of the processes used to improve the digestibility of pigeon pea flours.

The protein concentrates showed values of 11.5, 6.2 and 0.6 $\mu\text{g TId mg}^{-1}$ for PPC-C, PPC-S, and PPC-B, respectively (Table 3.1). PPC-C increased TIA by approximately 38% over its flour counterpart, close to values reported by Gaxiola et al. (2018) for soybean protein concentrate (increase of 32% in TIA compared to the initial activity in soybean flour). For PPC-S, however, TIA only increased around 9% in relation to FS, whereas PPC-B presented half of the TIA value determined for FB.

Legume trypsin inhibitors are classified into two families according to their molecular weight: Bowman-Birk (BBTIs) of 8 kDa and Kunitz (KTIs) of 20 kDa (Avilés-

Gaxiola, Chuck-Hernández, & Serna Saldívar, 2018). Low molecular weight proteins (< 37 kDa) showed less visible bands in the electrophoresis analysis (Fig. 3.2) even for the sample subjected only to the soaking, suggesting leaching of these proteins. A band below 10 kDa, maybe related to BBTIs, is visible in the flour produced with raw seeds, but not for the pre-treated flours. The same can be observed for the bands around 20 kDa, that can be associated with KTIs.

The results show that soaking pigeon pea seeds for 24 h followed by boiling for 30 min is enough to reduce the TIA by almost 86% in flours. An additive effect of the protein fractionation process was also observed, so that the combination of soaking + boiling pre-treatments followed by proteins aqueous fractionation can reduce the TIA by 95%. There is a clear positive effect in combining seed soaking + boiling pretreatments with the protein fractionation method adopted to mitigate the activity of trypsin inhibitors in protein concentrates.

The FC sample had the highest TSPC content (7.2 mg GAE/g), which is in the range of values reported for dried beans (Yang, Gan, Ge, Zhang, & Corke, 2018). Soaking reduced the content of TSPC by 16%, whereas boiling treatment led to further reduction (57%) of TSPC content in flour (Table 3.1). This may result from the leaching of phenolic compounds during soaking, as well as from degradation of these compounds by high temperature during the boiling treatment. Leaching of phenolic compounds during soaking depends on the rate of water absorption, coat permeability, hilum size and cotyledon composition. Soaking conducted for long hours (up to 16 h) considerably reduces the content of native phenolic compounds in pulses (Mba et al., 2019), and this loss can be intensified during seed boiling (Yang et al., 2018). According to Fleurit & Macheix (2003), phenolic acids can undergo oxidative processes during boiling, in addition to the release of free acids from conjugated forms and the formation of complex structures of phenolic compounds with other molecules, such as proteins. The reduction in TSPC levels observed for the samples has a positive impact on the digestibility of pigeon pea ingredients, since some phenolic compounds can form complexes with proteins, making them less soluble, in addition to potentially inhibiting the action of digestive enzymes (Chigwerede et al., 2019).

Regarding protein concentrates, all samples had the same TSPC content, around 5.7 mg GAE/g, this suggests that the process has little effect on the solubility of the phenolic compounds. It is important to highlight that although our results indicate the same TSPC content for all protein concentrates, their phenolic composition may change due to the different pre-treatments. Future studies should investigate the impact of pre-treatments on individual phenolic compounds. Also, the method used in our work only probed soluble phenolic compounds, so we do not have information about phenolic compounds that are chemically bound to proteins. The dark brown color of protein concentrates (Supplementary Figure 3.1) suggests the presence and/or modification of phenolic compounds during protein extraction at alkaline pH (Potin, Lubbers, Husson, & Saurel, 2019). The alkaline conditions of the plant-based protein fractionation process

cause rapid oxidation of phenolic compounds, which bind to protein side chains (Rashwan et al., 2023). Protein-polyphenol interactions can also alter the sensory characteristics and digestibility of protein concentrates (Rashwan et al., 2023).

Finally, the ash content decreased in pre-treated flours (approximately 33% for flours produced with soaked-boiled seeds), suggesting mineral leaching during seed hydration and boiling. Conversely, protein concentrates had an ash content of around 7.5 g 100 g⁻¹, showing no differences between samples ($p > 0.05$).

3.3 Endogenous fluorophores in flours and protein concentrates

In addition to the biochemical analysis of flours and concentrates, the exploration of intrinsic fluorescence may help in identifying additional minor compounds and/or provide a way to investigate chemical modifications in relation to the process (Locquet, Aït-Kaddour, & Cordella, 2018). The three-dimensional excitation-emission fluorescence matrices (3D-EEFM) (Fig. 3.3) showed two distinct regions for pigeon pea flours and protein concentrates when evaluated either in the solid or liquid state: one corresponding to emission of proteins ($\lambda_{Ex} = 270-290$ nm, $\lambda_{Em} = 300-360$ nm corresponding mainly to the tryptophan/tyrosine emission), and another region referring to the fluorescence emission of non-protein compounds ($\lambda_{Ex} \approx 400-440$ nm, $\lambda_{Em} = 450-500$ nm for solid state and $\lambda_{Ex} \approx 300-340$ nm $\lambda_{Em} = 440-480$ nm for liquid state).

The different configuration adopted for each measurement (front-surface vs. right-angle mode) does not allow a direct comparison between the results obtained in dry and liquid state in relation to the observed fluorescence intensity. However, the use of both methods allows to see the influence of the presence of a solvent on the configuration of the endogenous fluorophores present in the samples.

A lower intensity of tryptophan fluorescence emission region was observed in the pre-treated flours, both in dry and liquid state. This decrease in tryptophan fluorescence may be related to chemical degradation and/or complexation with other molecules during pre-treatments. The second region ($\lambda_{Ex} \approx 430$ nm for solid state) could be seen in the flours, having a higher intensity in FC and decreasing in FB and FS, respectively. The analysis of the suspensions also showed a second category of fluorophores with $\lambda_{Ex} \approx 340$ nm, implying that the solubilization of the samples in buffer may cause a large shift in the excitation wavelength from 430 nm to 340 nm. Another hypothesis is that a less visible peak in the dry state samples ($\lambda_{Ex} \approx 300-340$ nm $\lambda_{Em} = 440-480$ nm) may correspond to the second region observed in the liquid state samples, but more intense. For protein concentrates, the fluorescence intensity of this second region, both in solid and liquid state, increased with applying pre-treatments, in the order PPC-C < PPC-S < PPC-B. This region is associated with the fluorescence emission of several phenolic compounds (Locquet et al., 2018).

Since the concentration of samples in liquid state was standardized (absorbance of all samples ≈ 0.075), the same fluorescence intensity was expected for protein concentrates and flours. However, a slightly lower fluorescence intensity was observed

for protein concentrates, suggesting possible degradation of tryptophan (Lakowicz, 2006) with the fractionation process. Even so, in the liquid state we did not observe the almost complete extinction of the fluorescence intensity that was observed for protein concentrates when measured in the dry state. This almost complete extinction of fluorescence intensity may be related to the darker color of these powders. The presence of dark and/or highly light-absorbing compounds can lead to fluorescence extinction (Muller et al., 2011). The evaluation of food powders by 3D-EEFM is still scarce in the literature, and we did not find studies that evaluated brownish or darkish food ingredients. Yet, some studies were conducted to evaluate fluorescent compounds in soils in dry and liquid states. Mounier, Nicolodelli, Redon, & Milori (2017) also did not observe fluorescence for samples of a humic substance in solid state, but samples showed fluorescence when evaluated in solutions. Nakaya et al. (2020) studied the use of Al_2O_3 as a white diluent for soils to improve solid-state fluorescence measurements.

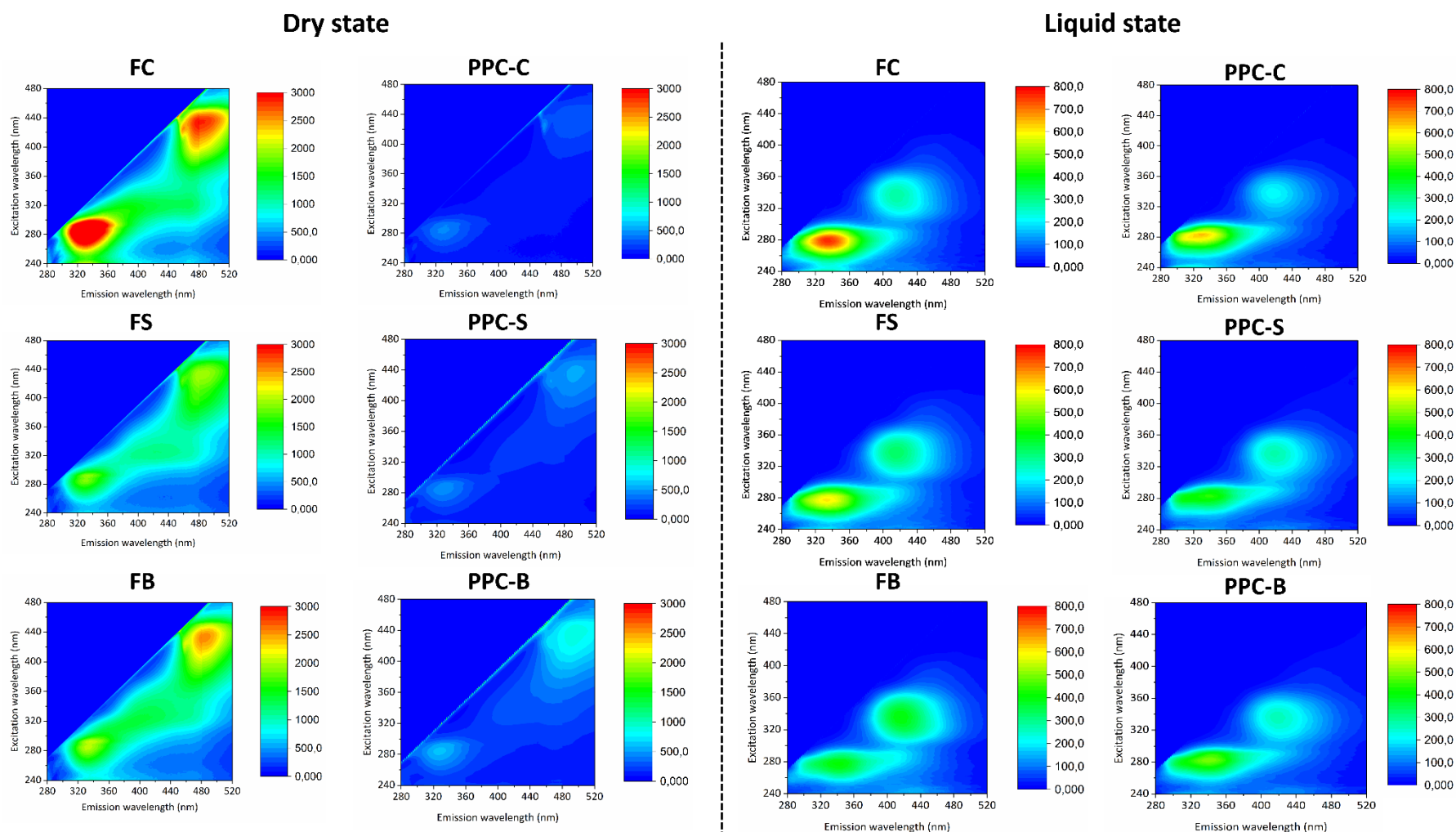


Figure 3.3. Three-dimensional excitation-emission fluorescence matrices (3D-EEFM) of pigeon pea flours and protein concentrates analyzed in dry and liquid state. FC: pigeon pea flour produced with raw seeds (control); FS: pigeon pea flour produced with soaked seeds; FB: pigeon pea flour produced with soaked+boiled seeds. PPC-C: protein concentrate produced with raw seeds flour (control); PPC-S: protein concentrate produced with soaked-seeds flour; PPC-B: protein concentrate produced with soaked+boiled seeds flour.

3.4 Protein concentrates obtained from soaked and soaked-boiled pigeon pea seeds: conformation and functionality

3.4.1 Protein conformation

Protein extraction from pre-treated flours allowed investigating aspects related to the conformation and functionality of pigeon pea proteins. The process yield (about 13%, w w⁻¹) and protein recovery (about 20%, w w⁻¹) were not affected by seed pre-treatments and their values are shown in Supplementary Table 3.2.

The deconvolution of the amide I region in the FT-IR spectrum (1600 to 1700 cm⁻¹) showed differences in the composition of the secondary structures of PPC-B, compared to the other two concentrates. The boiling treatment resulted in a decrease in β -sheet and α -helix structures and in an increase in disordered structures (random coil) (Supplementary Figure 3.3), which is an indication of higher molecular flexibility and chain unfolding provided by the heat treatment (Sun et al., 2020). On the other hand, the secondary structures of proteins were similar in PPC-C and PPC-S, with the β -sheet being the most prevalent structure (46-47%), followed by the α -helix (18-20%), turn (15 -16%), random coil (10-12%) and β -antiparallel (9%).

The tryptophan emission peaks for PPC-C and PPC-S had intensity maxima at 338 and 341 nm, respectively, whereas the peak of PPC-B had a lower intensity and a shift of the peak maximum to 357 nm (Fig. 3.4a) ($p < 0.05$). They could indicate an unfolding of the protein chains (Vivian & Callis, 2001). A second peak was observed for the three samples (from 402 to 408 nm), which may be indicative of the presence of phenolic compounds (Yan et al., 2021).

3.4.2 Solubility and electrostatic charge

Solubility is a key parameter for the performance of proteins functional properties such as emulsifying, foaming and gelling agents in food systems. In this work, the solubility of protein concentrates was defined as the amount of soluble proteins in the supernatant of a suspension after centrifugation under predefined conditions. The solubility at different pHs presented the characteristic U-shape curve of proteins (Fig. 3.4b), in which higher solubility values are observed at acid and alkaline extremes and lower solubility values are observed in regions close to the isoelectric point of globulins (pH 4-6) (Gravel & Doyen, 2023). PPC-C showed a higher protein solubility at pH 2.0 when compared to PPC-S and PPC-B ($p < 0.05$). Thermal processes can alter protein solubility due to denaturation that results in exposure of hydrophobic groups and formation of aggregates/precipitates. Irreversible changes in the structure of proteins can occur in processes that involve temperatures higher than the denaturation temperature of these proteins (Buhler et al., 2020). This is the case of the present work, in which the thermal process involved temperatures (100 °C) higher than the denaturation temperatures reported for pigeon pea proteins (90.4 to 96.7 °C) (Sosa, Chaves, Quiroga, & Avanza, 2021). This may be an indication that irreversible changes were caused in the structure

of the proteins, decreasing their solubility. Although PPC-S did not show evidence of changes in its conformation, its solubility was also affected.

At the alkaline extreme (pH = 12.0), PPC-B showed a higher solubility compared to the other protein concentrates, whereas PPC-S showed higher protein solubility at intermediate pHs. The causes of these differences in solubility are unclear. Pre-treatments that involve the use of heat can affect solubility in different ways. Wang et al. (2020) reported that the solubility of quinoa protein isolate pre-treated by boiling and microwaves had a solubility equivalent to that of the untreated protein isolate, while other heat treatment methods such as steaming and baking reduced its solubility.

The zeta potential of pigeon pea protein concentrates ranged from 22.3 mV to -32.5 mV (Fig. 3.4c). These values agree with the surface charge of several protein isolates as evaluated by Ge et al. (2021), such as protein isolates from pea, mung bean, black bean, kidney bean, cowpea, soybean, chickpea, etc. No difference was observed between treatments ($p < 0.05$), except for pH 3.0. Sun et al. (2020) also did not observe differences in the surface charge of non-treated vs microwave-heated pigeon pea proteins. All samples showed lower solubility and zeta potential close to zero between pH 3.0 and 4.0, comprising the region of the isoelectric point of the extracted proteins.

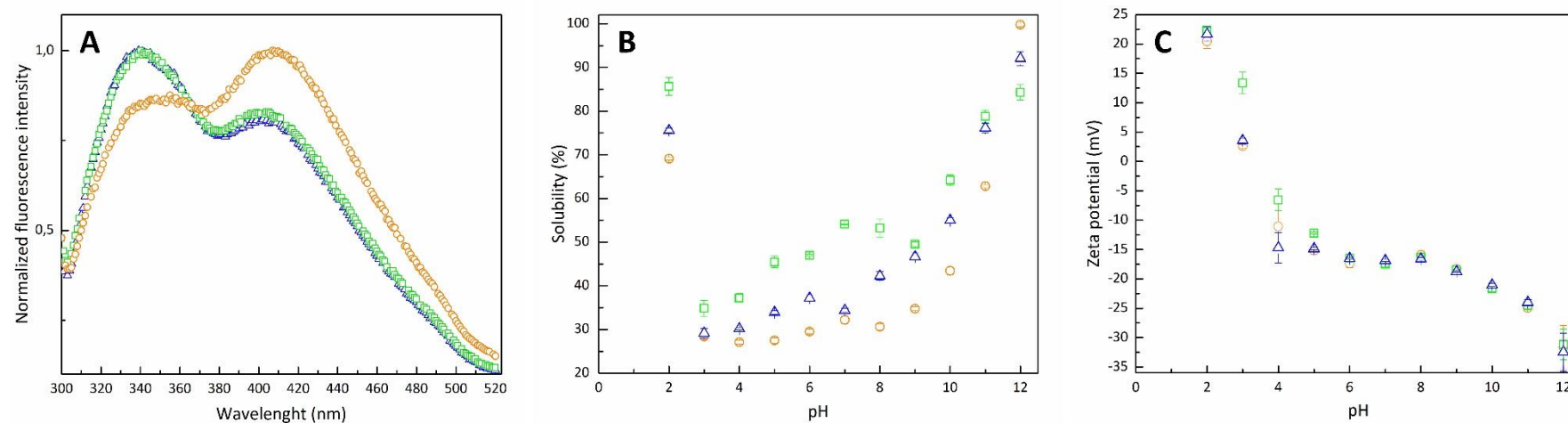


Figure 2.4. Intrinsic fluorescence spectra ($\lambda_{\text{Ex}} = 280 \text{ nm}$) (a), solubility (b) and zeta potential (c) of pigeon pea protein concentrates. Green square symbols: protein concentrate produced with raw seeds (PPC-C); blue triangle symbols: protein concentrate produced with soaked seeds (PPC-S); orange circle symbols: protein concentrate produced with soaked+boiled seeds (PPC-B).

3.4.3 Functional properties

The way water interacts with protein ingredients can determine the sensory and storage properties of various food products (Alfaro-Diaz et al., 2021). Determining the water holding capacity (WHC) is a way of evaluating how much water certain mass of protein ingredient can absorb. A high WHC is important for developing structured products and for reducing moisture losses in packaged products (Ge et al., 2021; Buhler et al., 2020). Soaking + boiling pre-treatment increased the WHC of PPC-B (3.7 g g^{-1}) when compared to PPC-C and PPC-S ($3.1\text{--}3.3 \text{ g g}^{-1}$) ($p < 0.05$) (Fig. 3.5a). The increase in WHC of PPC-B may be due to the partial denaturation of proteins caused by heating, thus exposing the peptide bonds and polar side chains (Arise, Aliyu, & Ajidagba, 2020), and the higher starch content presented by this sample (Table 3.2), since starch may have an additive effect with proteins on ingredient hydration.

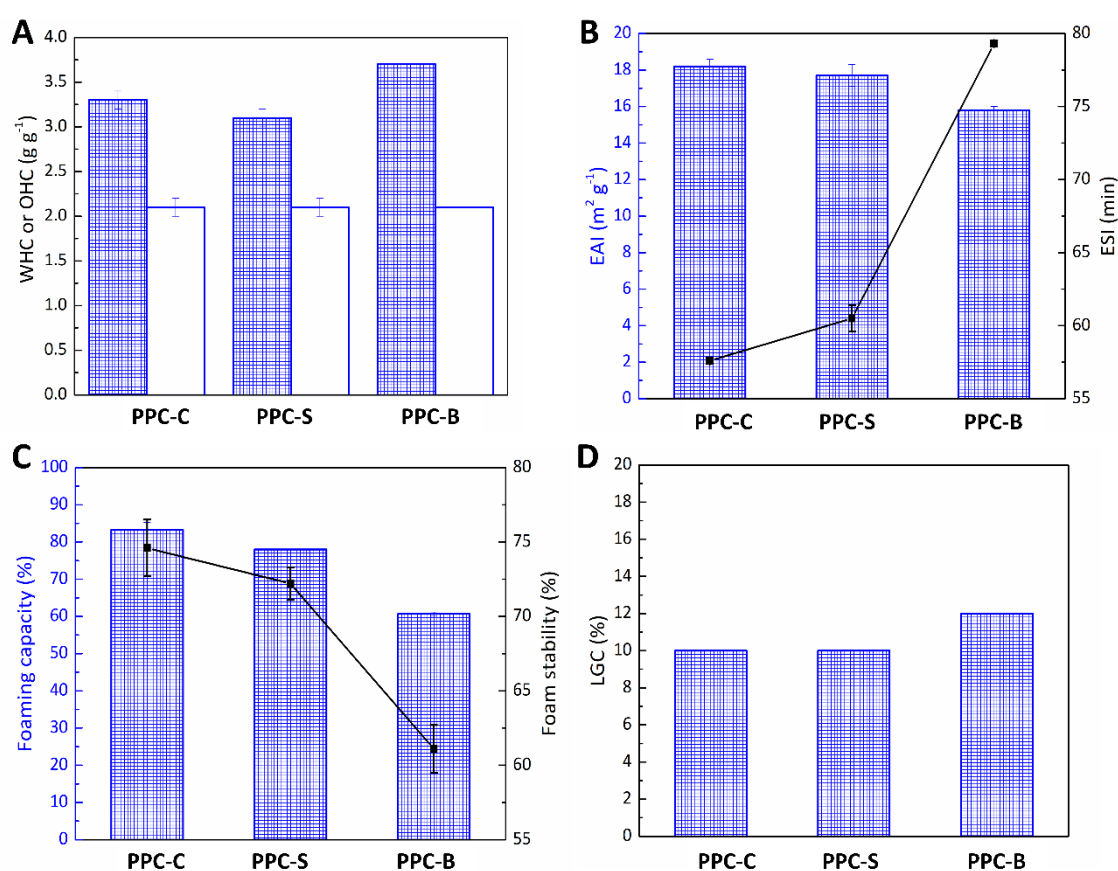


Figure 3.5. Functional properties of pigeon pea protein concentrates. **(a)** Water holding capacity (WHC, blue bar) and oil holding capacity (OHC, white bar); **(b)** emulsifying activity index (EAI, blue bar) and emulsion stability index (ESI, black line); **(c)** foaming capacity (FC, blue bar) and foam stability (FS, solid black line); **(d)** Least gelation concentration (LGC). PPC-C: protein concentrate produced with FC; PPC-S: protein concentrate produced with FS; PPC-B: protein concentrate produced with FB.

On the other hand, the oil holding capacity (OHC) was the only evaluated functional property that was not affected by either treatment. Wang et al. (2020) also did

not observe the impact of boiling treatment on OHC, which ranged from 2.4 to 2.8 g g⁻¹ for heat-treated or non-heat-treated quinoa protein concentrates. This is worth noticing, considering the variation in lipid content presented by the samples. These results suggest that the oil holding capacity is not necessarily linked to the amount of endogenous lipids in the ingredients.

The emulsifying properties of a protein ingredient are affected by several factors, such as: pH, surface charge, solubility, hydrophobicity, molecular flexibility, conformation state and protein composition (Ge et al., 2021). Among the indicators that can be used to describe the emulsifying properties are the emulsifying activity index (EAI) and the emulsion stability index (ESI). EAI is based on the turbidity measurement of a diluted emulsion, providing an estimate of the stabilized interfacial area per unit weight of protein (m² g⁻¹). The ESI measures the stability of the same diluted emulsion over a certain time, expressed in min (Karaca, Low, & Nickerson, 2011). PPC-B showed lower EAI (15.8 m² g⁻¹) when compared to samples PPC-C and PPC-S, which showed values of 18.2 and 17.7 m² g⁻¹, respectively ($p < 0.05$). A low emulsification and gel formation capacity of PPC-B can be explained by its lower solubility and conformational changes (Fig. 3.4). Phospholipids are compounds that also may play an important role in the emulsifying capacity of food ingredients. Phospholipids with a high PC content, for example, can contribute to oil droplet stabilization (Xia et al., 2022) and, of the pigeon pea protein concentrates evaluated, PPC-B was the only one that did not present significant amounts of phospholipids. The absence of phospholipids in PPC-B may also have contributed to the lower EAI observed for this sample compared to the others (Fig. 3.5b), highlighting the influence that lipids may have on the functional properties of these ingredients (Vondel, Janssen, Wouters, & Delcour, 2023).

It is interesting to note that, in spite of the fact that the boiling treatment decreased the emulsifying capacity of the pigeon pea protein concentrate, it had a positive impact on emulsion stability. The ESI of PPC-B (79.3 min) was higher than that of PPC-S (60.5 min) and PPC-C (57.6 min) ($p < 0.05$). Cai et al. (2020) observed similar ESI values for soy protein isolate and okara protein concentrate, which ranged from 54 to 61 min. The positive effect on emulsion stability caused by the boiling treatment, however, can be explained by stronger protein-protein interactions resulting from thermal processing, thus preventing coalescence of the droplets over time (Iyenagbe et al., 2017). The higher starch content presented by PPC-B may have also contributed to this result. The presence of polysaccharides such as cellulose, pectin and starches can affect the stability of the emulsion through interactions with proteins, modifying the viscosity of the interfacial film and reducing the size of the droplets, delaying or hindering destabilization mechanisms of emulsified systems (Locali-Pereira, Guazi, Conti-Silva, & Nicoletti, 2021; Albano, Cavallieri, & Nicoletti, 2019).

The foaming capacity was higher for PPC-C (83.3%), whereas the PPC-S and PPC-B showing reductions of 6.4 and 27.1% of this value, respectively (Fig. 3.5c). Regarding foaming stability, PPC-C and PPC-S showed similar values (74.6 and 72.2%, respectively),

which were higher than the observed for PPC-B (61.1%) ($p < 0.05$). Arise et al. (2020) also observed poor foaming properties for bambara protein isolate extracted from boiled-seeds. The decrease in foaming properties caused by the soaking treatment can be explained by the leaching of minor compounds that act in foam formation and stabilization. Saponins, for example, which are excellent foaming agents, are present in pigeon pea seeds in amounts that can vary from 2164 to 3494 mg/100 g (Singh, Singh, Singh, & Kaur, 2017) and their losses after soaking can reach 8%, whereas losses after cooking rise up to 66% (Duhan, Khetarpaul, & Bishnoi, 2001).

Protein concentrates produced from raw and soaked seeds presented LGC of 10 %, while the protein concentrate produced from soaked-boiled seeds presented LGC of 12 % ($p < 0.05$). (Fig. 3.5d). Wang et al. (2020) also obtained higher LGC (16%) for quinoa protein isolate extracted from boiled seeds, in relation to quinoa protein isolate extracted from raw seeds (12%).

4. Conclusions

Our study delved into the effects of soaking and boiling pre-treatments on pigeon pea seeds and their subsequent ingredients flour and protein concentrates. These pre-treatments modified the microstructure and size distribution within the resulting flours, primarily driven by solids leaching processes, starch gelatinization, and protein denaturation. The carbohydrate composition showed a greater accumulation of starch in protein concentrates produced with soaked-boiled seed flours and all protein concentrates showed mannose residues from oligo and/or polysaccharides. The total lipid content decreased in pre-treated flours, whereas the protein concentrates showed a significant accumulation of lipids. In addition, the use of soaked-boiled seeds for protein fractionation resulted in the total elimination of phospholipids in protein concentrates, which can affect the functionality of these ingredients due to the well-known action of these lipids in stabilizing emulsions and foams, for example. We also demonstrated remarkable efficiency in reducing trypsin inhibitor activity and raffinose family oligosaccharides using flour from soaked-boiled seeds for protein fractionation processes, a relevant aspect for pulse ingredients digestibility. Regarding functional properties, soaking reduced the foaming capacity of protein concentrates, possibly due to the elimination by leaching of compounds that have foaming ability, such as saponins. Since soaking can be used as a preliminary step in various pre-treatments, including dehulling, germination, and fermentation, these results are relevant to design several processes. On the other hand, boiling showed to be a favorable process for enhancing water holding capacity and emulsion stability in protein concentrates, thus offering advantageous prospects for food product applications. Molecular structural analysis indicated that boiling induced a degree of protein unfolding, that may explain the changes observed in its functional properties. Our results encourage the use of pre-treated seeds to produce protein concentrates due to the advantages provided for better digestion of these ingredients, such as reduction of anti-nutritional factors. However, the impacts on

functionality are aspects that must be taken into consideration, depending on the intended application of the protein concentrates.

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Supplementary material

Pre-treatment effects on the composition and functionalities of pigeon pea seed ingredients

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1.Methods

1.1 Process yield and protein recovery

The process yield (%) of pigeon pea protein concentrates was calculated by the mass ratio between the obtained powdered extract and the initial flour (Equation 1), whereas protein recovery (%), was calculated by the mass ratio between the protein contents in the freeze-dried extract and in the initial flour (Equation 2).

$$\text{Process yield (\%)} = \frac{\text{protein extract (g)}}{\text{initial flour (g)}} \times 100 \quad (1)$$

$$\text{Protein recovery (\%)} = \frac{\text{extract protein content (g)}}{\text{flour protein content (g)}} \times 100 \quad (2)$$

1.2 Attenuated total reflectance- Fourier transform infrared spectroscopy (ATR-FTIR)

The ATR-FTIR spectra of pigeon pea protein concentrates were recorded using a Fourier transform infrared spectrometer (VERTEX 70v, Bruker, USA) at 400-4000 cm^{-1} by placing the samples directly on the ATR crystal. The analysis of the secondary structures of proteins was performed through the second derivation and Gaussian deconvolution of the FTIR spectra in the amide I region (1600-1700 cm^{-1}), using the Peak Analyzer function of the OriginPro 2016 software (Massachusetts, USA.). The peaks generated by deconvolution were classified according to the following types of secondary structures: β -sheet (1610-1642 cm^{-1}), random coil (1643-1650 cm^{-1}), α -helix (1650-1659 cm^{-1}), turn (1660–1670 cm^{-1}) and β -antiparallel (1671–1692 cm^{-1}) (Sun et al., 2020; Sadat and Joye, 2020). The secondary structures of the proteins were quantified through the percentages of the peak areas.

2. Results



Supplementary Figure 3.1. Physical aspect of raw (control), soaked and soaked-boiled pigeon pea seeds and their respective flours and protein concentrates.

Supplementary Table 3.1. Size distribution parameters of pigeon pea flours.

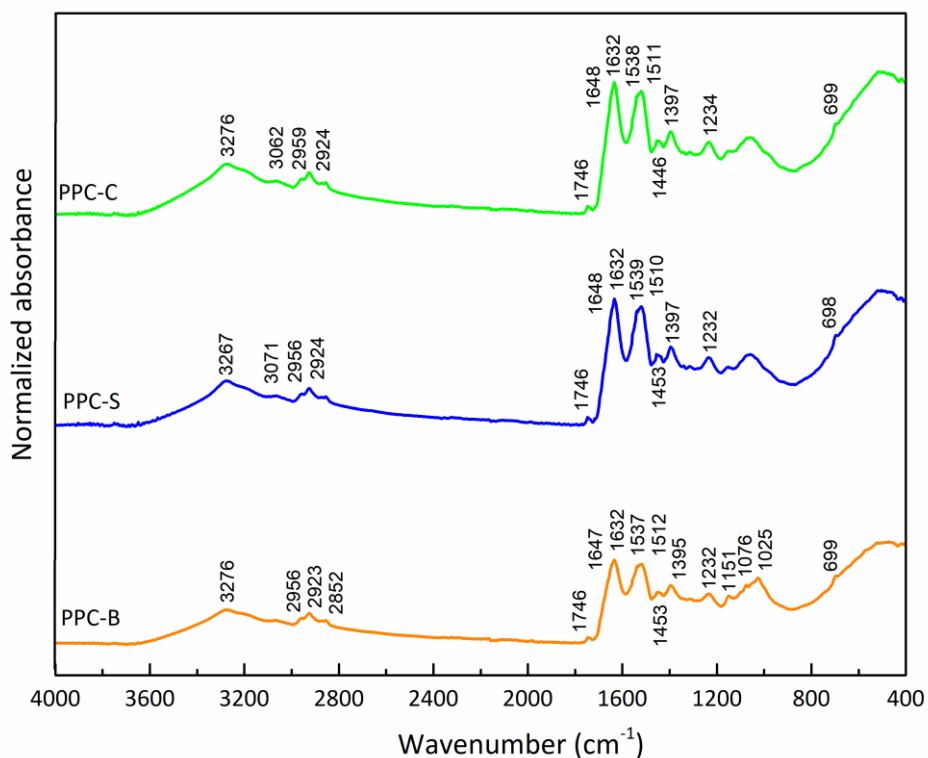
Size parameters	FC	FS	FB
$D[3,2]$ (μm)	59.5 ± 0.5^c	189.3 ± 9.5^a	145.7 ± 2.4^b
$D[4,3]$ (μm)	287.7 ± 3.5^b	557.0 ± 13.0^a	541.7 ± 8.4^a
d10 (μm)	22.0 ± 0.1^c	79.0 ± 6.8^a	61.6 ± 1.2^b
d50 (μm)	226.0 ± 0.5^b	489.7 ± 9.0^a	496.7 ± 9.8^a
d90 (μm)	664.3 ± 13.0^b	1123.3 ± 20.0^a	1080.0 ± 13.3^a

Different letters in the same line indicate statistical difference ($p < 0.05$). FC: pigeon pea flour produced with raw seeds (control) ; FS: pigeon pea flour produced with soaked seeds ; FB: pigeon pea flour produced with soaked-boiled seeds.

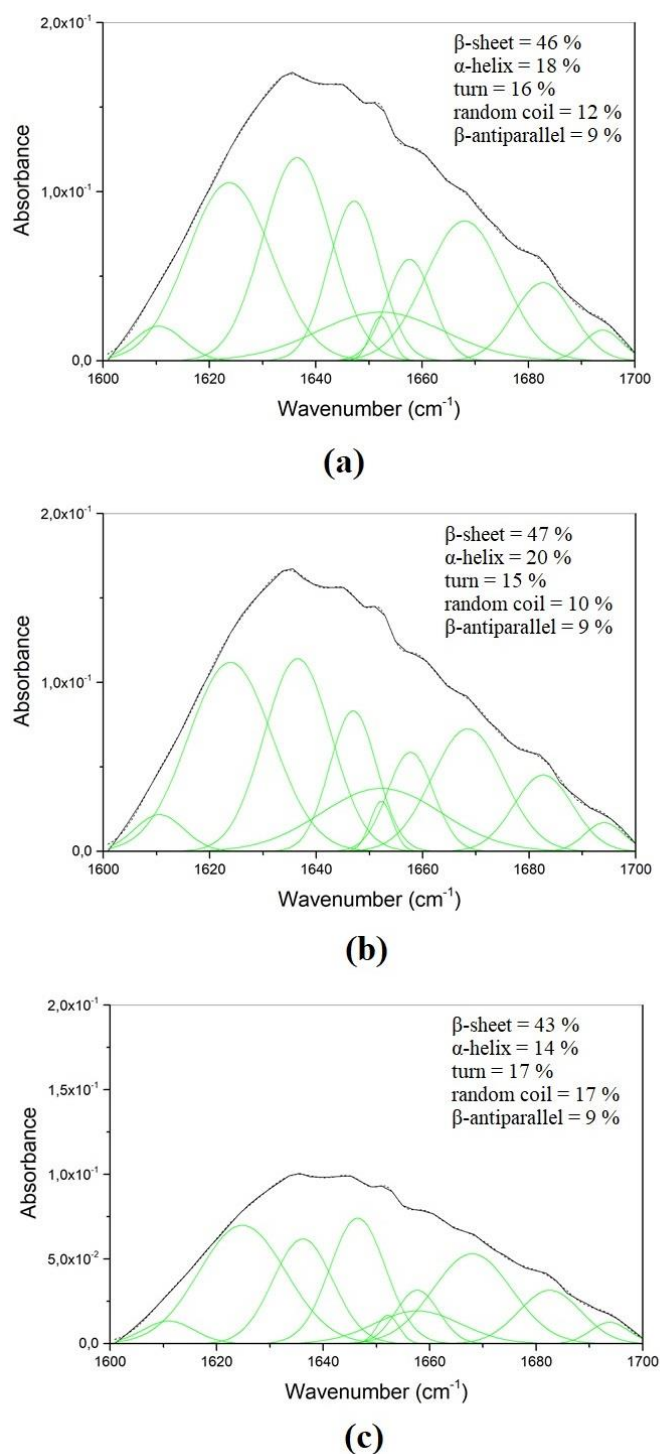
Supplementary Table 3.2. Yield and Protein recovery of pigeon pea protein concentrates.

Protein concentrate	Yield (% w w ⁻¹)	Protein recovery (% w w ⁻¹)
PPC-C	12.8 ± 0.2^a	21.3 ± 0.5^a
PPC-S	13.0 ± 0.1^a	20.2 ± 0.8^a
PPC-B	13.1 ± 0.2^a	20.0 ± 0.2^a

Different letters in the same column indicate statistical difference ($p < 0.05$). PPC-C: protein concentrate produced with raw seeds flour (control); PPC-S: protein concentrate produced with soaked-seeds flour; PPC-B: protein concentrate produced with soaked-boiled seeds flour.



Supplementary Figure 3.2. Fourier transform infrared (FT-IR) of pigeon pea protein concentrates. Green line: protein concentrate produced with raw seeds (PPC-C); blue line: protein concentrate produced with soaked seeds (PPC-S); orange line: protein concentrate produced with soaked+boiled seeds (PPC-B).



Supplementary Figure 3.3. Gaussian deconvolution of FTIR spectra of pigeon pea protein concentrates in the amide I region (1600-1700 cm^{-1}). (a) PPC-C: pigeon pea protein concentrate produced with raw seeds flour; (b) PPC-S: pigeon pea protein concentrate produced with soaked seeds flour and (c) PPC-B: pigeon pea protein concentrate produced with soaked-boiled seeds flour.

Chapter 4. Preparative-scale purification and physicochemical characterization of pigeon pea 7S globulin

Preamble

The previous chapter highlighted the highly complex composition of protein fractions, which implies the need for a simpler system to understand and characterize the intrinsic properties of individual proteins, like the 7S globulin. Liquid chromatography techniques can be used to set up a purification procedure to separate target proteins. By purifying proteins, it is possible to infer their role in the overall functionality of more complex food ingredients. The 7S globulin is the main protein in pigeon pea seeds, corresponding to around 60% of their composition. Previous studies have investigated the purification of pigeon pea 7S globulin on an analytical scale (Krishna et al., 1977). However, to probe functional properties of these proteins, it is necessary to migrate the purification process from the analytical to the preparative scale, to obtain purified fractions in sufficient amounts for analyzing their functionality. This section is dedicated to the protocol developed for preparative-scale purification of pigeon pea 7S globulin.

Main questions

- How to separate pigeon pea 7S globulin using a preparative scale purification process?
- Is the purification process yield suitable to probe functional properties?
- What are the physicochemical characteristics of pigeon pea 7S globulin?

This section is divided into two parts, the first part is dedicated to the development of a preparative-scale purification protocol of pigeon pea 7S globulin and the second part is dedicated to the characterization of the physicochemical properties of the resulting purified fractions. The preparative-scale purification process was developed with three steps: extract desalting, anion exchange chromatography and size exclusion chromatography. The purified fractions were characterized in terms of their UV-Vis spectrum, extinction coefficient, refractive index increment, solubility, electrophoretic mobility and size distribution by dynamic light scattering.

Main results

- The pigeon pea 7S globulin had a purity of around 89% and sugars are the major non-protein compounds;
- Native 7S globulin is composed of an α and β chains of 7S globulin, with molecular weights of around 64 and 49 kDa;
- A partial hydrolysis of 7S globulin was observed in one of the purification processes used;
- The partial hydrolysis of 7S globulin presented unconventional values for the physico-chemical parameters evaluated.

Part I. Preparative chromatography for purification of 7S globulin from pigeon pea seeds

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1. Introduction

Pigeon pea is a protein-rich pulse (18-28 wt %) native to emerging countries in Asia and Africa (Fuller et al., 2019). Globulins comprise the largest portion of pigeon pea proteins (around 60%), with the 7S fraction being the most abundant (Krishnan et al., 2017). Understanding the interplay between the physicochemical and the functional properties of individual globulins of pulse protein ingredients is of great importance for optimising their performance in food systems.

Krishna and co-workers were pioneers in investigating the composition of pigeon pea proteins using extraction methods followed by precipitation and/or purification by analytical chromatography (Krishna et al., 1977; Krishna & Bhatia, 1985, 1986). In their first study, pigeon pea proteins were extracted using solubilization/centrifugation/precipitation cycles (Krishna et al., 1977), which led to the identification of vicilin and legumin-type proteins. Then, they developed the first purification protocol for pigeon pea 7S vicilin on an analytical scale (Krishna et al., 1985). Firstly, a crude extract of globulins was obtained by alkaline extraction followed by isoelectric precipitation. The 7S globulin was then purified using zonal isoelectric precipitation and injection on a weak anion exchange column. According to the authors, 7S globulin has a molecular weight of around 184 kDa in its native state, with a 70 kDa subunit and two 57 kDa subunits. More recently, other studies have purified pigeon pea globulins (Bravo et al., 2022; Fernández Sosa et al., 2021). This was based on differential solubility and/or ammonium sulfate precipitation. While the purification of globulin

proteins has been a crucial step in elucidating their composition and structure, the analytical scale procedures reported in the literature yielded pigeon pea globulins on a milligram scale. However, to probe the functionality of purified proteins, it is necessary to purify them at the gram scale by upscaling from analytical to preparative chromatography (Gueguen et al., 1984; Tang & Sun, 2010). Moreover, milder processes, which do not use protein precipitation steps, have also not been investigated, and are more suitable for evaluating the functionality of proteins.

Preparative chromatography can be defined as a unity operation used to isolate a compound in sufficient purity and quantity for subsequent processes or experiments (Dapremont, 2014). Chromatographic methods are commonly used to isolate 7S fractions from globulin protein concentrates or isolates (Gravel & Doyen, 2023), and procedures on a preparative scale have been proposed for pea (Crévieu et al., 1996; Gueguen et al., 1984; Larré & Gueguen, 1986), rapeseed (Bérot et al., 2005), and bean globulins (Suchkov et al., 1990). The chromatographic steps may include: (i) desalting of the crude protein extract to remove small molecules that can form complexes with proteins (Bérot et al., 2005); (ii) ion exchange chromatography, in particular anion exchange chromatography, which is most used for globulin fractionation as it is suitable for proteins with a low isoelectric point (Gravel & Doyen, 2023; Gueguen et al., 1984); and (iii) gel filtration separation, which allows separating proteins based on molecular size, removing aggregates and minor compounds. Dialysis/ultrafiltration and/or gel filtration of protein fractions is commonly used before freeze-drying to remove salt and increase protein concentration (Gravel & Doyen, 2023).

Therefore, we developed a preparative scale purification procedure for pigeon pea 7S globulin using mild protein extraction process and the following chromatographic steps: desalting of the protein extract; separation on an anion exchange column, and subsequent separation on a gel filtration column. Two approaches were investigated: (i) salt removal by dialysis/ultrafiltration against ammonium carbonate buffer before and after separation in the gel filtration column and (ii) salt removal during the separation in the gel filtration column equilibrated with ammonium carbonate buffer (without dialysis/ultrafiltration). The biochemical properties of the resulting purified fractions were determined using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), size exclusion chromatography with multi-angle light scattering (SEC-MALS), fluorescence intrinsic measurements, and proteomic analysis. The foaming properties of the purified fractions were also investigated.

2. Materials and methods

2.1 Materials

Pigeon pea seeds (*Cajanus cajan*) were purchased from a local supplier (Sao Paulo, Brazil). Tris-glycine SDS running buffer, and Tris base were supplied by ThermoFisher (Massachusetts, USA). Sodium phosphate, sodium carbonate, sodium chloride, citric acid, MES free acid, ammonium carbonate, potassium phosphate monobasic, disodium phosphate, sodium hydroxide, hydrochloric acid, and 2- β -mercaptoethanol were supplied by Sigma-Aldrich (Missouri, USA). Acetic acid was provided by Carlo Erba (Emmendingen, Germany). Coomassie Blue staining G250 was supplied by Serva (Heidelberg, Germany).

2.2 Protein composition of pigeon pea flour by SE-HPLC

The protein composition of pigeon pea flour was investigated by size exclusion high-performance liquid chromatography (SE-HPLC) under denaturing conditions. To produce the flour (< 300 μ m), pigeon pea seeds were first ground using a disk mill (J.R. Araújo & Cia Ltda., Brazil) at 1730 rpm for 5 min. Proteins were separated according to their molecular weight and quantified by SE-HPLC, following the protocol described by Morel et al. (2000), with some modifications. Around 10 mg of flour or protein powder were used for protein extraction in 1 mL of 0.1 M sodium phosphate buffer pH 6.8 containing 1% SDS. Extraction was performed on a rotary shaker (Heidolph Reax) (60 rpm for 60 min at 20°C) and the supernatant with SDS-soluble proteins was recovered after centrifugation (20817 x g, 5 min, 20 °C). An additional extraction was performed from the pellet to recover SDS-insoluble proteins linked by disulphide bonds, using the previous solvent added with a thiol reducing agent, dithioerythritol (DTE) at 20mM. The SE-HPLC apparatus (Schimadzu) was equipped with an analytical column, TSK G4000-SWXL (Tosoh bioscience) (7.8 x 300 mm) and a TSK gel SW XL pre-column (4cm x6 mm) (Merck, Darmstadt, Germany). A volume of 20 μ L was loaded and eluted at 25°C with the 0.1 M sodium phosphate buffer pH 6.8, SDS 0.1% with at a flow rate of 0.7 mL min⁻¹. The absorbance spectra were recorded from 214 nm. Apparent molecular weights (MWs) were assessed with column calibration with protein standards with known MWs. The composition of SDS-soluble and SDS-DTE-soluble proteins was also investigated by SDS-PAGE.

2.3 Extractability of pigeon pea proteins

About 60 mg of flour were dispersed in 3 mL of each of the following buffer solutions (concentration = 20 mM): acetate buffer (pH 4.0, pK_a = 4.76), citrate buffer (pH 6.0, pK_a = 6.39), phosphate buffer (pH 8.0, pK_a = 7.2) and carbonate buffer (pH 10.0, pK_a = 10.25). Each buffer solution was evaluated for low (50 mM) and high (500 mM) ionic strength, using sodium chloride if necessary to achieve desired ionic strength. For the pH 6.0 buffer solution at low ionic strength, MES free acid (pK_a = 6.21) was used instead of

citric acid, due to the high contribution of citric acid to the ionic strength. The suspensions were agitated for 1 h at 20 rpm. After agitation, the tubes were centrifuged (10000 x g, 10 min, 20 °C) and the supernatant was collected. The nitrogen content in the protein extracts was determined by Dumas analysis; protein composition was assessed by SDS-PAGE, UV-visible absorbance spectra, intrinsic fluorescence and dynamic light scattering (DLS) (methodology presented in section 2.6).

2.4 Laboratory-scale purification of pigeon pea 7S globulin

2.4.1 Removal of small molecules from the protein extract

To remove small molecules from protein extracts, two approaches were used: membrane ultrafiltration and injection into a desalting column. First, 100 mg of pigeon pea flour were dispersed in 5 mL of 50 mM Tris buffer pH 8.0. The mixture was stirred for 1 h and centrifuged (10000 x g, 10 min, 20 °C) with supernatant collection. A 5-mL aliquot of buffer was added to the pellet and a new agitation and centrifugation were carried out, followed by collection of the supernatant. Finally, the two supernatants were pooled.

For ultrafiltration, 5 mL of protein extract were transferred to a membrane ultrafiltration tube (Amicon® Ultra Centrifugal Filters, 3K, Regenerated Cellulose, 3000 NMWL). The tube was centrifuged (5000 x g, 5 min, 20 °C) and the filtrate was collected. The retentate was added with buffer (up to 5 mL) and two additional centrifugations were performed with subsequent collections of the filtrates and the retentate. The three filtrates were pooled. At the end of the process, the retentate and the filtrate were evaluated by fluorescence measurements (as described in section 2.6.8).

For the desalting step, a desalting column (HiPrep 26/10, Cytiva) equilibrated in Tris 50 mM pH 8.0 was used at 12 mL/min. The injection was performed in two 10-mL runs and the volume of the collected fraction was 15 mL. The separated peaks were collected and evaluated by fluorescence measurements in the same way as for the ultrafiltered samples.

2.4.2 Optimization of fractionation conditions by anion exchange and size exclusion chromatography

For separation by anion exchange chromatography (AEC), the ultrafiltered and the desalted extracts were injected into a Q Sepharose FF column (HiTrap™ IEX Selection kit, GE HealthCare) coupled to an Akta Avant 25 chromatography system (GE HealthCare). Samples were injected at 1 mL/min and elution was conducted on a NaCl gradient (step 1 = 0 to 0.75 M in 20 mL and, then step 2 = 0.5 to 1 M in 5 min). The absorbance of the eluted proteins was recorded at 280 nm. The collected fractions were evaluated by polyacrylamide gel electrophoresis for identification of the 7S globulin fraction. Fractions with the same band composition of 7S globulin were pooled. To improve further separation, two approaches were used for the injection of the extracts in the Q Sepharose column: (i) increase of the NaCl elution gradient (step 1: 0 to 0.75 M in 40 mL and step 2:

0.5 to 1 M in 5 min) and (ii) addition of 1 M NaCl solution in the extract to obtain a final concentration around 100 mM before the injection + increase of the elution gradient of NaCl. For further purification by size exclusion chromatography (SEC), fractions were injected (100 μ L) into a Superose™ 6 column (Cytiva) equilibrated in Tris 50 mM pH 8.0 NaCl 150 mM at 0.5 mL/min connected to a HPLC system (1260 Infinity, Agilent). The ultrafiltered and the desalted extracts were also evaluated by gel filtration for comparison with the purified 7S globulin.

2.5 Preparative scale purification of pigeon pea 7S globulin

Chromatography was conducted onto fast protein liquid chromatography (FPLC) system (Akta pure 150, GE Healthcare). First, pigeon pea flour (200 g) was dispersed in 1 L of 50 mM Tris buffer pH 8.0 EDTA 5 mM and the suspension were stirred for 1 h in a mechanical stirrer (RZR 2041, Heidolph) at 225 rpm. Then, the suspension was centrifuged (10000 x g, 10 min, 20 °C) and the supernatant was collected. Another 1 L of buffer was added to the pellet with a new agitation and centrifugation with collection of the supernatant. The two supernatants were pooled, centrifuged again (10000 x g, 40 min, 20 °C) and filtered (8- μ m, mixed cellulose esters (MCE) membrane, Millipore®). The extract was injected into a desalting column (100 x 887 mm) filled with cellufine GH25 cellulose gel (Amicon-Millipore) equilibrated in 50 mM Tris buffer pH 8.0, to eliminate pigments and minor compounds. The injection was conducted at 50 mL/min. The collected volume of desalted extract was 2.8 L. After collection, 280 mL of 50 mM Tris buffer pH 8.0 + 1 M NaCl solution were added to the extract.

Then, the desalted extract (around 3.1 L) was injected in column (50 x 150 mm) filled with Q-Sepharose Fast Flow resin (GE Healthcare Bio-Sciences) at a flow rate of 13 mL/min. The elution was conducted in two steps: 35% of 50 mM Tris buffer pH 8.0 + 1 M NaCl solution for a time corresponding to an elution volume of 750 mL and 100% of 50 mM Tris buffer pH 8 + 1 M NaCl solution for a time corresponding to an elution volume of 750 mL. The absorbance of the eluted proteins was recorded at 280 nm and fractions were collected in 50 mL-tubes and evaluated with SDS-PAGE. After separation in the anion exchange column, purification was conducted following two distinct routes: (i) process with dialysis and (ii) process without dialysis (Figure 4.1).

In the process with dialysis, protein fractions were subjected to dialysis against ammonium carbonate buffer pH 8.0 (1 g/L) at 4 °C with four changes of buffer every 3 hours. After dialysis, the samples were freeze-dried for 72 h, and the powders were recovered. The purified fractions were solubilized in 0.1 M phosphate buffer pH 7.0 (30 g/L) and injected (flow rate = 12 mL/min) in a column (50 x 900 mm) filled with Cellufine GCL 2000 gel filtration resin equilibrated with 0.1 M phosphate buffer pH 7.0. Fractions were collected in 50 mL tubes, pooled and subjected to dialysis and freeze-drying as previously described.

In the process without dialysis, protein fractions were injected directly (flow rate = 12 mL/min) onto the Cellufine GCL 2000 gel filtration column equilibrated with

ammonium carbonate buffer pH 8 (1 g/L). Fractions were collected in 50-mL tubes, pooled and freeze-dried.

Powdered purified fractions were stored in a desiccator containing K_2CO_3 saturated solution (RH = 43.2%) at 20 °C for equilibration before further analysis.

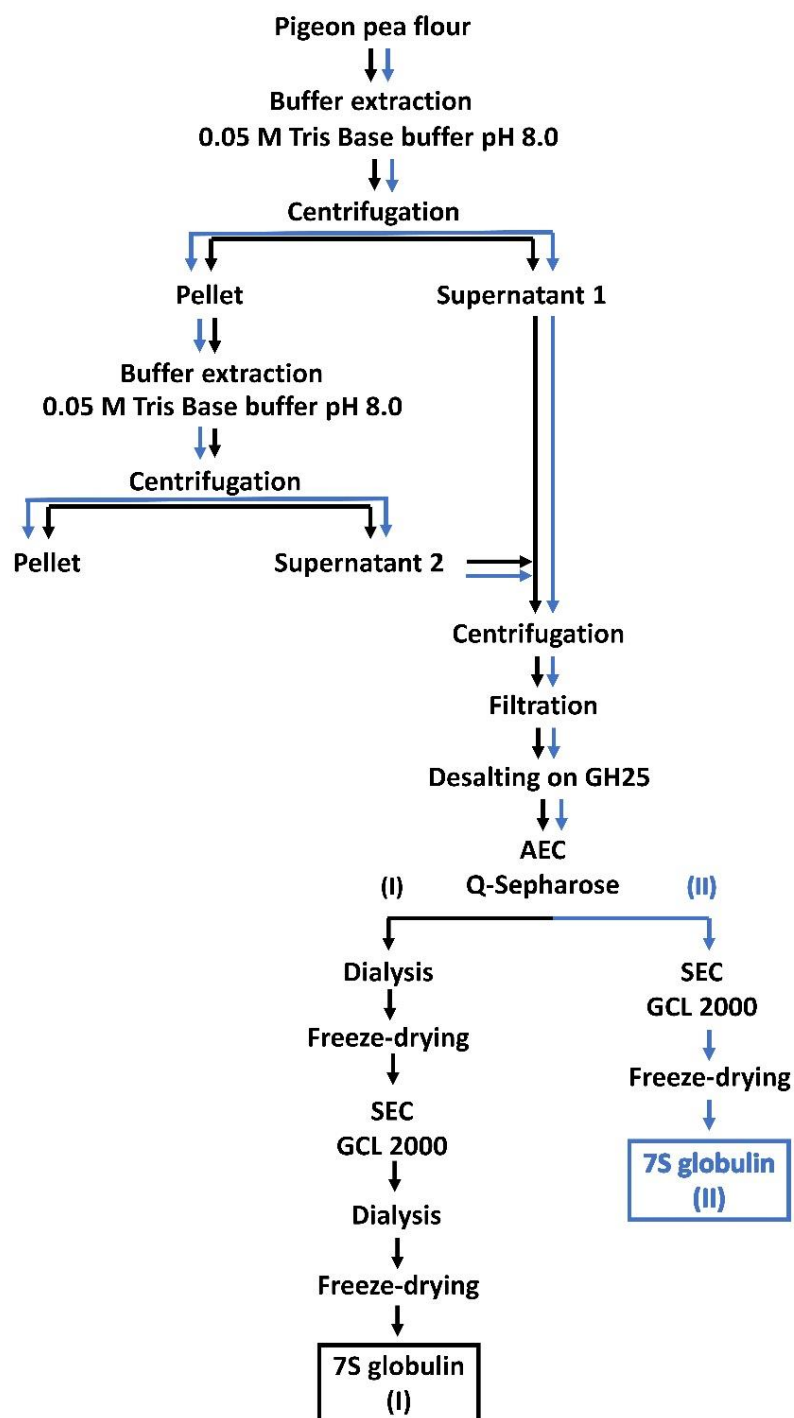


Figure 4.1. Schematic overview of the preparative-scale purification processes of pigeon pea 7S globulin.

2.6 Analytical characterization of the purified fractions

2.6.1 Process yield and protein recovery

The purification process yield (%) was calculated by the mass ratio between the obtained powdered purified fraction and the initial flour, whereas protein recovery (%), was calculated by the mass of protein recovered in the freeze-dried purified fraction and the mass of protein in the initial flour. The recovery of 7S globulin was calculated by the ratio of the mass of 7S globulin in the purified fraction and in the initial flour, based on data from the SE-HPLC analysis (Section 2.2).

2.6.2 Composition

The moisture content was determined using the gravimetric method under a nitrogen atmosphere (TGA 2050, TA-Instruments). Samples (20 mg) were heated from 20 to 105 °C (3 K/min) and held at 105 °C until constant weight.

The protein content was determined by the Dumas method using an elemental analyzer for nitrogen (Vario Micro Cube, Elementar, Frankfurt, Germany) with a nitrogen-to-protein conversion factor of 5.7 (Alamanou & Doxastakis, 1997).

Neutral sugars were determined by gas-liquid chromatography analysis, after hydrolysis of the samples to produce alditol acetate derivatives, according to the method described by Le Gall et al. (2016).

The trypsin inhibitor activity (TIA) was determined using the AOCS method Ba 12a- 2020 which determines the presence of tryptic inhibitors by a colorimetric reaction in the presence of trypsin and a substrate (N α -benzoyl-DL-arginine 4-nitroanilide hydrochloride, BAPNA) (Liu, 2021).

2.6.3 Proteomic analysis

For protein identification by proteomic analysis, the flour was first hydrolyzed with trypsin and then analyzed by liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) on a Q exactive HF set-up (Thermo Scientific™). The data were acquired in Top8 DDA with an exclusion delay of 5 seconds on a gradient of 50 min and were interpreted with X!Tandem pipeline against the Uniprot library restricted to taxon 3803 (Fabaceae; approx. 1200 k sequences). The peptide mass tolerance and the fragment mass tolerance were 5 ppm.

2.6.4 Polyacrylamide gel electrophoresis in sodium dodecylsulfate (SDS-PAGE)

First, samples were solubilized or diluted in Laemmli buffer (1 mg/mL). For reducing conditions 2- β -mercaptoethanol (5%, v v⁻¹) was added to the sample solutions. Then, all samples were heated at 95 °C for 5 min. Aliquots of 15 μ L were added to each well of the polyacrylamide gel (4-12% Bis-Tris Plus Gels, Bolt™, Invitrogen). The molecular weight marker (Precision Prestained Protein™ Standart, MW range 250-10 kDa, Bio-Rad) was also added to the gel. Electrophoresis was conducted (200 V/100 mA) until complete migration of the samples in the MES SDS running buffer (Novex). After complete migration, the gel was transferred to a container with a fixation solution (2% phosphoric

acid, 50% ethanol, v v⁻¹) and kept 1 h under agitation. Then, the gel was rinsed (2% phosphoric acid solution, v v⁻¹) for 40 min under agitation, transferred to a sensitization solution (17% ethanol, 2% orthophosphoric acid and 15% ammonium sulfate, v v⁻¹), and stained with Coomassie Brilliant Blue G250 after 20 min and kept overnight. After coloration, the gel was rinsed with water and scanned.

2.6.5 UV-visible spectroscopy

The UV-visible absorbance spectrum of samples was determined in a UV-visible microplate reader (Varioskan Lux, Thermo Scientific™), using a microplate (μDrop™, A2-3 model, Thermo Scientific™). Each well of the plate was filled with a drop of supernatant (3 μL). The samples were read in an absorbance range of 210-550 nm with a sampling interval of 2 nm.

2.6.6 Dynamic light scattering

The particle size distribution of the samples was determined by dynamic light scattering (DLS, Zetasizer Nano Series, Malvern Instruments). About 0.6 mL of each supernatant was filtered (pore size 0.45 μm, Minisart) added in a cuvette and placed in the equipment. Each sample was analyzed at 173° in triplicate at 25 °C and the refraction indices used was 1.45 for protein dispersions. For each reading, ten runs were performed by the equipment. The CONTIN model was used by the software for particle size calculations.

2.6.7 Intrinsic fluorescence measurements

Three-dimensional fluorescence measurements (excitation-emission fluorescence matrices, 3D-EFEM) were performed using a spectrofluorimeter (FP-8550, Jasco, Japan). Measurements were performed at excitation wavelengths range from 240 to 480 nm, and emission wavelengths range from 280 to 520 nm. A data step of 1 nm, as well as bandwidths of 2.5 nm for excitation and emission were set. For dry state measurements, samples were evaluated using a powder cell and performing the measurements in front-surface mode; in the liquid state, a quartz cuvette was used and the measurements were performed in right-angle mode. The liquid dispersions were prepared by dispersing the powders in buffer solutions (20 mg/mL of buffer) and centrifuging (10000×g, 10 min, 20 °C). Samples were diluted to reach an absorbance at 280 nm around 0.075.

2.7 Determination of foaming properties

Foaming properties were evaluated using protein solutions of 4 g L⁻¹. A 2 mL aliquot of each sample was added to 5 mL syringes. The syringe containing the protein solution was connected to another syringe containing 4 mL of air through a tube of 1 mm diameter. The foam was formed inside the syringes by making back and forth movements manually for 15 s. Then, the syringe containing the foam was disconnected, capped, and

inverted onto a support in front of a black background. Images were captured every 1 min by a camera (EOS, Canon, Tokyo, Japan). The total experiment time was 120 min.

The ImageJ software (NIH Image, Maryland, USA) was used to process raw foam images. The foam (white) was distinguished from the empty spaces (black) of the syringe by tuning the threshold of the gray values of pixels. The ratio between the volume occupied by the foam and the total volume of the syringe was calculated to determine the foam stability, considering the following times: 0, 1, 3, 5, 10, 15, 30, 45, 60, 90 and 120 min. All measurements were done in triplicate.

2.8 Statistical analysis

Data were analyzed using the OriginPro 2016 software. Variance analysis (ANOVA) followed by Tukey post hoc test was applied to determine the difference between samples at a 5% significance level, when relevant.

3. Results and discussion

3.1 Protein composition of pigeon pea flour

Pigeon pea flour was characterized based on its protein composition, using SE-HPLC in denaturing conditions, SDS-PAGE and proteomic analyses. Around 87% of pigeon pea proteins were soluble in SDS, 0.5% of the proteins in SDS with DTE while 13% corresponded to non-extractable proteins.

The SE-HPLC profile in denaturing conditions (Figure 3.2a) showed a broad range of molecular weights: most likely aggregated proteins with molecular weights higher than 100 kDa; groups of proteins with molecular weights around 112, 52, 38, 21 and 16 kDa; and low molecular weight non-protein compounds (< 10 kDa). Proteins with molecular weights of 52 and 38 kDa correspond to the most abundant proteins in pigeon pea flour. The SDS-PAGE analysis (Figure 4.2b) of SDS-soluble proteins showed two main subunits: one between 50 and 75 kDa and another between 37 and 50 kDa. These values are quite different from the 72 and 57 kDa subunits identified by Krishna & Bratia (1985) for pigeon pea 7S globulin. In other studies (Fernandez Sosa et al., 2021; Krishna et al., 2017) subunits of 65, 52 and 47 kDa were also reported to be characteristic of pigeon pea 7S globulin. Also, the protein bands were not sensitive to the reducing agent in our study, being compatible with 7S globulins due to the lack of cysteine residues (Duranti et al., 1981). The same protein bands were visualized in lower intensity in SDS-insoluble proteins.

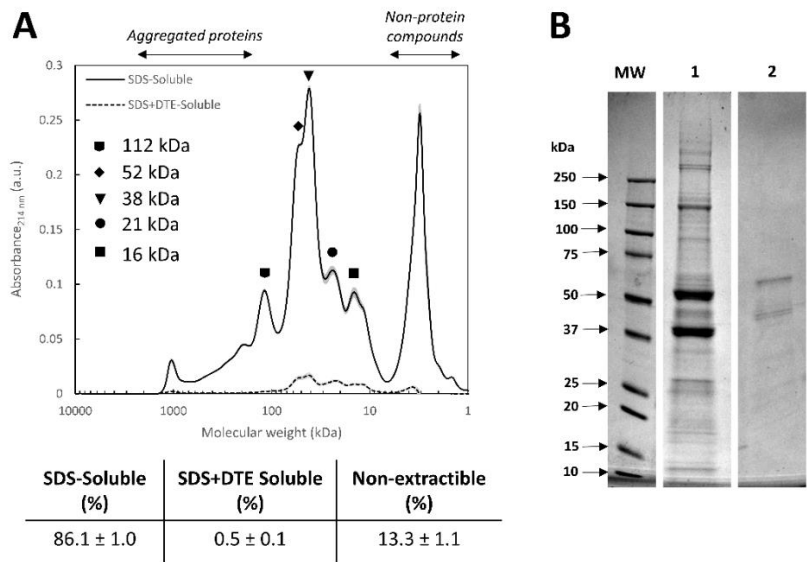


Figure 4.2. (a) SE-HPLC in denaturing conditions at 214 nm (upper) and protein extraction yields (lower) panels (b) SDS-PAGE of pigeon pea flour 1: SDS-soluble proteins, 2: SDS+DTE soluble proteins, MW: molecular weight marker (Biorad) loaded in a 4-20% gradient gels. The sample SDS-soluble has been diluted 10x as compared to SDS+DTE-soluble sample. Grey area on the SE-HPLC profile stands for the standard deviation of the three repeats.

Therefore, the characterization of pigeon pea flour showed that approximately 48 % of soluble proteins correspond to 7S globulin, that is expected to be a heterotrimer, (Krishnan et al., 1977). The ratio between the two main protein bands identified of 52 and 38 kg mol⁻¹ was 0.59 (Fig 4.2a).

Proteomic analysis of pigeon pea flour showed the presence of around 125 groups of proteins (Table 4.1). The main protein identified was the α -chain of 7S β -conglycinin (64 kDa), with 661 spectra and 64 peptides and β -chain of 7S β -conglycinin (48 kDa) with 338 spectra and 45 peptides compatible with the *Cajanus cajan* species database. Basic 7S globulin (21 kDa) were identified, in agreement with a study by Krishnan et al. (2017). A sucrose-binding protein was also identified in the flour, probably due the fact that pigeon pea 7S globulins are classified as glycoproteins (Krishnan et al., 1977). The identification of glycinin indicates the presence of 11S globulins in pigeon pea flour, although in a smaller proportion when compared to the 7S fraction (205, 256 spectra). In the albumin group, only 2S albumin (16 kDa) was identified. Among the oleosins, an oleosin of 18.5 kDa was identified, in agreement with predictions using pigeon pea amino acid sequences reported by Locali-Pereira et al. (2023). An oil body-associated protein of approximately 28 kDa was also recognized. Various protease inhibitors such as cysteine proteinase inhibitor (27 kDa), trypsin inhibitor 1A (21 kDa), Kunitz-type trypsin inhibitor KTI1 (16 kDa), Bowman-Birk type proteinase inhibitor (12 kDa), and subtilisin inhibitor (11 kDa), were identified, as well as different enzymes, such as lipoxygenase, peroxidase, peroxygenase, and aspartic proteinase. These accessions may be related to the molecular weight groups of 21 and 16 kDa identified in the SE-HPLC analysis (Fig 4.2a) and the various bands between 25 and 10 kDa identified in the SDS-PAGE analysis (Fig 4.2b).

Table 4.1. Proteins identified on pigeon pea (*Cajanus cajan*) flour by MALDI-TOF-TOF-MS/MS analysis.

Group	Protein name	Uniprot accession	Coverage (%)	MW	Spectra	Peptides
1a	Beta-conglycinin, alpha chain	A0A151U6Q2	59.61	64269	661	64
1b	Beta-conglycinin, beta chain	A0A151S2A5	56.67	48585	388	45
2	Sucrose-binding protein	A0A151RL88	55.30	54728	205	48
3	Glycinin G3	A0A151TUL0	79.15	50145	252	43
4	Glycinin	A0A151RNW3	73.02	55550	266	43
5	Alpha-1,4 glucan phosphorylase	A0A151T017	41.27	109838	63	33
6	Uncharacterized protein	A0A151RC24	72.53	24641	128	30
7	Low-temperature-induced 65 kDa protein	A0A151RHC5	44.75	71752	45	26
8	Protein disulfide-isomerase	A0A151SQ13	59.42	58231	36	24
9	Embryonic protein DC-8	A0A151U337	35.83	40489	48	21
10	Urease	A0A151SDR3	31.16	89113	76	19
11	Basic 7S globulin	A0A151TW66	76.04	21274	31	19
12	Trypsin inhibitor 1A	A0A151SYZ1	64.32	21513	57	18
13	Late embryogenesis abundant protein	A0A151SSD0	28.38	46859	50	18
14	Seed biotin-containing protein SBP65	A0A151SEU4	42.94	53463	52	17
15	Vicilin-like antimicrobial peptides 2-2	A0A151T151	33.26	49306	44	17
16	Vicilin-like antimicrobial peptides 2-2	A0A151TN63	36.75	52448	42	17
17	Peroxiredoxin	A0A151S664	70.18	24441	42	17
18	Glyceraldehyde-3-phosphate dehydrogenase	A0A151TR34	57.99	36602	32	16
19	Formate dehydrogenase, mitochondrial	A0A151R9F0	59.48	42561	41	16
20	Peroxidase	A0A151 RKT1	30.83	70075	24	15
21	UTP--glucose-1-phosphate uridylyltransferase	A0A151S0L7	31.58	52103	19	13
22	Glucose and ribitol dehydrogenase	A0A151STP5	56.90	31614	32	13
23	Poly [ADP-ribose] polymerase	A0A151TNV1	20.00	91077	17	12
24	phosphopyruvate hydratase	A0A151TV65	39.07	48666	21	12
25	Glucose and ribitol dehydrogenase	A0A151U4W9	41.43	30240	21	11
26	CobW/HypB/UreG nucleotide-binding domain-containing protein	A0A151S4D2	57.19	30493	34	11
27	Uncharacterized protein	A0A151SXY6	49.67	16106	30	11
28	Fructose-bisphosphate aldolase	A0A151R1P9	34.92	38255	18	11
29	Embryonic protein	A0A151U337	40.43	20548	31	11
30	18.2 kDa class I heat shock protein	A0A151SX67	64.47	17405	29	10
31	Annexin	A0A151TPX1	31.44	34445	17	10
32	EMB-1 protein	A0A151S2P2	62.63	10913	26	10
33	18.2 kDa class I heat shock protein	A0A151S1P1	67.53	17156	24	10
34	Nucleoside diphosphate kinase	A0A151RIQ5	56.38	16488	11	9
35	Lipoxygenase	A0A151SUB6	12.30	97316	8	8
36	Oil body-associated protein 2B	A0A151RE06	40.00	27919	14	8
37	Uncharacterized protein	A0A151U0F1	21.43	75605	10	8
38	Kunitz-type trypsin inhibitor KT11	A0A151SZ51	41.29	16773	18	8
39	Luminal-binding protein 4	A0A151RID0	12.93	73319	15	7
40	Peptidyl-prolyl cis-trans isomerase	A0A151RAJ7	52.91	18185	9	7
41	Heat shock cognate protein 80	A0A151S4L4	12.16	80833	9	7
42	Alcohol dehydrogenase 1	A0A151TSB7	21.86	43053	7	7
43	5-methyltetrahydropteroyltrimethylglutamate--homocysteine S-methyltransferase	A0A151SX55	14.51	84830	14	7
44	Cytochrome P450 71A1	A0A151TR99	11.88	94943	11	7

Table 4.1. continued.

Group	Protein name	Uniprot accession	Coverage (%)	MW	Spectra	Peptides
45	Elongation factor 1-alpha	A0A151RR52	16.11	49331	9	7
46	Phosphoglycerate kinase	A0A151QRU3	25.19	42549	10	7
47	Bowman-Birk type proteinase inhibitor 2	A0A151RI37	43.93	12006	10	6
48	Uncharacterized protein ECU03-1610	A0A151RZ44	29.02	24329	8	6
49	Desiccation protectant protein Lea14 isogeny	A0A151UCY8	39.47	16784	6	6
50	Cysteine proteinase inhibitor	A0A151RC35	35.29	26727	11	6
51	Triosephosphate isomerase, cytosolic	A0A151SUT3	36.76	27196	7	6
52	Cell division cycle protein 48 isogeny	A0A151SGI9	13.36	84474	7	6
53	Elongation factor 2	A0A151S2W2	10.35	92537	30	6
54	Protein IN2-1 isogeny B	A0A151TUS7	24.71	29406	29	6
55	Oleosin 18.5 kDa	A0A151TOX9	30.37	14622	10	6
56	Calmodulin	P17928	39.60	16851	17	5
57	14-3-3-like protein E	A0A151TDK8	27.86	29760	10	5
58	Heat Shock protein 101 family	A0A151SPH8	8.91	100920	9	5
59	Defensin-like protein 4	A0A151S2H7	29.73	8151	8	5
60	Uncharacterized protein	A0A151RHY5	31.01	14045	8	5
61	UPF0098 protein MTH-273	A0A151RGP8	54.12	18729	6	5
62	2S albumin	A0A151QQ22	30.35	16695	6	5
63	Annexin	A0A151SY78	17.76	36738	6	5
64	Malate dehydrogenase	A0A151T1Y5	27.71	35331	6	5
65	Aminopeptidase	A0A151R7C2	9.05	98738	6	5
66	Aquaporin TIP-type alpha	A0A151RKJ8	19.92	27113	5	5
67	Uncharacterized protein	A0A151QU84	44.16	8160	5	5
68	superoxide dismutase	A0A151UDT1	32.67	15136	15	5
69	Glutaredoxin-dependent peroxiredoxin	A0A151TIT0	48.15	17287	13	5
70	Reticulon-like protein	A0A151SV55	21.65	28677	10	5
71	Peroxygenase	A0A151T132	18.33	27336	7	5
72	Thioredoxin H-type 1	A0A151TC84	43.22	12998	6	4
73	Calreticulin	A0A151RF02	13.35	50444	6	4
74	Selenium-binding protein	A0A151SQ99	17.13	51415	7	4
75	Translationally-controlled tumor protein isogeny	A0A151TSX4	36.53	18963	6	4
76	Dehydrin COR47	A0A151TD44	35.19	24274	5	4
77	UDP-arabinopyranose mutase	A0A151TRX5	23.01	41314	4	4
78	Outer envelope pore protein 16-2, chloroplastic	A0A151QR91	19.79	20244	4	4
79	UPF0098 protein MTH-273	A0A151RH57	30.25	17897	4	4
80	EC protein isogeny 1	A0A151S071	46.67	8624	4	4
81	Subtilisin inhibitor 1	A0A151RL22	46.39	10859	9	4
82	Histone H4	A0A151RKC5	31.07	11402	7	3
83	Eukaryotic translation initiation factor 5A	A0A151SL27	12.50	17475	5	3
84	Gamma-interferon-inducible lysosomal thiol reductase	A0A151SXY3	9.20	28642	4	3
85	Uncharacterized protein	A0A151TCQ9	59.77	9277	13	3
86	Lactoylglutathione lyase	A0A151T6U3	12.89	32390	4	3
87	Laminin-like protein epi-1	A0A151TM15	4.89	98292	5	3
88	Seed biotin-containing protein SBP65	A0A151TPG0	17.49	34956	5	3
89	18 kDa seed maturation protein	A0A151TUD4	16.56	15538	5	3
90	Alcohol dehydrogenase 1	A0A151UBN7	12.25	37135	4	3
91	Pectinesterase	A0A151UCA3	8.16	54510	4	3

Table 4.1. continued.

Group	Protein name	Uniprot accession	Coverage (%)	MW	Spectra	Peptides
92	Caffeoyl-CoA O-methyltransferase At4g26220 family	A0A151RGJ5	24.79	26707	4	3
93	SMP domain-containing protein	A0A151RL90	20.00	26783	4	3
94	Leucine aminopeptidase 1	A0A151RXM3	7.12	59642	4	3
95	Gibberellin receptor GID1L3	A0A151S0I2	13.39	53233	4	3
96	40S ribosomal protein S8	A0A151S1X3	17.20	24956	3	3
97	Seed maturation protein PM36	A0A151ST20	13.10	26112	3	3
98	Superoxide dismutase	A0A151TQZ9	16.94	26656	3	3
99	ATP synthase subunit beta	A0A151U754	9.11	59870	3	3
100	Uncharacterized protein	A0A151PJF4	17.81	16147	3	3
101	Ribulose biphosphate carboxylase large chain	A4GG89	4.23	52358	3	2
102	Ferritin	A0A0L9UVI2	11.77	28416	3	2
103	Argininosuccinate synthase	A0A0R0JQZ9	10.73	48248	3	2
104	PPM-type phosphatase domain-containing protein	A0A151RIE8	9.57	30945	3	2
105	Universal stress protein A-like protein	A0A151RYQ8	12.57	19950	3	2
106	40S ribosomal protein 5S	A0A151S1X3	7.88	36765	8	2
107	60S acidic ribosomal protein P2B	A0A151R6V4	47.79	11493	8	2
108	22.7 kDa class IV heat shock protein	A0A151SIV6	13.27	22237	5	2
109	Aspartic proteinase	A0A151SU18	4.12	55422	5	2
110	Bifunctional dihydrofolate reductase-thymidylate synthase	A0A151R969	4.55	56959	4	2
111	Non-specific lipid-transfer protein	A0A151R3S3	16.95	12328	4	2
112	Late embryogenesis abundant protein	A0A151R986	38.89	13367	4	2
113	Glycine-rich protein 2b	A0A151RNE5	19.39	18710	4	2
114	Calnexin isogeny	A0A151SY44	7.58	61873	3	2
115	protein disulfide-isomerase	A0A151SQ85	4.83	55628	3	2
116	Serine hydroxymethyltransferase	A0A151SQI9	6.58	51828	3	2
117	Endo-1,3,1,4-beta-D-glucanase	A0A151STR4	10.46	25513	2	2
118	Mitochondrial outer membrane protein porin of 36 kDa	A0A151SX94	6.27	45043	2	2
119	Calnexin isogeny	A0A151SY44	4.74	62338	2	2
120	Superoxide dismutase	A0A151T157	8.84	28324	2	2
121	Elongation factor 1-gamma	A0A151T893	8.05	44107	2	2
122	Uncharacterized protein	A0A151TIA9	9.43	32116	2	2
123	Uncharacterized protein	A0A151TR40	5.16	54150	2	2
124	Glutathione S-transferase DHAR2	A0A151U811	15.57	23172	2	2
125	Intracellular protease 1	A0A151U9X1	8.53	41196	2	2

3.2 Extractability of pigeon pea proteins

The extractability of pigeon pea proteins was investigated as a first step to the purification process. To find the right conditions of extraction we used different pHs (4.0, 6.0, 8.0 and 10.0) and low (50 mM) and high (500 mM) ionic strength. Protein extracts obtained using the different solvents were evaluated through nitrogen content determination, SDS-PAGE, UV-visible absorbance measurements, intrinsic fluorescence measurements and size distribution by DLS.

Protein extraction was affected by the pH and ionic strength of the buffer used. At low ionic strength, protein extraction increased with pH, being approximately 17, 48, 50 and 60% for pHs 4.0, 6.0, 8.0 and 10.0, respectively. At high ionic strength, the amount extracted was approximately 39, 45, 45 and 52% for pHs 4.0, 6.0, 8.0 and 10.0, respectively (Figure 4.3a).

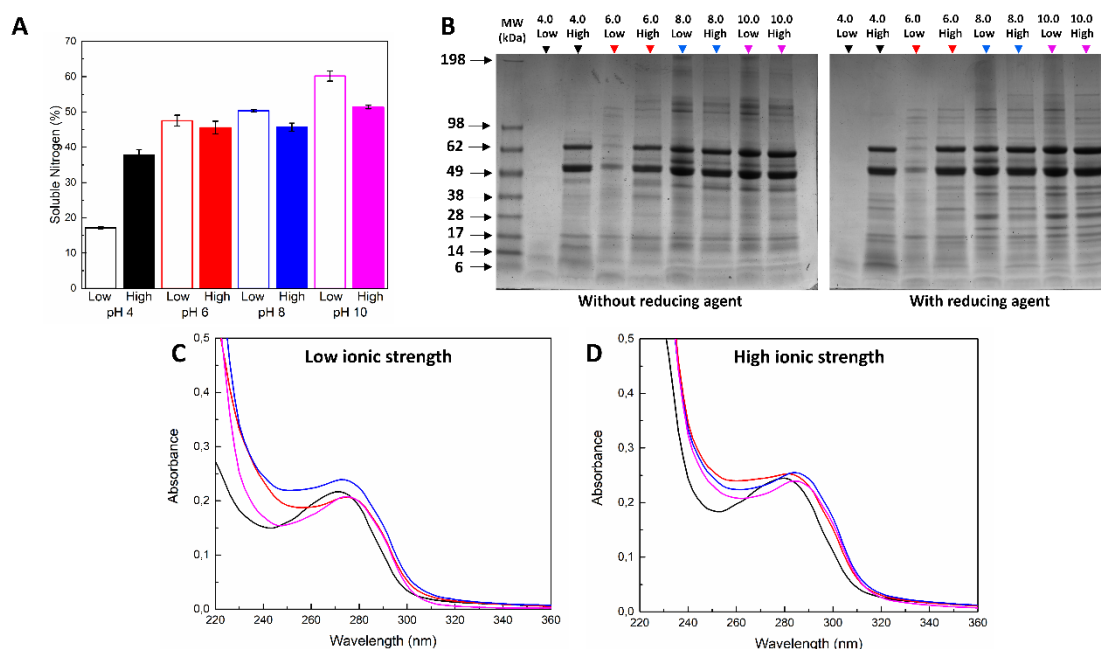


Figure 4.3. Extractability of pigeon pea proteins with flour suspensions (20 mg/mL) in buffers at different pHs and ionic strength. **(a)** Nitrogen solubility in the resulting supernatants (mean values with standard deviation bars, $n = 3$), **(b)** SDS-PAGE with and without reducing agent, and **(c, d)** UV-visible absorption spectra of supernatants. The lines and markers in black, red, blue, and pink corresponds to pHs 4.0, 6.0, 8.0, and 10.0, respectively. Low and High refers to low ionic strength (50 mM) and high ionic strength (500 mM), respectively.

SDS-PAGE analysis showed two main bands in the protein extracts: one around 64 kDa and other around 49 kDa (Figure 4.3b). No bands were observed in acidic condition (pH 4.0) and at low ionic strength. However, at high ionic strength, the same two bands as identified for the other extracts were visible. A similar result was observed for pH 6.0, with only the band close to 49 kDa being identified, yet with the recovery of the two main bands at high ionic strength. For these conditions, the extraction of proteins with subunits

of approximately 64 and 49 kDa showed high dependence on ionic strength. We identified that a minimum ionic strength of 100 mM is required to recover both bands under these conditions (data not shown).

The UV-visible absorption spectra also showed an intriguing result for the extract at pH 4.0 and low ionic strength (Figure 4.3c-d). Despite the low nitrogen content of this condition in relation to the other pHs evaluated, the UV-visible spectra were similar for all conditions evaluated. This result suggests that the tryptophan concentration is the same for different nitrogen contents, which may indicate a different extracted protein group. It is also surprising that the UV-visible spectrum is similar to those obtained in the other conditions, since no protein bands were identified in SDS-PAGE analysis for the extract at pH 4.0 and low ionic strength.

The emission-excitation fluorescence matrices of the protein extracts measured in right-angle mode showed two distinct regions: one referring to the emission of proteins, mainly to tryptophan/tyrosine emission ($\lambda_{\text{Ex}} = 270\text{-}290\text{ nm}$, $\lambda_{\text{Em}} = 300\text{-}360\text{ nm}$), and the other referring to the fluorescence of unknown compounds ($\lambda_{\text{Ex}} \approx 300\text{-}340\text{ nm}$, $\lambda_{\text{Em}} = 440\text{-}480\text{ nm}$) (Supplementary Figure 4.1). For pHs 4.0 and 6.0 at low ionic strength, lower fluorescence intensity was observed in relation to the other protein extracts, whereas the absorbance at 280 nm was similar. These results may be due to the different composition of the proteins extracted under these conditions and/or the change in their conformation. For pH 10.0, the fluorescence intensity was the highest among the protein extracts evaluated. It is important to highlight that the absorbance at 280 nm was normalized for all extracts and since the protein composition seems similar for pH 6.0-10.0, this normalization may correspond to constant protein content in these extracts. The higher fluorescence intensity may be purely due to the effect of pH on the quantum yield of tyrosine/tryptophan. While no changes in the quantum yield of tyrosine are expected in this pH range, tryptophan may show an increase in its quantum yield for pH 8.0 and 10.0 (De Lauder & Wahl, 1970; White, 1959). Therefore, the increase in fluorescence observed for 10.0 may be related to changes in tryptophan quantum yield.

In relation to the fluorescence region of unknown compounds, a lower fluorescence intensity was observed for pHs 8.0 and 10.0, regardless of the ionic strength. These results may indicate a lower extraction of these compounds at these pHs, but studies were conducted by extracting the proteins at pH 10.0 and acidifying the extract to pH 8.0, 6.0 and 4.0 with intrinsic fluorescence measurements at each step, and it was found that this is a pH-dependence response to the fluorescence of these compounds (data not shown).

The extracts were also investigated for size distribution by DLS (Supplementary Figure 4.2). The size distribution of samples showed two populations: a small population around 15-20 nm and another larger population around 100-130 nm. Ruiz-Henestrosa et al. (2012) reported a size distribution of 10.1-11.7 nm for soybean 7S globulin. The first population may therefore be attributed to the 7S globulin trimer. The larger population ($\approx 100\text{ nm}$) suggests the presence of aggregates in the protein extracts. For extracts at

low ionic strength, the contribution of the population around 15-20 nm to the signal was lower than in extracts at high ionic strength. At high ionic strength, the aggregation state of pigeon pea proteins was reduced.

Altogether, the results showed that all extracts, except extracts at pH 4.0 and 6.0, presented the same protein profile. Therefore, pHs above 6.0 showed to be suitable for the extraction of 7S globulin. The low effect of ionic strength on the extractability is suitable for subsequent separation using ion exchange chromatography. Also, all extracts showed the presence of aggregates and minor compounds, regardless of pH or added salt. However, increasing salt after ion exchange chromatography can reduce protein aggregation.

3.2 Laboratory-scale purification

3.2.1. Desalting vs UF

The elimination of small molecules contained in protein extracts was investigated using two approaches: membrane ultrafiltration and injection on a desalting column. Samples were evaluated by emission-excitation fluorescence matrices to monitor the presence of the compounds of interest (tryptophan/tyrosine fluorescence emission) and the elimination of unknown compounds (small molecules).

The use of membrane ultrafiltration proved to be an efficient technique for separating small molecules, which were concentrated in the extract filtrate, whereas proteins were concentrated in the retentate (Supplementary Figure 4.3). Only a low intensity of fluorescence could be seen in the region of unknown compounds in the retentate, suggesting that some low molecular weight compounds were still present in the extract.

In parallel, the desalting of the protein extract in a HiPrep desalting column showed three chromatographic peaks (Figure 4.4a). The size exclusion chromatograms (SEC) of the protein extract and of the fractions separated by the desalting column shows the predominance of the target protein in the first peak and smaller molecules in peaks 2 and 3 (Figure 4.4b). Also, the fluorescence signal in the region attributed to proteins was observed only in the first peak, while the other fractions showed low fluorescence in the protein region (Figure 4.4c).

The ultrafiltered and desalted extracts were also compared by analytical size exclusion chromatography (Figure 4.5). This showed that desalting contains less aggregates (eluted around 15 min) and less small molecules (eluted between 40 and 55 min), evidenced by the peak intensity ratio (30 min / 42 min), which was 0.6 for the initial protein extract and 19.6 for the desalted protein extract. On the other hand, the peak intensity ratio (30 min/15 min) was 2.6 for the initial protein extract and 2.8 for the desalted protein extract. This difference between both techniques could be related to the higher concentration on the membrane caused by ultrafiltration, which can lead to irreversible aggregation. Despite this, membrane ultrafiltration showed to be a fast and

efficient technique for removing the minor compounds present in the protein extract, which can be interesting for studies that aim to separate them for identification and quantification. For protein purification purposes, desalting showed to be the most suitable technique and was used in the protocol described in this work for the purification of pigeon pea 7S globulin.

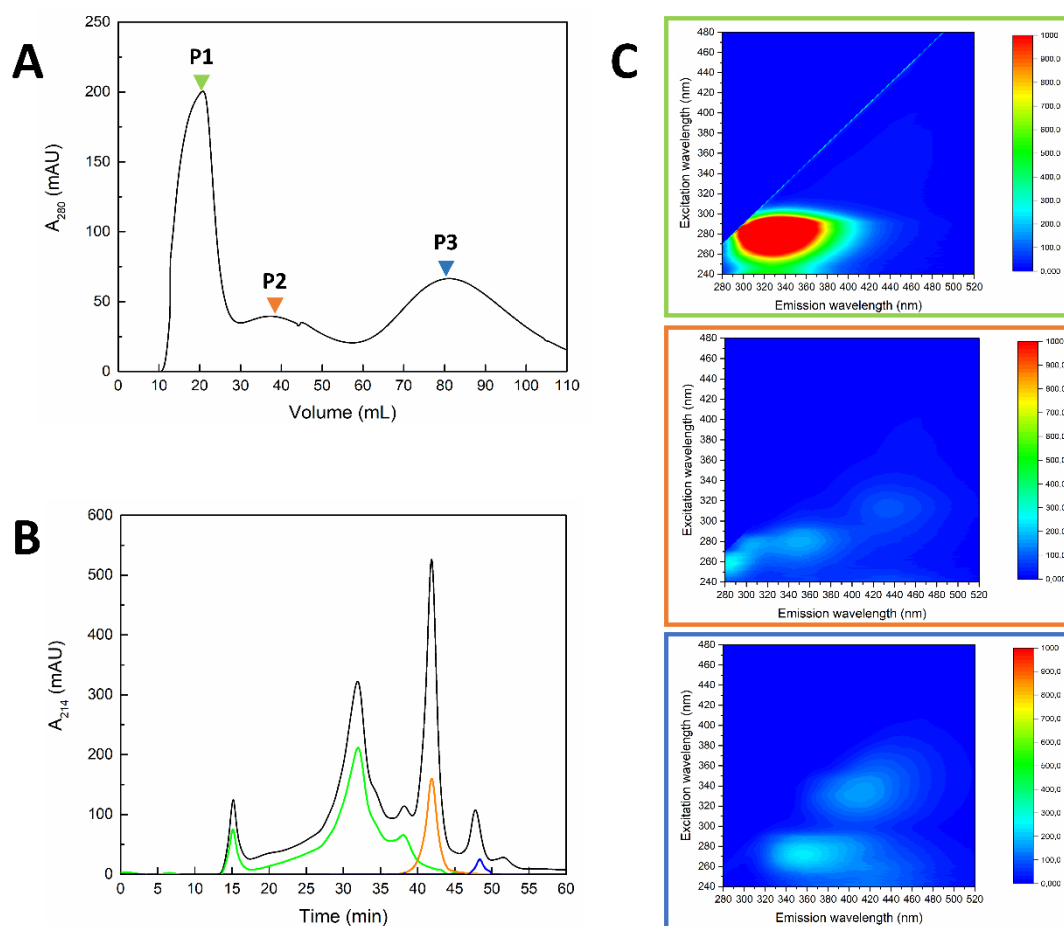


Figure 4.4. Injection of pigeon pea protein extract into a desalting column to remove small molecules. **(a)** Elution profile on the HiTrap desalting column; **(b)** Size exclusion chromatogram of the pigeon pea protein extract and the fractions separated in the desalting column; **(c)** 3D Emission-Excitation fluorescence matrices of the fractions separated in the desalting column. Black, green, orange and blue lines refer to the initial protein extract and peaks P1, P2 and P3, respectively.

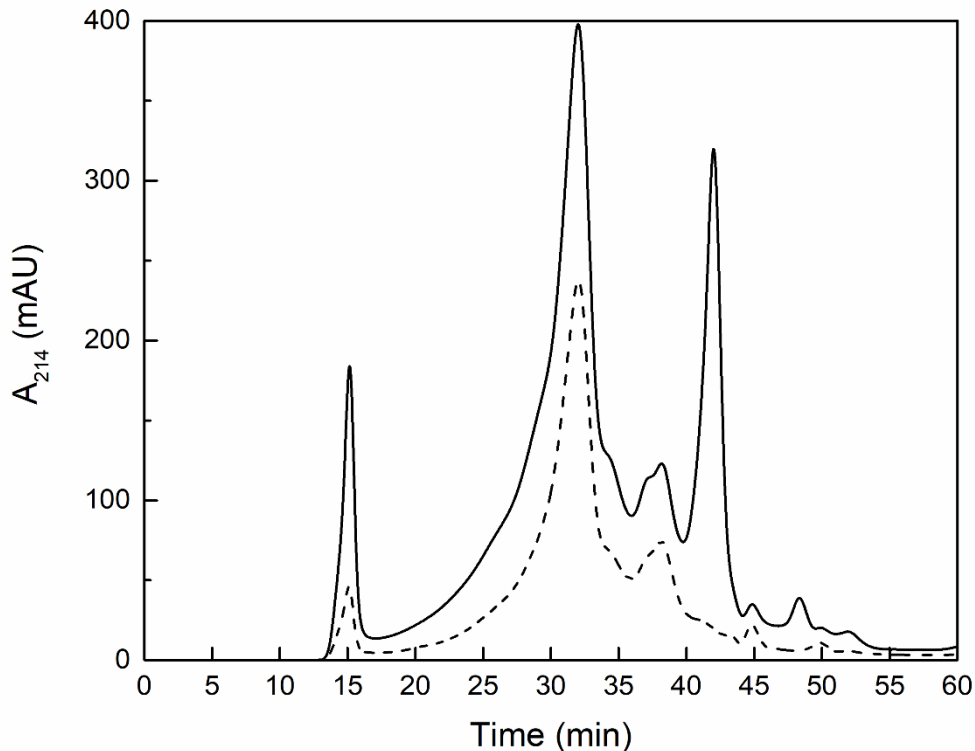


Figure 4.5. Size exclusion chromatograms of pigeon pea protein extract after membrane ultrafiltration (solid line) and injection in a desalting column (dashed line).

3.2.2 Separation by anion exchange and size exclusion chromatography

A screening of different chromatography columns showed that anion exchange columns performed better in retaining pigeon pea proteins compared to cation exchange columns. Pigeon pea protein extracts at pH 8.0 and injected into the Q column showed better results in protein separation (Supplementary Figure 4.4), so these conditions were used to compare both protein extracts.

To optimize the separation using the desalted extract, two procedures were investigated for anion exchange chromatography: (i) increasing the duration of NaCl elution gradient from 0 to 0.75 M NaCl and (ii) increasing the NaCl concentration at 0.1 M in the extract to avoid fixation of weakly charged proteins onto the column. In the first procedure, the AEC separation showed three peaks (Figure 4.6a). SDS-PAGE analysis of the fractions showed the presence of weak protein bands characteristic of 7S globulin in the fraction that was not-retained in the column. The first separated peak showed a mixture of different proteins; the second peak showed the characteristic bands of the 7S globulin; and the third peak showed no protein bands. The second procedure used showed a better separation of the target protein (Figure 4.6b), eliminating the first peak and enabling better separation of the 7S globulin peak. SDS-PAGE analysis of the non-retained fraction from this procedure also showed that most non-retained proteins were depleted with the second procedure. The 7S globulin fractions separated by AEC were collected, pooled and further purified by SEC (Figure 4.6c), which showed a single major peak of the 7S fraction and small molecule peaks.

3.3 Preparative scale purification

The preparative scale purification process of pigeon pea 7S globulins was carried out using the same extraction procedures as the optimized process on an analytical scale, but at higher concentration. The extract desalting step on the GH25 column presented results similar to those obtained at the analytical scale. Three peaks were observed, the first peak referring to the protein-rich fraction and the two subsequent peaks referring to minor compounds such as phenolic compounds and salts (Figure 4.7a).

The desalted extract was then injected onto the Q-Sepharose column to be fractionated by AEC with an elution step (Figure 4.7b). Three peaks were observed, with peak 1 referring to the fraction not retained on the column, whereas peaks 2 and 3 corresponded to proteins bound on the column eluting at 0.35 M NaCl and 1 M NaCl, respectively. SDS-PAGE analysis showed that the fraction that was not retained on the column is composed of protein bands different from those of 7S globulin, whereas fraction 2 presented two main bands close to the region of pigeon pea 7S globulin subunits (around 47 and 64 kDa), and peak 3 presented the same bands as peak 2, but less intense. Proteins not retained in the Q-Sepharose column were also analyzed for their composition. The non-retained fraction was diluted and reinjected into the Q-Sepharose and GLC 2000 column for further separation (Supplementary Figure 4.5). The main protein band was identified as a trypsin inhibitor 1A (Uniprot A0A151SYZ1_CAJCA), by proteomic analysis (Supplementary Table 4.1). This shows that part of the trypsin inhibitors is potentially eliminated at this stage of the process.

After fractionation by AEC, two different processes were adopted: (i) dialysis and freeze-drying of the fraction eluted at 0.35 M NaCl with subsequent separation by SEC and new dialysis and freeze-drying, and (ii) further fractionation of the fraction eluted at 0.35 M NaCl by SEC and freeze-drying. The strategy of equilibrating the GLC 2000 column with ammonium carbonate buffer pH 8.0 allows for circumventing the dialysis step. In this case, salt elimination was conducted at the same time as protein size separation during SEC.

The fractionation by SEC on the GLC 2000 column presented a similar profile for both processes used (Figure 4.7c-d), with elution of 7S globulins between 1000 and 1500 mL of volume and small peaks eluted between 1500 and 2500 mL of volume, referring to small compounds. However, SDS-PAGE analysis showed unexpected results for the band profiles of the fractions after SEC separation. Using the first process, 7S globulin showed bands around 50 and 20-25 kDa, while using the second process, 7S globulin showed bands around 47 and 64 kDa, characteristic of pigeon pea 7S globulin in native state. These results show that a possible hydrolysis of proteins occurred during the process with the dialysis steps. In addition, SDS-PAGE analysis of aliquots of the protein fraction after the first dialysis, conducted before freeze-drying and subsequent fractionation by SEC,

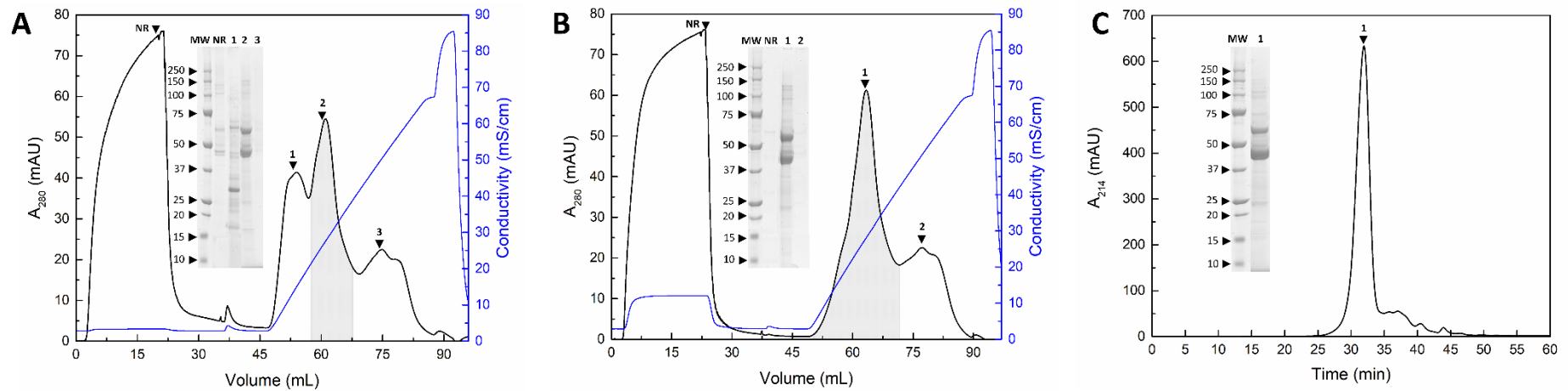


Figure 4.6. Analytical-scale purification of pigeon pea 7S globulin. **(a)** Ion exchange chromatography with NaCl elution gradient (step 1 = 0 to 0.75 M in 40 mL, step 2 = 0.5 to 1 M in 5 min) and **(b)** with the combination of NaCl elution gradient and 1 M NaCl solution addition in the protein extract before injection. **(c)** Size exclusion chromatography of the pigeon pea 7S globulin fraction. NR: not-retained fraction; 1, 2, 3: fraction peaks. In the inset the SDS-PAGE gels of the separated peaks. Gray areas indicate pooled fractions for subsequent SEC analysis.

already showed this protein bands' profile, confirming that hydrolysis occurred during the dialysis step (data not shown).

The use of the process without the dialysis steps, in addition to reducing the total purification time (from 15 to 7 days, approximately) and having a lower consumption of water and salts, also showed to be suitable for maintaining the pigeon pea 7S globulins in their native state.

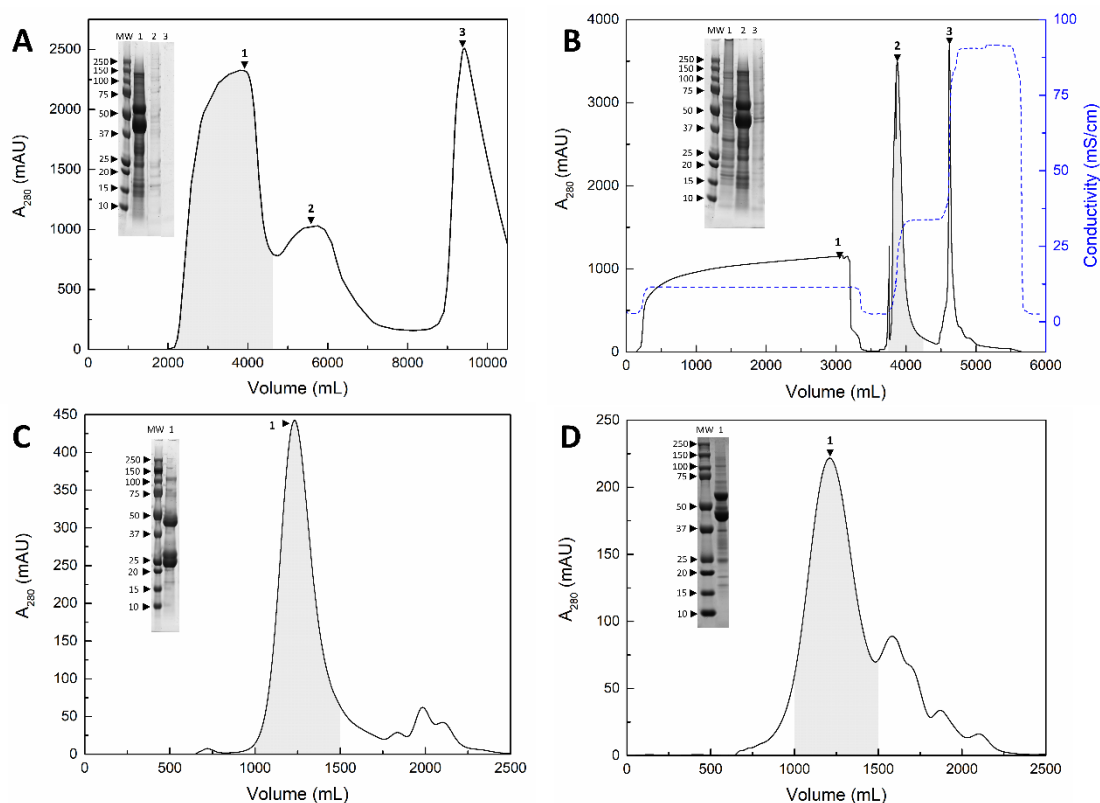


Figure 4.7. Preparative scale purification of pigeon pea 7S globulin. **(a)** Elution profile in the desalting column. **(b)** Anion exchange chromatography of the desalted protein extract. **(c)** Size exclusion chromatograms of pigeon pea 7S globulin in the partially hydrolyzed state (process I) and **(d)** in the native state (process II). The inset shows SDS-PAGE analysis of numbered fractions. The gray filled peak corresponds to the 7S globulin fraction.

3.4 Analytical characterization of the pigeon pea 7S globulin

3.4.1 Yield and purity

The process yield is not usually reported by authors in pulse protein purification studies, but it is expected to be around 10 mg of 7S globulin/ 1 g of flour (1%) (Gravel and Downen, 2023). Figure 4.8 shows the distribution of dry matter and protein content throughout the purification process of pigeon pea 7S globulin.

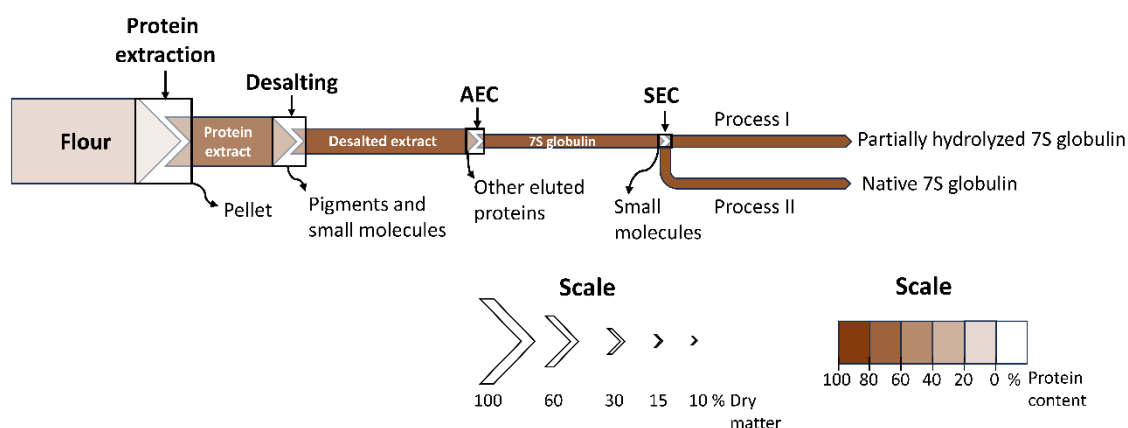


Figure 4.8. Distribution of dry matter and protein content during preparative-scale purification of pigeon pea 7S globulin. AEC: anion exchange chromatography. SEC: size exclusion chromatography.

Around 200 g of flour were used in both processes, with a total soluble protein content of approximately 33.6 g. The protein content of both native and partially hydrolyzed 7S globulin fractions was about 89% w/w dry matter (DM). The dry matter obtained, however, differed between the processes used, being 4.7 g for the first process and 3.8 g for the second process. Considering that all purified proteins are 7S globulins and that the initial content of 7S globulins in the flour was 16.3 g, based on SE-HPLC analysis, the recovery of 7S globulins was 28.8 and 23.3% for the first and second process, respectively. Therefore, our protocol showed to be efficient for recovering a reasonable amount of the 7S globulin.

3.4.2 Protein composition

The protein composition of purified pigeon pea 7S globulin fractions was characterized by SDS-PAGE and proteomic analysis. SDS-PAGE analysis revealed two main bands in the purified fraction in native state: one between 75 and 50 kDa and two other bands between 50 and 37 kDa (Figure 4.9). Such bands correspond to the subunits of 7S globulin from pigeon pea, as described by Krishna & Bhatia (1985). As observed by the authors, the banding pattern did not show changes in the presence of the reducing agent 2- β -mercaptoethanol (subunits not linked by disulphide bonds). For the well with the highest concentration of proteins, it is possible to visualize other bands (minor contaminants), whereas the well with the lowest concentration of proteins shows the purity of the two main bands referring to pigeon pea 7S globulin. For the 7S globulin that was partially hydrolyzed, four subunits were observed: two bands between 50 and 37 kDa and two bands around 25 kDa.

The protein bands identified in SDS-PAGE for the purified 7S globulin fractions were evaluated by proteomic analysis (Table 4.2), as well as the crude protein extract (powder) (Supplementary Table 4.2). The two main bands identified in native 7S globulin of 64 and 49 kDa were identified as the α - and β -chain of 7S β -conglycinin, respectively. Glycinin was also identified in the purified fraction, suggesting the presence of 11S globulin residues. The two bands around 25 and 20 kDa identified for partially hydrolyzed 7S globulin were shown to be composed of a mixture of the α - and β -chain of 7S β -conglycinin, according to the proteomic analysis. This may be due to the high sequence homology of the two chains. However, according to SDS-PAGE of the fractions the partial hydrolysis products come from the α -chain.

To our knowledge, no work in literature has shown the proteolysis of pigeon pea 7S globulin. However, proteolysis of 7S globulin from other species has been reported, including soybean, which has high homology with pigeon pea. Partial proteolysis of soybean 7S globulin has been observed during the early stages of seed maturation and in mature seeds (Asano et al., 1977; Bond & Bowles, 1983). Morita et al. (1994) observed proteolysis of the α chain of 7S globulin in mature soybean seeds, while the β chain was resistant to hydrolysis, in agreement with the phenomenon observed in our study. However, the hydrolysis reported by the authors resulted in a product with a molecular weight of around 47 kDa, unlike the subunits of around 25 kDa observed for pigeon pea. According to the authors, hydrolysis possibly occurred due to the action of a neutral/alkaline serine protease. Although no serine protease was identified in pigeon pea protein fractions, other endogenous proteases may have caused partial hydrolysis.

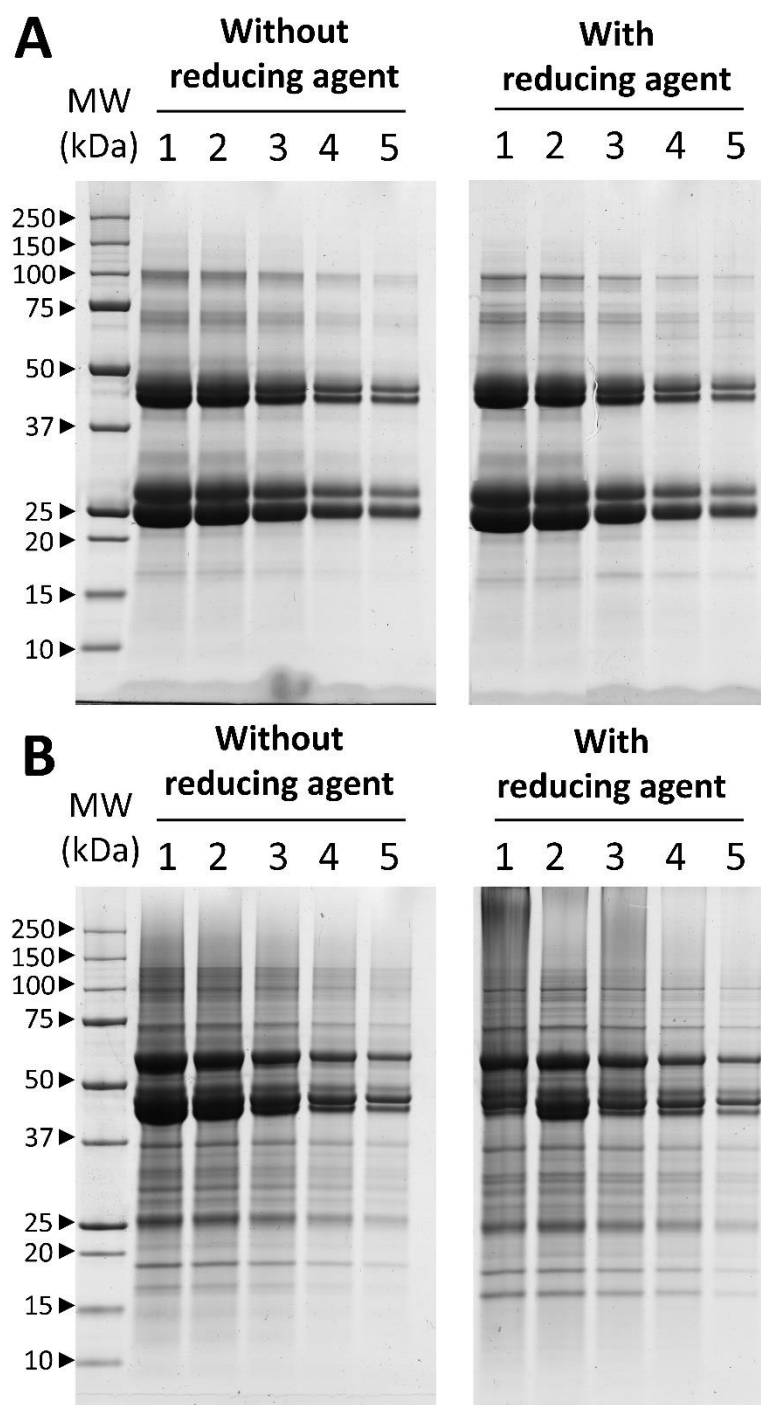


Figure 4.9. SDS-PAGE of pigeon pea 7S globulin in the presence or absence of reducing agent (2- β -mercaptoethanol). **(a)** partially hydrolyzed state, **(b)** native state. Concentrations of 2.0, 1.5, 1.0, 0.5, and 0.25 mg/mL of proteins are indicated by the numbers 1, 2, 3, 4, and 5, respectively.

Table 4.2. Proteins identified on pigeon pea purified fractions by MALDI-TOF-TOF-MS/MS analysis.

Native 7S globulin, band 64 kDa						
Group	Protein name	Uniprot accession	Coverage (%)	MW	Spectra	Peptides
1	Beta-conglycinin, alpha chain	A0A151U6Q2	44.7	64269	659	38
2	Glucose and ribitol dehydrogenase	A0A151U4W9	16.2	31614	8	4
3	Glycinin	A0A151RNW3	11.4	55550	4	3
4	Heat shock 70 kDa protein	P26413	4.5	70835	2	2
Native 7S globulin, band 49 kDa						
Group	Protein name	Uniprot accession	Coverage (%)	MW	Spectra	Peptides
1	Beta-conglycinin, beta chain	A0A151S2A5	64.8	48585	883	60
2	Guanosine nucleotide diphosphate dissociation inhibitor	A0A151UC16	10.6	49691	6	4
3	ATP-dependent RNA helicase	A0A072URR3	5.6	48555	2	2
4	Glycinin	A0A151RNW3	8.7	55550	2	2
Partially hydrolyzed 7S globulin, band 25 kDa						
Group	Protein name	Uniprot accession	Coverage (%)	MW	Spectra	Peptides
1	Beta-conglycinin, alpha chain	A0A151U6Q2	42.0	64269	443	40
2	Glucose and ribitol dehydrogenase	A0A151U4W9	62.4	31614	75	24
3	14-3-3-loke protein E	A0A151TDK8	59.2	29760	69	22
4	Glycinin	A0A151RNW3	37.1	55550	41	12
5	Glucose and ribitol dehydrogenase	A0A151STP5	45.7	30240	13	7
6	Heat shock 70 kDa protein isoform X2	A0A8B8JHE8	12.4	65453	12	6
7	Aspartic proteinase	A0A151SU18	9.2	55421	6	4
8	Alpha-1,4 glucan phosphorylase	A0A151T017	8.7	109838	5	5
9	Sucrose-binding protein	A0A151RL88	7.5	54728	4	3
10	Vicilin-like antimicrobial peptides 2-2	A0A151TN63	4.9	52447	3	2
11	Glycinin G3	A0A151TULO	7.2	50145	2	2
Partially hydrolyzed 7S globulin, band 20 kDa						
Group	Protein name	Uniprot accession	Coverage (%)	MW	Spectra	Peptides
1	Beta-conglycinin, beta chain	A0A151S2A5	59.7	48585	740	50
2	Vicilin-like antimicrobial peptides 2-2	A0A151TN63	28.2	52447	40	15
3	Superoxide dismutase	A0A151T157	23.7	28324	9	4
4	Glucose and ribitol dehydrogenase	A0A151STP5	30.4	30240	8	4
5	Alpha-1,4 glucan phosphorylase	A0A151T017	9.8	109838	7	6
6	Glucose and ribitol dehydrogenase	A0A151U4W9	16.6	31614	7	4

From the data obtained in the proteomic analysis (Table 4.1, Table 4.2 and Supplementary Table 4.2), accessions exhibiting enzymatic activity were investigated. The identification of the aspartic protease enzyme was evidenced by the presence of a basic 7S globulin (A0A151TW66) and aspartic proteinase (A0A151SU18) in the flour, total protein extract and in the hydrolysis product of approximately 25 kDa. Also, the identification of the leucine aminopeptidase enzyme was evidenced by the accessions of an aminopeptidase (A0A151R7C2) and leucine aminopeptidase 1 (A0A151RXM3) in the flour and total protein extract. Aspartic proteases have an optimum pH of 3.0 to 5.5 and are inhibited by pepstatin (1 μ M) (Szecsi, 1992). Given the alkaline pH conditions used in our extraction method, it is not expected that aspartic proteases would find optimal conditions of action. On the other hand, leucine aminopeptidase has an optimum pH of around 8, and its action is inhibited by bestatin (1 μ M) and EDTA (5 mM) (Mucha et al., 2010). Although leucine aminopeptidase may have an action on pigeon pea protein extract, the addition of EDTA in our process weakens this hypothesis. In parallel to this, we also tested extracting the proteins under the optimal conditions of both enzymes, but no hydrolysis was observed (data not shown).

The molecular weight of the hydrolysis products found in our work also differs from the products reported in studies such as that of Morita et al. (1994). In contrast, Wilson & Tan-Wilson (2018) observed the cleavage of pea 7S globulin around the cupin domain, generating products with molecular weights around 19 and 28 kDa, values closer to the hydrolysis products of pigeon pea 7S globulin.

It is important to highlight that this is the first report of partial hydrolysis of pigeon pea 7S globulin and more studies should be conducted in the future to understand the mechanisms behind this phenomenon. The fact that this hydrolysis occurred during the purification process of the main protein fraction highlights the importance of understanding this phenomenon in the processing of ingredients based on pigeon pea seeds. Although the causes of the observed proteolysis are not clear, our process without dialysis steps (process II) showed to be efficient in avoiding the cleavage of 7S globulin, which could be of great interest for the purification of proteins from other species that may be susceptible to proteolysis during processing.

Another important aspect is that hydrolysis was not observed in the purification process on an analytical scale, suggesting that aspects linked to the upscaling of the process (higher concentration, longer process time, etc.) may be related to optimal conditions for the action of this proteinase.

3.4.3 Minor components

The minor components present in the purified 7S globulin fractions were mainly small sugars. Around 3 and 2.3% of polysaccharides were identified in the 7S globulin in its native and partially hydrolyzed state (Supplementary Table 4.3). Mannose and uronic acid were the main neutral sugars in both purified fractions with values of, respectively, 1.4 and 0.9 g 100g⁻¹ for the 7S globulin in native state and 1.7 and 0.5 g 100 g⁻¹ for partially hydrolyzed state. The high mannose content is surprising and may be linked to the glycosylation of pigeon pea 7S globulin.

As shown by proteomic analysis, trypsin inhibitors were present in the crude protein extract (Supplementary Table 4.2) and the trypsin inhibitor activity (TIA) was investigated in the purified fractions (Supplementary Table 4.4). The trypsin inhibitor activity (TIA) was 3.6 µg Tld mg⁻¹ sample for 7S globulin in the native state and 1.4 µg Tld mg⁻¹ sample for partially hydrolyzed 7S globulin. Pigeon pea flour and protein concentrate presented TIA values of 8.3 and 11.5 µg Tld mg⁻¹ in the previous work (Chapter 3). Therefore, the purified fractions had a significant reduction in TIA in relation to these values, probably due to the elimination of trypsin inhibitors during the chromatographic separation steps. The difference observed between the samples can be explained by the procedure used (dialysis vs. without dialysis), suggesting that dialysis of the samples favors the elimination of remaining trypsin inhibitors.

The occurrence of minor compounds was also checked by monitoring the endogenous fluorophores present in the purified fractions. Fluorescence emission-excitation matrices were measured in front-surface and right-angle mode for pigeon pea flour and purified 7S globulin fractions (Figure 4.10). While the flour presents two distinct regions of fluorescence emission (in both measurement methods), only the region referring to the fluorescence emission of proteins can be seen for the 7S globulin samples. In the latter samples, the emission intensity is also considerably higher. This is in line with the fair purity of the 7S globulin fraction, in addition to suggesting the complete elimination and/or degradation of the other endogenous fluorophores observed in the flour. The elimination of these compounds was demonstrated during the desalting step of the protein extract, and it probably corresponds to phenolic compounds (Jakubíková et al., 2022; Murugesan et al., 2023; Wu et al., 2022). A difference in the location of the fluorescence emission region of the unknown compounds was observed for the two measurement methods investigated. While in front-surface mode the fluorescence emission region of the unknown compounds was at $\lambda_{Ex} \approx 420$ nm and $\lambda_{Em} \approx 500$ nm, in right-angle mode the region was shifted to $\lambda_{Ex} \approx 340$ nm and $\lambda_{Em} \approx 410$ nm. The shape observed for this region also changed, being more circular for the measurement taken in right-angle mode. It is important to highlight that the partially hydrolyzed 7S globulin showed the peak of maximum fluorescence emission shifted to the left in relation to 7S globulin in its native state, suggesting a change in the tryptophan conformation and/or chemical environment (Lakowicz, 2006).

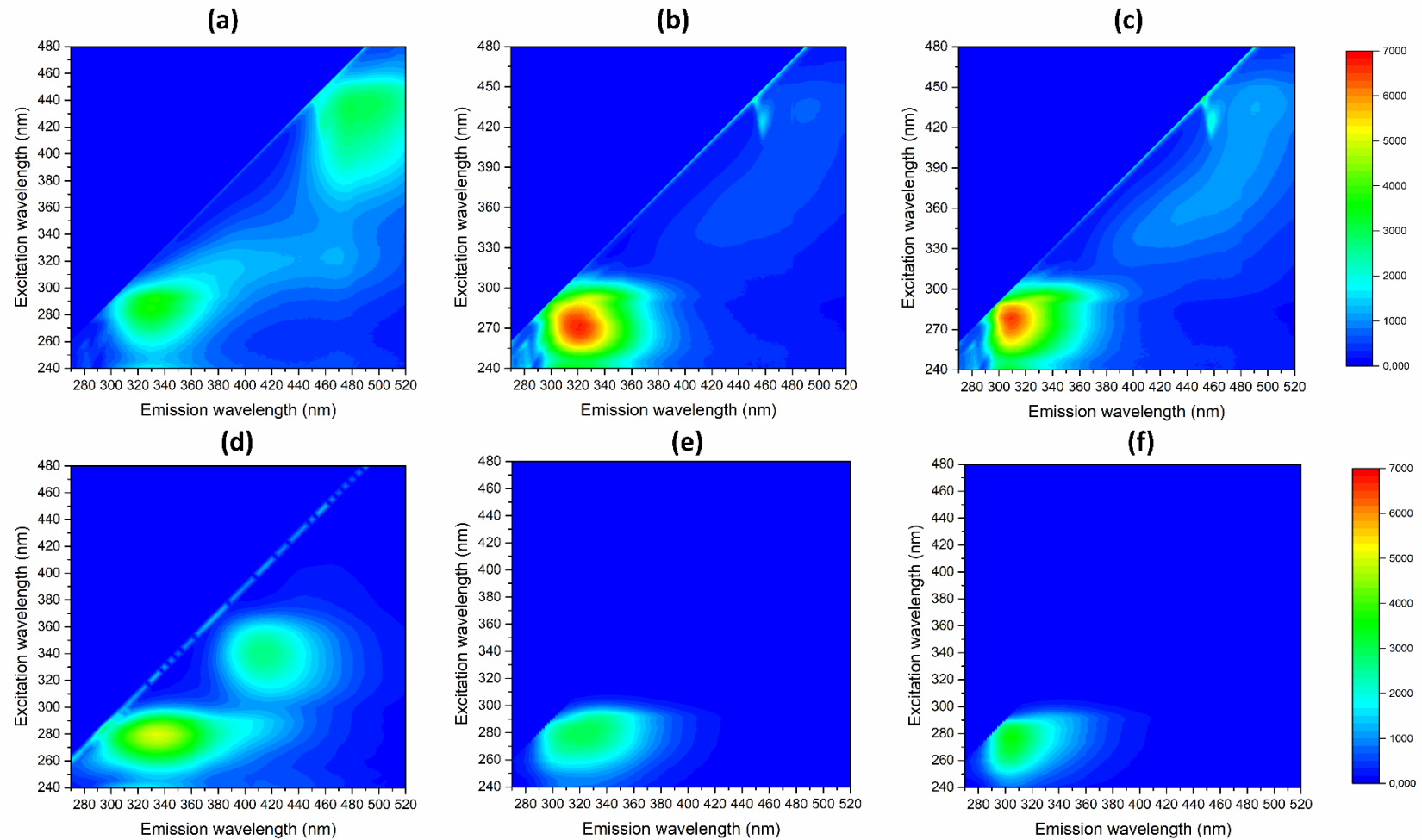


Figure 4.10. Emission-excitation fluorescence matrices of pigeon pea flour (**a, d**), native 7S globulin (**b, e**), and partially hydrolyzed 7S globulin (**c, f**). (a, b, and c) Measurements performed in front-surface mode (dry state) and (d, e, and f) measurements performed in right-angle mode (liquid sta

3.5 Foaming properties

Among the functionalities that proteins performed in food systems, foaming properties are one of the main ones, being important for several products that have foam-type structures, such as mousses, meringues, cakes, breads, ice cream, beverages, among others (Janssen et al., 2023). The globulins stand out for being abundant in pulse seeds and having good water solubility, which facilitates their adsorption at the air-water interface (Janssen et al., 2023).

The evaluation of the foaming properties of purified pigeon pea 7S globulin fractions showed better foam stability (FS) for foams produced with protein suspensions of native 7S globulin in relation to foams produced with suspensions of partially hydrolyzed 7S globulin (Fig. 4.11a). After 120 min, the foam stabilized by native 7S globulin showed a FS value of 69%, while the foam stabilized by partially hydrolyzed 7S globulin showed a FS value of 59% ($p < 0.05$). The physical appearance of the foams produced also differed (Fig. 4.11b), showing more destabilization for the foam produced with partially hydrolyzed 7S globulin.

The enzymatic hydrolysis of proteins results in a reduction in the size of the polymer and can affect physicochemical properties related to the stability of foams, such as cohesiveness, elasticity, surface viscosity and surface adsorption (Bombara et al., 1994). The degree of hydrolysis (DH) is important for improving or decreasing foaming properties. While proteins that undergo extensive hydrolysis can have their foam stability improved, partial hydrolysis can reduce the ability of proteins to form stable foams (Vioque et al., 2000; Zhao et al., 2011). Jamdar et al. (2010) reported that peanut protein hydrolysates of 10 to 40% DH had poor foam stability compared to peanut protein isolate. A decrease in foam stability may occur due to the smaller size of hydrolyzed proteins reducing their ability to interact at the air-water interface (Martinez et al., 2005). Changes in protein surface hydrophobicity can also reduce the foaming properties of partially hydrolyzed proteins (Jung et al., 2005). The behavior of pigeon pea 7S globulin at the air-water interface has not yet been reported in the literature and should be evaluated in future work to better interpret the results. Comparison of the foaming properties of pigeon pea proteins with other proteins of animal origin is also of great interest.

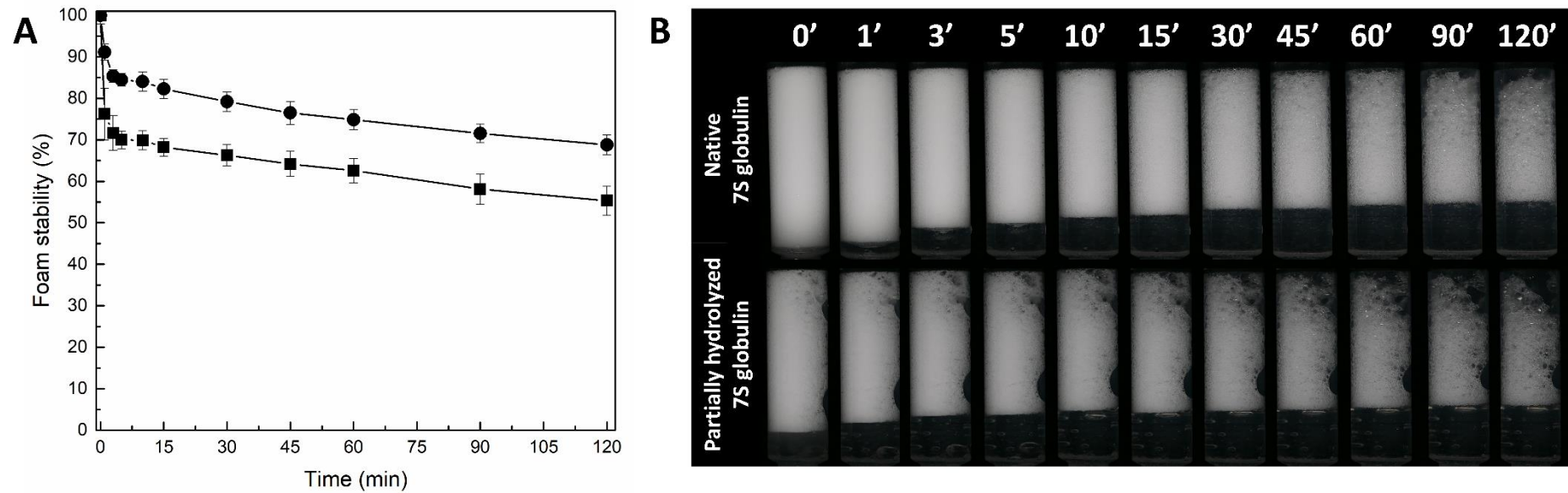


Figure 4.11. Foaming properties of pigeon pea 7S globulin. **(a)** Foam stability of native 7S globulin (circles) and partially hydrolyzed 7S globulin (squares) foams over time. **(b)** Physical appearance of foams over time.

4. Conclusion

In this work, a preparative scale purification method of pigeon pea 7S globulins was developed. The protocol was first developed and optimized on a laboratory scale, showing reproducibility when transferred to the preparative scale. The use of a process without dialysis/ultrafiltration allowed the recovery of 7S globulin in its native state, whereas the use of dialysis/ultrafiltration resulted in an unexpected partial hydrolysis of 7S globulin. The causes of the observed hydrolysis are unclear, but it may have been caused by endogenous proteases. In native state, the pigeon pea 7S globulin is mainly composed of α - and β -chains of 7S β -conglycinin, with subunits of molecular weight around 64 and 49 kDa. Partial hydrolysis cleaved the α chain, resulting in two products with a molecular weight of around 25 kDa, while the β chain remained unchanged. Carbohydrate residues, mainly mannose, were identified in both purified fractions, suggesting the presence of glycosylated proteins. The developed processes are suitable for purifying pigeon pea 7S globulin in sufficient amounts for protein characterization and functional properties studies. Partial hydrolysis decreased the foaming properties of pigeon pea 7S globulin compared to native 7S globulin. The method without dialysis showed to be a suitable process for the preparative scale purification of pigeon pea 7S globulin, maintaining its native state and preserving its functionality.

Acknowledgments

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Supplementary material

Preparative chromatography for purification of 7S globulin from pigeon pea seeds

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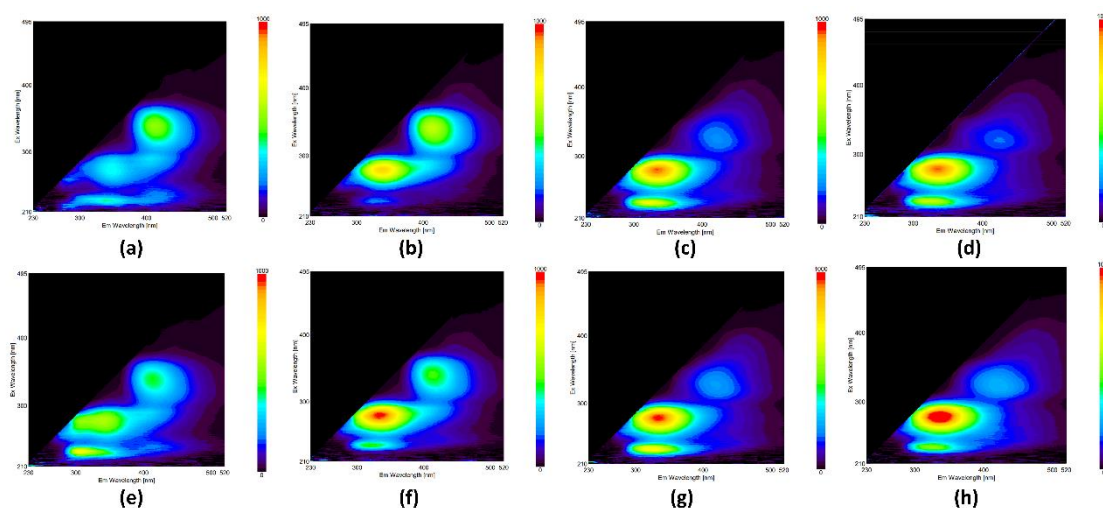
1. Methods

1.1 Ion exchange chromatograph (IEX) screening

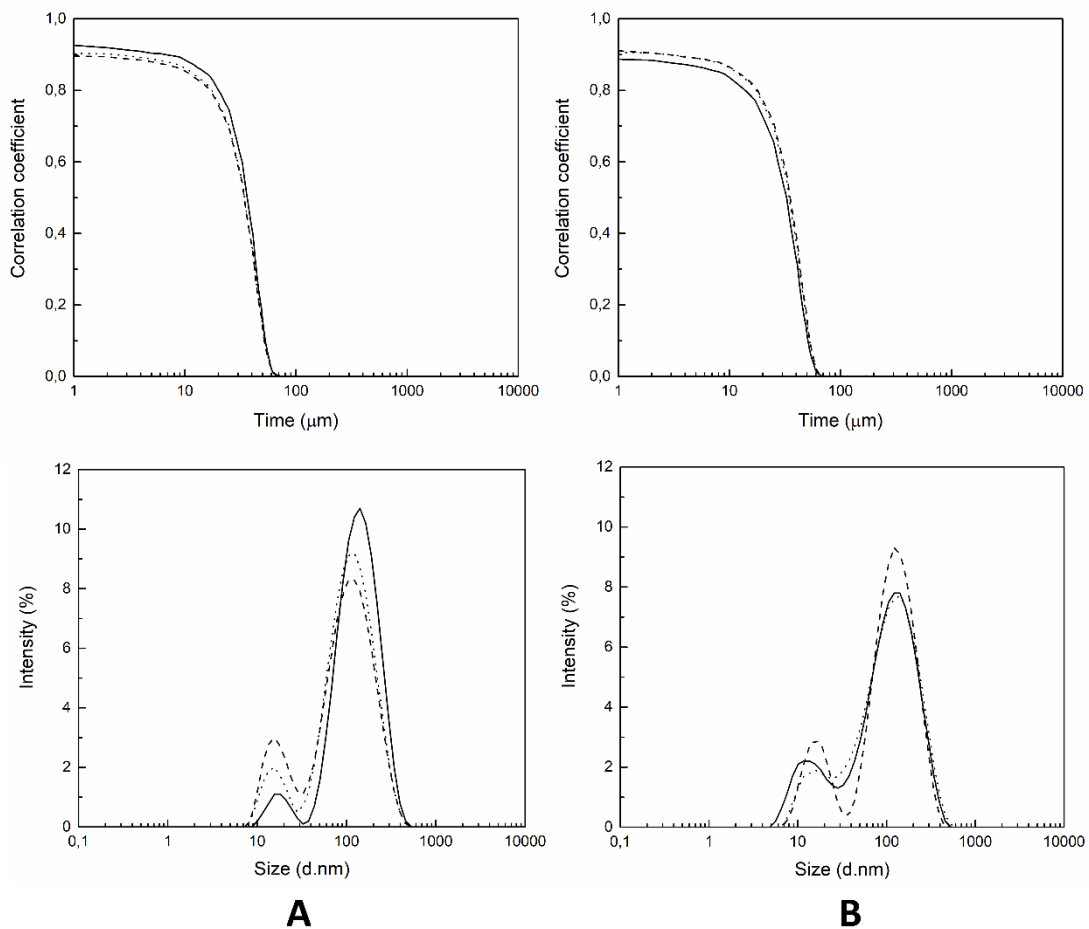
Four different ion exchange chromatography resins were tested: SP Sepharose FF, CM Sepharose FF, DEAE Sepharose FF and Q Sepharose FF (HiTrap™ IEX Selection kit, GE HealthCare) coupled to an Akta Avant 25 chromatography system (GE HealthCare). Pigeon pea flour (625 mg) was dispersed in 32 mL of ultrapure water. The tubes were shaken for 1 h at 20 rpm and centrifuged (10000 g x 10 min x 20°C). After collecting the supernatant, 32 mL of ultrapure water were added to the pellet for further extraction. Then, the two supernatants were mixed. The solution was divided into three parts with pH adjustment to 7.0, 7.5 and 8.0 (using 0.1 M NaOH), to evaluate the effect of the pH on the fixation of proteins in the resin.

Samples were injected onto the columns at 1 mL/min and elution was conducted on a NaCl gradient (step 1 = 0 to 0.75 M in 20 mL and, then step 2 = 0.5 to 1 M in 5 min). The absorbance of proteins was recorded at 280 nm. Fractions (1 mL) were collected and evaluated with polyacrylamide gel electrophoresis.

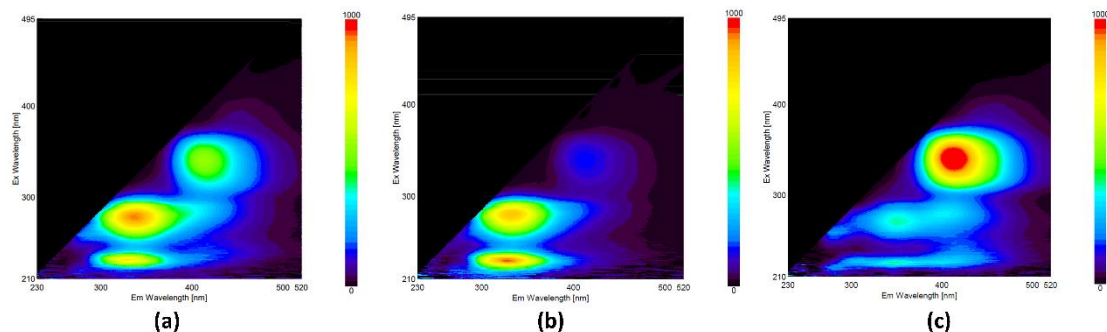
2. Results



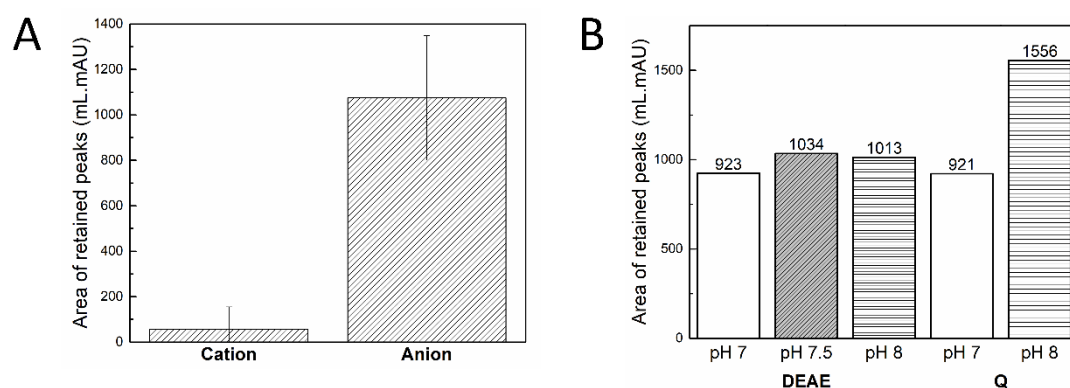
Supplementary Figure 4.1. 3D Emission-Excitation fluorescence matrices of pigeon pea flour supernatant (20 mg/mL) normalized to $A_{280} = 0.075$. (a) 50 mM and (e) 500 mM acetic acid buffer extract, pH 4; (b) 50 mM and (f) 500 mM citric acid buffer extract, pH 6; (c) 50 mM and (g) 500 mM phosphate buffer extract, pH 8; (d) 50 mM and (h) 500 mM carbonate buffer extract, pH 10.



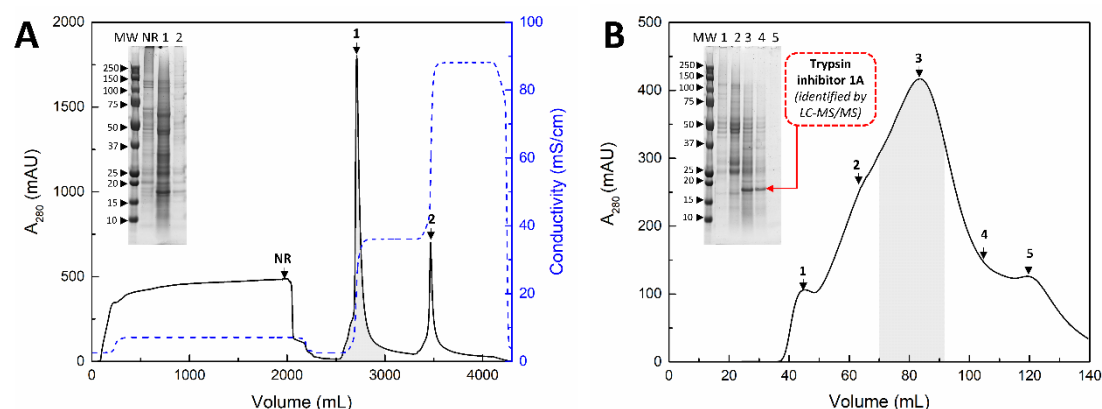
Supplementary Figure 4.2. Correlation function (in the top) and size distribution (in the bottom) of pigeon pea extracts. **(a)** citric acid (pH 6), phosphate (pH 8), and carbonate (pH 10) buffers extracts in low ionic strength (50 mM), **(b)** and citric acid (pH 6), phosphate (pH 8) and carbonate (pH 10) buffers extracts in high ionic strength (500 mM). Solid, dashed, and dotted line corresponds to pH 6, 8, and 10, respectively.



Supplementary Figure 4.3. 3D Emission-Excitation fluorescence matrices of pigeon pea protein extract **(a)** and retentate **(b)** and filtrate **(c)** after three cycles of membrane ultrafiltration.



Supplementary Figure 4.4. Screening of ion exchange chromatography columns and pH of pigeon pea extract for better protein retention. **(a)** Total area of retained peaks (%) in the anion and cation chromatography columns. **(b)** Total area of retained peaks (%) in the anion chromatography columns (DEAE and Q) as a function of extract pH.



Supplementary Figure 4.5. Isolation of trypsin inhibitor 1A (AOA151SYZ1) from pigeon pea from reinjection of the non-retained fraction diluted 10x from the 7S globulin purification by AEC into the Q column **(a)** followed by separation by size exclusion chromatography **(b)**. The gray filled peak corresponds to the collected fraction. NR: not-retained fraction; 1, 2, 3, 4, and 5: fraction peaks. In the inset the SDS-PAGE gels of the separated peaks.

Supplementary Table 4.1. Proteins identified on not-retained fraction by MALDI-TOF-TOF-MS/MS analysis.

Group	Protein name	Uniprot accession	Coverage (%)	MW	Spectra	Peptides
1	Trypsin inhibitor 1A	A0A151SYZ1	73.4	21512	830	61
2a	Beta-conglycinin, alpha chain	A0A151U6Q2	28.0	64269	237	15
2b	Beta-conglycinin, beta chain	A0A151S2A5	43.6	48585	101	15
3	Desiccation protectant protein Lea14 isogeny	A0A151UCY8	48.0	16783	25	8
4	Glutaredoxin-dependent peroxiredoxin	A0A151TIT0	66.7	17287	23	10
5	Kunitz-type trypsin inhibitor KTI1	A0A151SZ51	41.2	16772	22	8
6	Oil body-associated protein 1A	A0A151NLL8	12.5	27664	18	4
7	Basic 7S globulin	A0A151TW66	34.4	21273	13	7
8	Oil body-associated protein 2B	A0A151RE06	29.8	27919	11	6
9	Activator of 90 kDa heat shock protein ATPase isogeny 1	A0A151TU91	34.1	28286	10	5
10	PPM-type phosphatase domain-containing protein	A0A151RJE8	24.8	30944	9	4
11	Uncharacterized protein	A0A151TW63	29.7	16793	7	4
12	Glycinin G3	A0A151TUL0	7.9	50145	5	2
13	3-oxoacyl-[acyl-carrier-protein] reductase	A0A151VJZ4	7.9	31759	4	2
14	Glycinin	A0A151RNW3	5.7	55550	4	2
15	VOC domain-containing protein	A0A151TMA9	20.1	16526	3	2
16	Beta-conglycinin, beta chain	A0A151U6V8	16.2	21210	3	2
17	Formate dehydrogenase, mitochondrial	A0A151QPT4	10.8	42832	2	2
18	Lectin 2	P93456	10.5	28228	2	2

Supplementary Table 4.2. Proteins identified on pigeon pea crude protein extract by MALDI-TOF-TOF-MS/MS analysis.

Group	Protein name	Uniprot accession	Coverage (%)	MW	Spectra	Peptides
1a	Beta-conglycinin, alpha chain	A0A151U6Q2	59.61%	64270	964	65
1b	Beta-conglycinin, beta chain	A0A151S2A5	60.42%	48585	646	49
2	Glycinin G3	A0A151TUL0	79.15%	50146	400	53
3	Glycinin	A0A151RNW3	73.02%	55550.6	396	42
4	Sucrose-binding protein	A0A151RL88	54.05%	54728.8	157	44
5	Uncharacterized protein	A0A151RC24	72.96%	24641.3	143	34
6	Embryonic protein DC-8	A0A151U337	33.16%	40489.1	110	26
7	Alpha-1,4 glucan phosphorylase	A0A151T017	45.89%	109839	90	39
8	Trypsin inhibitor 1A	A0A151SYZ1	66.33%	21513	88	25
9	Basic 7S globulin	A0A151TW66	76.04%	21273.8	64	23
10	Vicilin-like antimicrobial peptides 2-2	A0A151T151	42.31%	52447.6	59	20
11	Urease	A0A151SDR3	38.65%	89113.4	57	30
12	Peroxiredoxin	A0A151S664	73.39%	24441.4	56	19
13	Vicilin-like antimicrobial peptides 2-2	A0A151TN63	38.30%	49306.4	56	20
14	18.2 kDa class I heat shock protein	A0A151SX67	69.08%	17404.8	51	19
15	Protein disulfide-isomerase	A0A151SQ13	56.54%	58231.1	48	31
16	Glucose and ribitol dehydrogenase	A0A151U4W9	56.90%	31614	48	16
17	Bowman-Birk type proteinase inhibitor 2	A0A151RI37	43.93%	12006.3	44	7
18	Late embryogenesis abundant protein D-29	A0A151R986	23.80%	46858.2	39	15
19	Uncharacterized protein	A0A151U0F1	50.33%	16105.7	35	13
20	Seed biotin-containing protein SBP65	A0A151SEU4	38.24%	53462.7	31	17
21	Embryonic protein DC-8	A0A151U337	29.26%	20548.4	31	10
22	Glyceraldehyde-3-phosphate dehydrogenase	A0A151TR34	49.70%	36602	30	12
23	Formate dehydrogenase, mitochondrial	A0A151R9F0	59.48%	42560.7	29	17
24	Nucleoside diphosphate kinase	A0A151RIQ5	65.10%	16487.6	28	12
25	CobW/HypB/UreG nucleotide-binding domain-containing protein	A0A151S4D2	66.55%	30493.3	27	16
26	Peroxidase	A0A151 RKT1	33.93%	70074.7	26	18
27	18.2 kDa class I heat shock protein	A0A151SX67	64.29%	17155.8	26	10
28	Fructose-bisphosphate aldolase	A0A151R1P9	49.16%	38255	24	14
29	Peptidyl-prolyl cis-trans isomerase	A0A151RAJ7	52.91%	18185	24	10
30	Kunitz-type trypsin inhibitor KTI1	A0A151SZ51	38.19%	21602.1	23	8
31	Annexin	A0A151TPX1	40.47%	34445.3	22	13
32	Low-temperature-induced 65 kDa protein	A0A151RHC5	24.96%	71752.3	22	16
33	Triosephosphate isomerase, cytosolic	A0A151SUT3	54.15%	27196.2	22	13
34	Poly [ADP-ribose] polymerase	A0A151TNV1	27.65%	91076.6	21	16
35	Lipoxygenase	A0A151SUB6	21.46%	97315.9	21	15
36	UTP--glucose-1-phosphate uridylyltransferase	A0A151SOL7	40.21%	52103.5	19	12
37	Heat shock cognate protein 80	A0A151S4L4	19.80%	80833.2	19	14
38	phosphopyruvate hydratase	A0A151TV65	35.32%	48665.8	19	10
39	Oil body-associated protein 2B	A0A151RE06	47.76%	27919.6	19	11
40	Desiccation protectant protein Lea14 isogeny	A0A151UCY8	48.03%	16783.7	19	8
41	Elongation factor 2	A0A151S2W2	17.45%	92536.7	18	11
42	14-3-3-like protein E	A0A151TDK8	40.46%	29760.1	17	11
43	Elongation factor 1-alpha	A0A151RR52	23.04%	49330.8	17	8
44	Gamma-interferon-inducible lysosomal thiol reductase	A0A151SXY3	37.20%	28642.1	16	6

Supplementary Table 4.2. continued.

Group	Protein name	Uniprot accession	Coverage (%)	MW	Spectra	Peptides
45	Luminal-binding protein 4	A0A151RID0	15.04%	73319	15	9
46	Protein IN2-1 isogeny B	A0A151TUS7	41.31%	29406.2	14	9
47	Phosphoglycerate kinase	A0A151QRU3	30.42%	42548.7	13	11
48	Thioredoxin H-type 1	A0A151TC84	52.54%	12997.8	13	5
49	Uncharacterized protein	A0A151U0F1	44.16%	8159.93	13	6
50	EMB-1 protein	A0A151S2P2	39.39%	10913.3	13	4
51	Annexin	A0A151TPX1	26.17%	36738.2	12	9
52	Alcohol dehydrogenase 1	A0A151TSB7	19.60%	43052.7	12	8
53	Glutaredoxin-dependent peroxiredoxin	A0A151TIT0	48.15%	17287.1	12	6
54	Aquaporin TIP-type alpha	A0A151RKJ8	21.48%	27113.2	12	5
55	superoxide dismutase	A0A151UDT1	32.67%	15135.5	12	6
56	2S albumin	A0A151QQ22	44.83%	16695	11	8
57	Seed maturation protein PM36	A0A151ST20	33.19%	26112.1	10	6
58	Malate dehydrogenase	A0A151T1Y5	31.93%	35331.2	10	6
59	Cytochrome P450 71A1	A0A151TR99	7.15%	94943.2	10	5
60	Aspartic proteinase	A0A151SU18	14.51%	55422	9	7
61	Cell division cycle protein 48 isogeny	A0A151SGI9	12.70%	84474.1	8	7
62	Calreticulin	A0A151RF02	29.41%	50444.5	8	7
63	Calmodulin	P17928	42.28%	16850.9	8	5
64	Uncharacterized protein ECU03-1610	A0A151RZ44	20.09%	24328.7	8	4
65	Peroxygenase	A0A151T132	19.58%	27336.4	8	5
66	Alcohol dehydrogenase 1	A0A151UBN7	15.16%	37134.8	8	5
67	Ribulose biphosphate carboxylase large chain	A4GG89	7.40%	52358.5	8	4
68	Uncharacterized protein	A0A151RHY5	27.13%	14044.8	8	3
69	Defensin-like protein 4	A0A151S2H7	29.73%	8151.02	8	2
70	Uncharacterized protein	A0A151TV88	13.10%	75604.9	7	6
71	Glutathione S-transferase DHAR2	A0A151U811	45.76%	23172.2	7	7
72	Endo-1,3,1,4-beta-D-glucanase	A0A151STR4	27.62%	25513.2	7	5
73	Pectinesterase	A0A151UCA3	10.82%	54509.9	7	5
74	Aminopeptidase	A0A151R7C2	10.88%	98738.1	6	6
75	Elongation factor 1-gamma	A0A151T893	23.96%	43822.4	6	6
76	Heat shock protein 101 family	A0A151SPH8	10.45%	100920	6	6
77	Translationally-controlled tumor protein isogeny	A0A151T5X4	36.53%	18962.5	6	4
78	Eukaryotic translation initiation factor 5A	A0A151SL27	24.38%	17474.7	6	4
79	Cysteine proteinase inhibitor	A0A151RC35	21.85%	26726.5	6	3
80	UPF0098 protein MTH-273	A0A151RGP8	30.25%	17896.8	6	4
81	Elongation factor 1-beta 1	A0A151T4L0	16.74%	23978.1	6	4
82	Seed biotin-containing protein SBP65	A0A151TPG0	17.49%	34956.3	6	5
83	UDP-arabinopyranose mutase	A0A151TRX5	24.66%	41313.7	5	5
84	Intracellular protease 1	A0A151U9X1	17.31%	41195.7	5	4
85	Leucine aminopeptidase 1	A0A151RXM3	9.90%	59641.8	5	4
86	Caffeoyl-CoA O-methyltransferase At4g26220 family	A0A151RGJ5	29.83%	26707.3	5	4
87	Subtilisin inhibitor 1	A0A151RL22	72.17%	10858.5	5	4
88	formate--tetrahydrofolate ligase	A0A151RWZ9	9.69%	57952	5	5
89	Superoxide dismutase	A0A151T157	20.48%	28324.2	5	4
90	S-(hydroxymethyl)glutathione dehydrogenase	A0A151TUM6	27.44%	40386.5	5	4
91	ADP-ribosylation factor	A0A151RYT4	22.96%	22655.7	5	3

Supplementary Table 4.2. continued.

Group	Protein name	Uniprot accession	Coverage (%)	MW	Spectra	Peptides
92	Cold and drought regulatory protein (Fragment)	A0A1B5FL35	31.18%	10730	5	4
93	Aldose reductase	A0A151T987	15.31%	36055.3	5	3
94	60S acidic ribosomal protein P2B	A0A151R6V4	47.79%	11492.8	5	3
95	UPF0098 protein MTH-273	A0A151RGP8	27.65%	18728.5	5	3
96	Uncharacterized protein	A0A151TCQ9	24.14%	9277.54	5	4
97	Selenium-binding protein	A0A151SQ99	11.56%	51414.6	4	4
98	Elongation factor 1-delta	A0A151TR87	27.16%	25438.8	4	4
99	Universal stress protein A-like protein	A0A151RYQ8	18.29%	19949.9	4	3
100	superoxide dismutase	A0A151UDT1	37.59%	14965.8	4	3
101	Outer envelope pore protein 16-2, chloroplastic	A0A151QR91	19.79%	20244.3	4	3
102	Serine hydroxymethyltransferase	A0A151SQI9	8.28%	51828.4	4	4
103	Reticulon-like protein	A0A151SV55	18.50%	28677.3	4	4
104	Dehydrin COR47	A0A151TD44	26.39%	24274.3	4	4
105	ATP synthase subunit beta	A0A151U754	9.64%	59869.3	4	3
106	Lactoylglutathione lyase	A0A151T6U3	9.06%	32389.6	4	2
107	22.7 kDa class IV heat shock protein	A0A151SIV6	13.27%	22236.8	4	2
108	Uncharacterized protein	A0A0R0KF19	17.81%	16147.1	4	3
109	Proteasome subunit alpha type	A0A151TQ46	21.89%	25430.7	3	3
110	Sucrose synthase	A0A151SKF0	4.04%	84857.1	3	3
111	glutathione transferase	A0A151RGJ3	24.19%	24750	3	3
112	SMP domain-containing protein	A0A151RL90	21.92%	26782.6	3	3
113	60S acidic ribosomal protein P0	A0A151S1K9	21.12%	34282	3	3
114	Protein thiJ	A0A151S9C5	13.45%	41792.1	3	3
115	phosphoglucomutase (alpha-D-glucose-1,6-bisphosphate-dependent)	A0A151T3C9	10.48%	59288.2	3	3
116	Ferritin	A0A0L9UVI2	13.33%	28415.6	3	3
117	Histone H4	A0A151RKC5	21.36%	11402.4	3	2
118	ATP-dependent RNA helicase	A0A151SRZ9	5.81%	46791.9	3	2
119	RuBisCO large subunit-binding protein subunit alpha	A0A151TNC7	5.24%	58295.4	3	2
120	4-alpha-glucanotransferase	A0A151TQK3	6.21%	63515.3	3	3
121	Guanosine nucleotide diphosphate dissociation inhibitor	A0A151UC16	6.98%	49691	3	2
122	MLP-like protein 43	A0A151SFR7	16.05%	18911.4	3	3
123	40S ribosomal protein S3-3	A0A151SP15	12.77%	26235.1	2	2
124	60S ribosomal protein L10	A0A151QVNO	10.18%	25418.3	2	2
125	40S ribosomal protein S9-2	A0A151RZH1	12.18%	23052.4	2	2
126	40S ribosomal protein S5	A0A151S1X3	8.49%	36765.3	2	2
127	malate dehydrogenase	A0A151T1Y5	5.85%	43163.4	2	2
128	argininosuccinate synthase	A0A0R0JQZ9	5.71%	48247.7	2	2
129	Calnexin isogeny	A0A151SY44	4.92%	62338.1	2	2
130	Bifunctional dihydrofolate reductase-thymidylate synthase	A0A151R969	4.36%	56958.6	2	2
131	Glucose-6-phosphate isomerase	A0A151TC03	4.02%	60260	2	2
132	Desiccation protectant protein Lea14 isogeny	A0A151UCY8	6.23%	41182.3	2	2
133	Hyoscyamine 6-dioxygenase	A0A151QRI4	11.05%	37780.1	2	2
134	Protein IN2-1 isogeny B	A0A151TUS7	8.03%	28580	2	2
135	PPM-type phosphatase domain-containing protein	A0A151RJE8	7.09%	30944.8	2	2
136	Protein usf	A0A151RM14	13.53%	26493.3	2	2
137	aldehyde dehydrogenase (NAD(+))	A0A151RMA7	10.15%	50649.9	2	2

Supplementary Table 4.2. continued.

Group	Protein name	Uniprot accession	Coverage (%)	MW	Spectra	Peptides
138	Co-chaperone protein p23	A0A151S880	17.34%	19231.3	2	2
139	Glutaredoxin-C1	A0A151S9S1	28.93%	13177.7	2	2
140	5'-AMP-activated protein kinase subunit gamma-3	A0A151SPV7	10.57%	40572.9	2	2
141	Eukaryotic peptide chain release factor GTP-binding subunit ERF3B	A0A151T323	3.58%	56231.9	2	2
142	Malic enzyme	A0A151T3P8	4.26%	67499.1	2	2
143	Pectin acetyltransferase	A0A151TFB9	8.52%	43993.6	2	2
144	60S acidic ribosomal protein P2B	A0A151U3E9	26.67%	12174.3	2	2

Supplementary Table 4.3. Polysaccharide composition in purified pigeon pea 7S globulin.

	Neutral and acid monosaccharides (g 100 g ⁻¹ , d.b)								NSP (g 100 g ⁻¹ , d.b)
	Rha	Fuc	Ara	Xyl	Man	Gal	UA	Glu*	
7S globulin (hydr.)	n.d	n.d	0.1 ± 0.1	n.d	1.7 ± 0.1 ^a	0.1 ± 0.0	0.3 ± 0.0 ^b	n.d	2.3 ± 0.1 ^b
7S globulin (native)	n.d	n.d	0.2 ± 0.0	0.2 ± 0.0	1.4 ± 0.1 ^b	0.0 ± 0.0	0.9 ± 0.1 ^a	n.d	2.9 ± 0.0 ^a

Different letters in the same column (for flours and protein concentrates) indicate statistical difference ($p < 0.05$). Rha, Fuc, Ara, Xyl, Man, Gal, UA, Glu, NSP are rhamnose, fucose, arabinose, xylose, mannose, galactose, uronic acid, glucose and non-starch polysaccharides, respectively. Values below the detection limit are expressed as n.d.

Supplementary Table 4.4. Trypsin inhibitors activity (TIA) in purified pigeon pea 7S globulin.

	µgTId mg ⁻¹ sample	µgTId mg ⁻¹ protein
7S globulin (hydr.)	1.4 ± 0.0 ^b	1.6 ± 0.0 ^b
7S globulin (native)	3.6 ± 0.0 ^a	4.1 ± 0.0 ^a

Different letters in the same column (for flours and protein concentrates) indicate statistical difference ($p < 0.05$).

Part II. Physicochemical characterization of purified pigeon pea 7S globulin

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1.Introduction

Pigeon pea (*Cajanus cajan* (L.) Millsp.) is a drought-resistant and protein-rich pulse (18-28%) whose global production corresponds to around 6% of total pulse production around the world (FAO, 2019). Native to emerging countries in Africa and Asia, its cultivation is almost entirely restricted to small-scale farmers despite its great potential for expansion. A greater understanding of its protein composition and its possible applications in the food industry is one way to boost interest in this underused pulse (Locali-Pereira et al., 2023; Sosa et al., 2021; Boachie et al., 2019).

Globulin is the main storage protein in pigeon pea (about 61%) and according to the Svenberg sedimentation coefficient (S) classification, the 7S fraction is the most abundant in its seeds (Gravel & Doyen, 2023; Krishnan et al., 2017). A member of the 7S globulin family, globulin is a trimer of molecular weight of around 180 kDa and is composed of two subunits, α and β , in the ratio of 1:2 and not linked by disulphide bonds (Krishna & Bhatia, 1985). Krishnan et al. (2017) demonstrated that pigeon pea 7S globulin has high homology with the amino acid sequence of soybean 7S globulin. Furthermore, it has been shown that like soybean 7S globulin, pigeon pea 7S globulin is also classified as a glycoprotein (Krishna et al., 1977).

Some studies have developed protocols for the analytical-scale purification of 7S globulin from pigeon pea (Fernández Sosa et al., 2021; Krishna et al., 1977; Krishna & Bhatia, 1985) using ion exchange and size exclusion chromatography techniques. Despite the fundamental knowledge that these studies brought regarding the composition of pigeon pea 7S globulin, there is a need for a more in-depth physicochemical characterization of this protein, which, to our knowledge, has never been conducted.

In parallel, bioinformatics techniques can be used to make predictions of various physicochemical properties of proteins, based on their amino acid sequence. The

physicochemical properties of each of the 20 amino acids that make up proteins are well known and each of their residues, individually, contribute to the global physicochemical properties of proteins (Anfinsen et al., 1961; Baxevanis & Ouellette, 2001). To our knowledge, *in silico* studies performed with pigeon pea protein sequences have only been used to predict bioactive properties of peptides released by enzymatic hydrolysis (Boachie et al., 2019). However, no study has been reported to predict the physicochemical properties of purified globulins from pigeon pea. Therefore, the question that arises is: is it possible to accurately predict the physicochemical properties of pigeon pea 7S globulin using bioinformatics?

The present study aimed at the physicochemical characterization of pigeon pea 7S globulin obtained by a preparative-scale purification protocol developed by our research group. The 7S globulin from pigeon pea was investigated in its native and partially hydrolyzed state. The characterization of the physicochemical properties was conducted by a combination of biochemical, spectroscopic and chromatographic techniques. Experimental data obtained to determine extinction coefficients, refractive index increment, UV-visible absorption spectra, among others, were compared with theoretical values computed from known sequences of the α - and β -chains of 7S globulin from pigeon pea. Differences between predicted and experimental data are discussed, as well as the impact of partial protein hydrolysis on these values.

2. Materials and Methods

2.1 Materials

Pigeon pea seeds were purchased from a local supplier (São Paulo, Brazil). Sodium hydroxide, hydrochloric acid, sodium phosphate, were purchased from Sigma-Aldrich. Acetic acid was purchased from Carlo Erba. Citric acid was purchased from Merck. Sodium chloride was purchased from Analar NormaPur.

2.2 *In silico* analyses

The sequence analysis of 7S β -conglycinin from pigeon pea were retrieved from the public UniProt Knowledgebase, using the α - (A0A151U6Q2) and β -chains (A0A151S2A5) sequences. The amino acid sequences were processed on the ProteinPred webserver (<https://www.proteinpred.fr/foodProteinDB/>) to predict the physicochemical properties.

2.3 Pigeon pea protein extraction and purification

Pigeon pea seeds were milled ($< 300 \mu\text{m}$) in a grinder (J.R. Araújo & Cia Ltda., Brazil) at 1730 rpm. Then, the flour was dispersed in 50 mM pH 8 Tris buffer (1:10, w/w) and stirred for 1 h in a mechanical stirrer (RZR 2041, Heidolph) at 225 rpm. After stirring, the solution was centrifuged ($10000 \text{ g} \times 10 \text{ min} \times 20^\circ\text{C}$) and the supernatant was collected. The pellet was redispersed in 50 mM Tris pH 8 buffer solution, with subsequent stirring, centrifugation, and collection of the new supernatant. The two supernatants were pooled and filtered ($8 \mu\text{m}$ MCE membrane, Millipore).

The procedures for purifying 7S globulin from pigeon pea were presented in Part I of this chapter. Briefly, to purify 7S globulin in native state, the extract was first injected into a desalting column (GH25, Cellufine). Then, the desalted extract was injected into a Q-Sepharose Fast Flow column (GE Healthcare). The fractions were pooled and injected into a gel filtration column (Cellufine GCL) to further purification. After separation in the gel filtration column, the fractions were pooled, frozen, and freeze-dried for 72h. Purified partially hydrolyzed 7S globulin were produced using the same process, but with dialysis of the protein fractions after separation in the Q-Sepharose column. Dialysis was conducted against ammonium carbonate buffer (pH 8.0) in three cycles of 8h each. After dialysis, the fractions were lyophilized for 72h, and the powders were redispersed in phosphate buffer pH 7.0 (30g/L) and injected into the gel filtration column. After separation, the fractions were collected, pooled, and subjected to dialysis and freeze-dried again.

The purified fractions were stored at 20°C in a desiccator containing a K_2CO_3 saturated solution (RH = 42%) until analysis. The purified protein fractions had a purity of 87% d.b and approximately 3% carbohydrates.

2.4 Amino acid profile

To determine the amino acid profile of the purified fractions, the protocol AOAC 2018.06 was used (Jaudzems et al., 2019). The determination of tryptophan content was performed under accreditation ((ISO/IEC 17043:2010).

2.5 Determination of the specific extinction coefficient

The specific extinction coefficients of pigeon pea 7S globulin dispersion were determined at pH 4.0, 5.0, 6.0, 7.0, and 8.0. Acetic acid ($pK_a = 4.76$) was used to prepare the pH 4.0 and pH 5.0 buffer solutions, citric acid ($pK_a = 6.39$) was used to prepare the pH 6.0 buffer solution, and sodium phosphate ($pK_a = 7.20$) was used to prepare the pH 7.0 and pH 8.0 buffer solutions. Different proportions of acids and bases were used to obtain the desired pHs and sodium chloride was added to reach an ionic strength of 500 mM. All buffer solutions were prepared to have a concentration of 20 mM. Protein stock solutions (2 mg mL^{-1}) were shaken at 20 rpm for 2h, centrifuged ($10000 \text{ g} \times 20^\circ\text{C} \times 10 \text{ min}$), filtered ($2 \mu\text{m}$, Minisart) and added to hydrated dialysis membranes. Dialysis was conducted against the buffer solution (1:20, volume sample/volume buffer) to remove any residual salt. The buffer solution was changed 3 times after 8 h each dialysis cycle. The total nitrogen content of supernatant was determined using the Kjeldahl procedure and a nitrogen-to-protein factor of 5.85 and 5.74 for native and hydrolyzed 7S globulin, respectively, based on amino acid composition. The stock solution was then diluted (20, 10, 6.6, 5.0, 4.0, 2.6, 2.0, 1.60, 1.30 and 1.15 times). The UV–visible spectra were recorded from 250 to 800 nm with a step of 1 nm. All samples were measured using a quartz cuvette ($L = 1 \text{ cm}$) and a spectrophotometer (Shimadzu UV1800, Shimadzu corporation, Kyoto, Japan). The specific extinction coefficients at 280 nm were determined by the slope of the curve of absorbance at 280 nm vs protein concentration.

2.6 Refractive index increment (dn/dc)

The same stock solutions and dilutions prepared for the specific extinction coefficients were used to determine the refractive index increment (dn/dc). The dn/dc was determined at 589.3 nm using a multi-wavelength Abbe refractometer (Anton Paar GmbH, Graz/AUSTRIA) at 20°C . Aliquots of $50 \mu\text{L}$ were used for each dispersion and measurements were performed in triplicate.

2.7 Protein solubility

To probe protein solubility, the purified fractions were dispersed in buffer solutions (5 mg mL^{-1}) for each evaluated pH. Suspensions were evaluated at low and high ionic strength (50 or 500 mM). After stirring for 2 hours at 20 rpm, the suspensions were centrifuged ($10,000 \text{ g} \times 10 \text{ min} \times 20^\circ\text{C}$) and the absorbance of the supernatant was recorded. The extinction coefficients were used to calculate the protein concentrations in the supernatants. For samples at low ionic strength (50 mM), a 4 M NaCl solution was used to dilute the samples to reach an ionic strength of 500 mM before absorbance

measurement, so the same specific extinction coefficients could be used. The solubility (%) was calculated by the ratio between the protein concentration in the supernatant and the protein concentration in the initial sample mass.

2.8 Electrophoretic mobility

The purified fractions were dispersed in distilled water (5 mg mL^{-1}), shaken for 2 h at 20 rpm and centrifuged ($10000 \text{ g} \times 10 \text{ min} \times 20^\circ\text{C}$). Supernatants were collected and dialyzed against distilled water (1:20, volume of supernatant/volume of water). After three cycles of dialysis (8h each), 1 mL aliquots of supernatant had their pH adjusted to 4.0, 5.0, 6.0, 7.0 or 8.0, using 0.1 M HCl and 0.1 M NaOH solutions. Electrophoretic mobility was measured in triplicate for each pH using a Zetasizer Nano Series (Nano-ZS, Malvern instrument, Germany).

2.9 Dynamic light scattering (DLS)

To evaluate the effect of pH on size distribution, stock solutions of purified fractions (2 mg mL^{-1}) prepared for determination of specific extinction coefficients were measured using a Zetasizer Nano Series (Nano-ZS, Malvern instrument, Germany). Aliquots (0.6 mL) were filtered (0.2 μm , Minisart, High-Flow, Sartorius Stedim Biotech GmbH, Goettingen, Germany), added to cuvettes and equilibrated for 120s at 20°C before measurements. Measurements were performed in manual mode conducted in 10 runs of 30 sec and were repeated three times for each sample.

2.10 Statistical analysis

Data were analyzed using the OriginPro 2016 software. Variance analysis (ANOVA) followed by Tukey post hoc test was applied to determine the difference between samples at a 5% significance level. The experiments were carried out in three independent replicates.

3. Results and discussion

Proteomic analysis identified the α and β chains of pigeon pea 7S globulin in the purified fractions (Part I of this chapter). Both sequences were retrieved from UniProtKB and used to calculate theoretical values for several physicochemical properties determined experimentally in this work. The amino acid composition of purified fractions was determined (Table 4.3) and also used to calculate theoretical values. Therefore, the next sections discuss the experimental results obtained in the physicochemical characterization of pigeon pea 7S globulin in comparison with predicted values based on the sequences and amino acid composition of the purified fractions.

3.1 Amino acid composition, N-to-P factor and chemical score

Glutamic acid, phenylalanine, glycine, leucine and alanine were the predominant amino acids in pigeon pea flour and in the purified fractions of 7S globulin, in agreement with the values predicted from the sequences (Table 4.3). On the other hand, all samples showed low levels of sulphur-containing amino acids (cysteine and methionine), which was also observed by Zheng et al. (2009) for soybean 7S globulin. The amino acid composition of native and partially hydrolyzed 7S globulin showed similarity in the contents of all amino acids, with more significant differences in the contents of phenylalanine and leucine, which showed reductions of 13 and 9% in partially hydrolyzed 7S globulin, in relation to the native protein.

The N-to-P factors determined from the amino acid composition were 5.83, 5.85 and 5.74 for pigeon pea flour, native 7S globulin and partially hydrolyzed 7S globulin (Table 1). In comparison, the N-to-P factor calculated from the sequences of the α and β chains of 7S globulin was 5.75. These values are below the N-to-P factor of 6.25 that is commonly used for proteins. Therefore, calculating the N-to-P factor based on the amino acid composition is more suitable to obtain N-to-P factors with greater accuracy. It was noted that partial hydrolysis of 7S globulin resulted in a decrease in the N-to-P factor, possibly due to changes in the content of some amino acids.

Analysis of the chemical score showed that tryptophan is the first limiting amino acid for 7S globulin, while methionine and cysteine are, together, the secondary limiting amino acids (Figure 4.12). The chemical scores of pigeon pea 7S globulin for leucine, isoleucine, lysine and phenylalanine + tyrosine were higher than the values reported by Zheng et al. (2009) for soybean 7S globulin, especially for the value of phenylalanine + tyrosine, in which the chemical score for pigeon pea was approximately double that of soybeans. Finally, the histidine chemical score showed the greatest difference between native 7S globulin (179), partially hydrolyzed 7S globulin (226) and the predicted value (295), due to the different histidine contents observed in the amino acid composition.

Table 4.3. Experimental and predicted amino acid composition and N-to-P factors of pigeon pea 7S globulin.

Amino acids	Composition (% nb total aa)					
	Experimental			Theoretical		
	Pigeon pea Flour	Native 7S globulin	Hydrolyzed 7S globulin	α -chain	β -chain	7S- globulin*
Alanine	6.9	6.5	6.8	6.2	6.0	6.1
Cystein	1.5	0.3	0.5	0.9	0.0	0.5
Aspartique acid	3.6	4.6	4.3	4.1	3.7	3.9
Glutamique acid	11.4	10.2	10.2	11.8	9.5	10.6
Phenylalanine	7.5	7.6	6.6	6.4	8.0	7.2
Glycine	7.5	5.6	6.3	4.7	5.0	4.8
Histidine	2.8	2.5	3.2	6.2	2.2	4.2
Isoleucine	3.9	4.7	4.6	4.3	5.5	4.9
Lysine	5.9	6.2	6.4	6.0	6.5	6.2
Leucine	7.2	10.2	9.3	7.9	10.5	9.2
Methionine	1.1	0.8	0.9	0.6	0.5	0.5
Asparagine	6.1	7.7	7.3	7.0	6.5	6.7
Proline	5.8	5.0	5.2	5.1	5.0	5.0
Glutamine	6.3	5.7	5.7	6.6	6.2	6.4
Arginine	4.5	4.6	4.9	5.6	5.0	5.3
Serine	6.2	7.5	7.1	7.0	8.2	7.6
Threonine	4.0	3.3	3.6	2.4	3.7	3.1
Valine	5.2	5.0	5.1	4.5	5.5	5.0
Tryptophane	0.4	0.1	0.2	0.2	0.0	0.1
Tyrosine	2.1	2.1	2.1	2.4	2.7	2.6
N-to-P factor	5.83	5.85	5.74	5.60	5.90	5.75

* Theoretical values for 7S β -conglycinin were calculated considering a 1:1 ratio between the α and β chains.

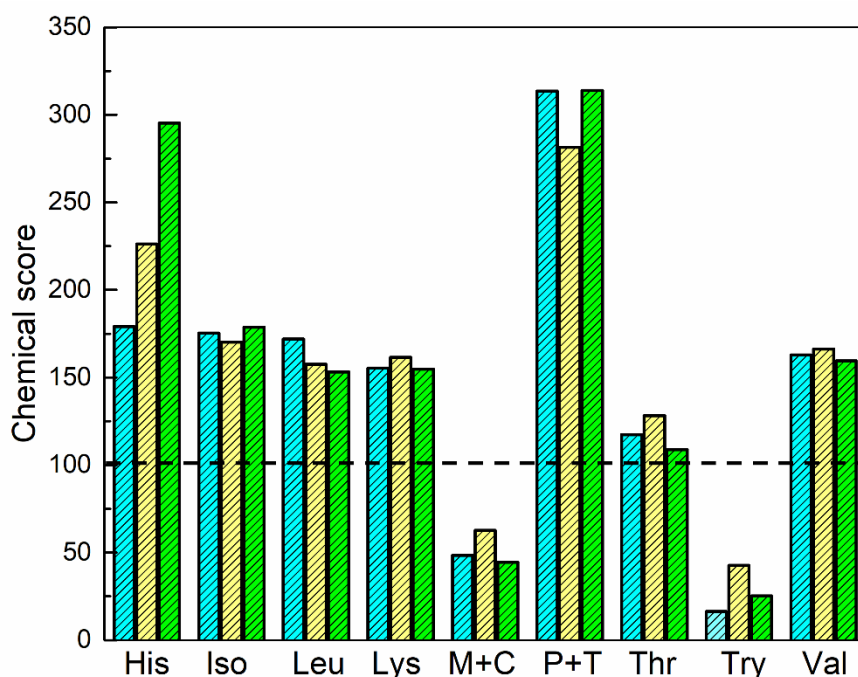


Figure 4.12. Chemical score of pigeon pea 7S globulin. Blue, yellow and green bars indicate 7S globulin in native state, partially hydrolyzed state and theoretical values obtained by predictive study, respectively. His, Iso, Leu, Lys, M+C, P+T, Thr, Try and Val indicate Histidine, Isoleucine, Leucine, Lysine, Methionine + Cysteine, Phenylalanine + Tyrosine, Threonine, and Tryptophane, respectively.

3.2 UV-visible spectra and refractometric parameters

The absorption of protein solutions depends mainly on the contribution of three UV-visible absorbing amino acids: tyrosine, tryptophan, and phenylalanine. While tyrosine and tryptophan show maximum absorbance at 280 nm, phenylalanine has a maximum absorption around 240-265 nm (Hendricks & Julian, 2015; Pace et al., 1995). The UV-visible absorbance spectrum plotted from pigeon pea globulin sequences (Fig 4.13a) showed a higher absorbance of the α chain in relation to the β chain. The maximum absorption for predicted globulin was 274 nm, corresponding mainly to tyrosine and tryptophan absorption. However, the absorption profile before the peak of maximum absorbance also suggests the contribution of phenylalanine.

In fact, the phenylalanine content of the native, partially hydrolyzed and predicted 7S globulin was about 7.6, 6.6 and 7.2 %, respectively. The tyrosine and tryptophan contents are lower than those of phenylalanine, being around 2.1 and 0.4, 2.1 and 0.1, and 2.6 and 0.1 for native, partially hydrolyzed and predicted 7S globulin, respectively. The UV-visible spectrum plotted from the experimental data and based on the amino acid composition of the purified 7S globulin from pigeon pea in native (Fig 4.13b) and partially hydrolyzed state (Fig 4.13c) presented the same profile as the predictive data. However, a shift of 3-4 nm was observed for the fractions purified in the laboratory, which showed maximum absorptions equal to 277 and 278 nm for partially hydrolyzed and native 7S globulin, respectively. The maximum absorbance value of

proteins did not vary with pH, which was also reported by Ducrocq et al. (2022) for leaf protein concentrate and by Schmidt et al. (2004) for napin protein. It is also noted that partial hydrolysis increased the absorbance of pigeon pea 7S globulin, making the spectrum more similar to that of the α chain, while in its native state the purified fraction is more closely resembled the spectrum of the β chain. In fact, the contents of tyrosine, tryptophan and phenylalanine of partially hydrolyzed 7S globulin showed values very close to the contents predicted by the sequence of the α chain of 7S globulin (Table 4.3). In contrast, the phenylalanine content of native 7S globulin was closer to the content predicted by the sequence of the β chain of 7S β -conglycinin.

The extinction coefficients determined at 280 nm ranged from 0.33 to 0.47 $\text{cm}^{-1} \text{g}^{-1} \text{L}$ for 7S globulin in its native state and from 0.38 to 0.57 $\text{cm}^{-1} \text{g}^{-1} \text{L}$ for its partially hydrolyzed state (Table 4.4). These values agree with the extinction coefficient at 280 nm calculated from the globulin sequences (0.34 $\text{cm}^{-1} \text{g}^{-1} \text{L}$) but are quite higher than the values calculated from the amino acid composition (0.27 and 0.32 $\text{cm}^{-1} \text{g}^{-1} \text{L}$) (Table 4.4). This may be related to an underestimation of tryptophan content which is difficult to assess experimentally. The effect of pH on the extinction coefficients was the same for both fractions, in native and partially hydrolyzed states, being lower for pHs 4.0 and 5.0 and higher for pH 6.0. Partial hydrolysis increased the extinction coefficients of 7S globulin for all pH conditions evaluated ($p < 0.05$).

Regarding the refractive index increment (dn/dc), the values ranged from 0.183 to 0.233 for pigeon pea 7S globulin in the native state and from 0.222 to 0.368 for the partially hydrolyzed state (Table 4.4).

According to the theoretical values from sequence and amino acid composition of 7S globulin, the dn/dc value was around 0.190-0.192 (Table 4.4). The refractive index increment value generally used in consensus for proteins is 0.185 (Zhao et al., 2011). The experimental values are, for most conditions, slightly above the theoretical values. Higher dn/dc values were obtained for pH 6.0, while the lowest values were those for pH 5.0, for both samples. It was observed that partial hydrolysis caused an increase in the dn/dc value in all conditions evaluated ($p < 0.05$). A higher dn/dc value may be related to the presence of non-protein compounds in protein fractions. However, given the level of purity of the fractions, it is unlikely that small contaminants would have a significant effect on the values.

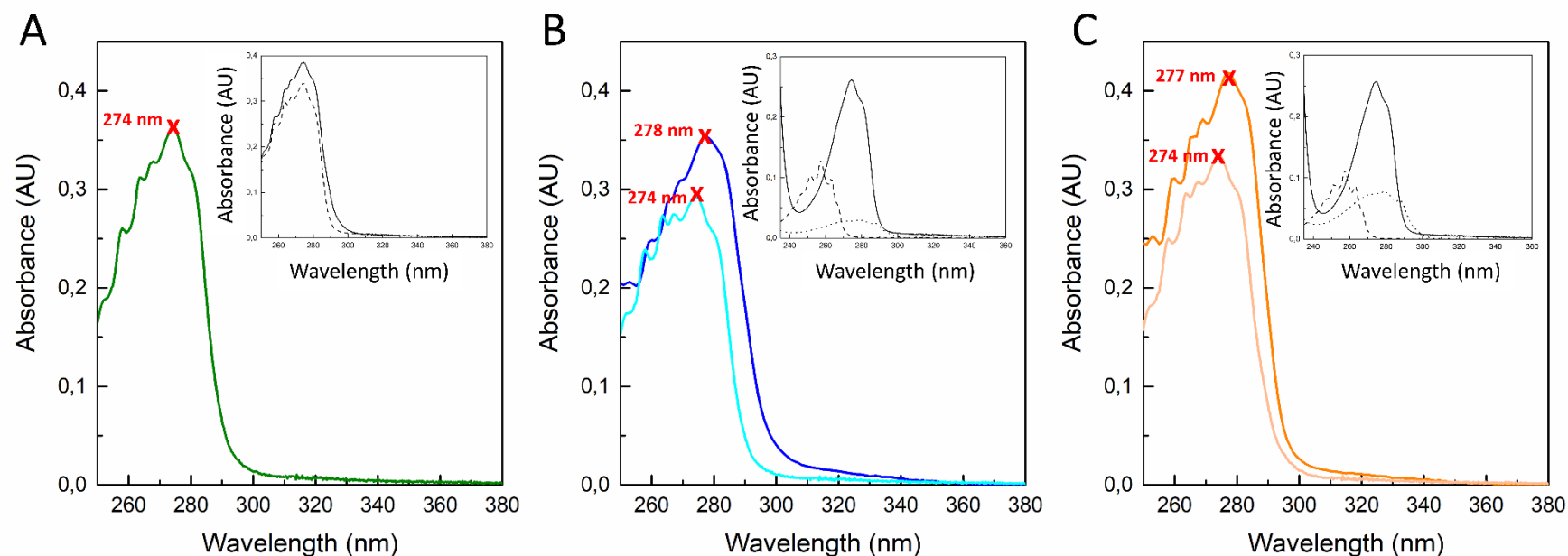


Figure 4.13. Absorption spectra of 1 g/L pigeon pea 7S globulin. **(a)** Theoretical UV-visible spectrum of 7S globulin obtained from the α - and β -chain sequences (1:1) of 7S globulin from pigeon pea. **(b)** UV-visible spectra of native 7S globulin obtained experimentally (dark blue line) and from the amino acid composition (light blue line). **(c)** UV-visible spectra of partially hydrolyzed 7S globulin obtained experimentally (dark orange line) and from the amino acid composition (light orange line). The inset in (a) shows the contributions from the α - (solid line) and β -chain (dashed line) of 7S β -conglycinin, while the insets in (b) and (c) show the contributions from tyrosine (solid line), phenylalanine (dashed line) and tryptophan (dotted line).

Table 4.4. Extinction coefficients and refractive index increment of pigeon pea 7S globulin in native and partially hydrolyzed state.

Purified fraction	pH	Extinction coefficient (280 nm)	Refractive index increment (dn/dc)
Native 7S globulin	Theoretical*	0.27	0.191
	4	0.33 ± 0.00^b	0.194 ± 0.004^b
	5	0.34 ± 0.01^b	0.187 ± 0.003^b
	6	0.47 ± 0.00^b	0.233 ± 0.006^b
	7	0.38 ± 0.01^b	0.212 ± 0.001^b
	8	0.38 ± 0.01^b	0.183 ± 0.005^b
Partially hydrolyzed 7S globulin	Theoretical*	0.32	0.190
	4	0.38 ± 0.00^a	0.247 ± 0.002^a
	5	0.38 ± 0.00^a	0.222 ± 0.001^a
	6	0.57 ± 0.01^a	0.368 ± 0.004^a
	7	0.46 ± 0.00^a	0.281 ± 0.004^a
	8	0.49 ± 0.00^a	0.281 ± 0.006^a
Theoretical values from sequence [†]		0.34	0.192

Different letters in the same column and evaluated pH indicate difference between samples ($p < 0.05$).

[†]Theoretical values calculated from the sequences of the alpha and beta chains of 7S beta-conglycinin from pigeon pea. *Theoretical values calculated from the amino acid composition of native 7S beta-conglycinin.

*Theoretical values calculated from the amino acid composition of 7S beta-conglycinin from pigeon pea.

3.3 Effect of pH on protein solubility and surface charge

The solubility of purified pigeon pea 7S globulin fractions was evaluated at low (50 mM) and high (500 mM) ionic strength (Figure 4.14). At low ionic strength, a clear effect of pH on protein solubility was observed: at pH 4.0, protein solubility was around 41-50%, decreasing to 7-17% at pH 5.0 and then gradually increasing with pHs until reaching a maximum solubility at pH 8.0, which ranged from 67-82% (Fig 4.14a). At all pHs observed, 7S globulin in its native state showed higher solubility than the partially hydrolyzed state. At high ionic strength, the solubility of proteins was higher than the solubilities determined at low ionic strength. Furthermore, at high salt concentrations, 7S globulin in its native state showed solubility > 90% at almost all pHs evaluated, except for pH 6.0 where solubility was around 70% (Fig 4.14b). The 7S globulin partially hydrolyzed in high salt concentrations showed higher solubility at more acidic pHs (83-85% for pHs 4.0 and 5.0) than at alkaline pHs (65-71% for pHs 7.0 and 8.0). For pH 6.0, the solubility of the hydrolyzed protein was also lower (around 57%). From experimental data, minimal solubility was observed at pHs equal to 5.0 and 6.0 for low and high ionic strength conditions, respectively, due to the proximity to the isoelectric point of 7S globulin.

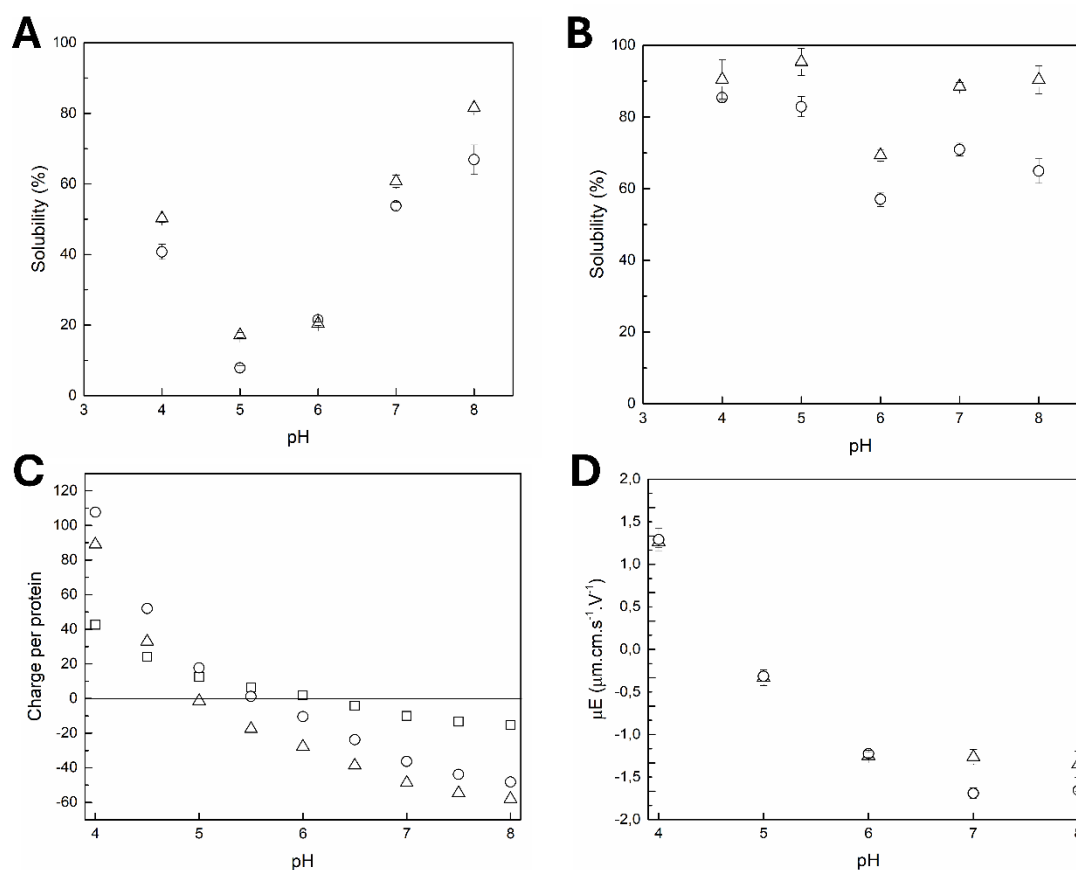


Figure 4.14. Solubility and charge as a function of pH of pigeon pea 7S globulin. Solubility at 50 mM (a) and 500 mM (b) ionic strength; Predicted charge per protein using sequences and amino acid composition of 7S globulin (c); and electrophoretic mobility of purified 7S globulin (d). Triangle, circle, and square indicate native, partially hydrolyzed, and predicted 7S globulin.

The isoelectric point predicted from the sequences and amino acid composition of the purified fractions showed different values (Fig 4.14c). Considering the amino acid composition of the samples, the predicted isoelectric point is around 5.0 for native 7S globulin, while for partially hydrolyzed 7S globulin it is around 5.5. On the other hand, considering the sequence of the α and β chains of 7S β -conglycinin, the isoelectric point is around 6.0.

According to the data obtained for electrophoretic mobility, the isoelectric point of 7S pigeon pea globulin is closer to the values predicted using the amino acid composition, as showed by the electrophoretic mobility value close to zero for pH 5.0 (Fig 4.14d). Differences between experimental and predicted values for the isoelectric point were also observed by Ducrocq et al. (2022) for leaf protein concentrate. This difference in values may be related to the presence of non-protein compounds in the purified fractions. The electrophoretic mobility of 7S globulin in its native state ranged from 1.3 to $-1.3 \mu\text{m cm s}^{-1} \text{V}^{-1}$ and from 1.3 to -1.7 for its partially hydrolyzed state. There was no effect of pH on the electrophoretic mobility of samples at pHs 4.0-6.0 ($p > 0.05$). For pHs

7.0 and 8.0, 7S globulin in its native state showed greater electrophoretic mobility than 7S globulin in a partially hydrolyzed state.

3.4 Effect of pH on the size distribution of proteins

The effect of pH on the size distribution of purified pigeon pea 7S globulin fractions was investigated by DLS measurements (Figure 4.15). The size distribution of 7S globulin presented a bimodal distribution for both evaluated states, native and partially hydrolyzed, with a population around 10-20 nm and another population between 100-300 nm. While 7S globulin in its native state had a greater contribution from the population between 100-300 nm, partial hydrolysis made the population at 15-20 nm more significant. The native 7S globulin showed a larger population around 300 nm when evaluated at pH 6.0. On the other hand, more alkaline pHs (7.0-8.0) shifted this population to around 100 nm. A similar effect was observed for the partially hydrolyzed 7S globulin. In addition, the population around 15-20 nm suffered a greater impact from pH for partially hydrolyzed 7S globulin, with this population being larger for pH 5.0 and gradually decreasing for pHs 4.0, 6.0, 7.0, and 8.0. On the other hand, the larger population showed a significant impact due to pH 4.0, being significantly reduced under these conditions.

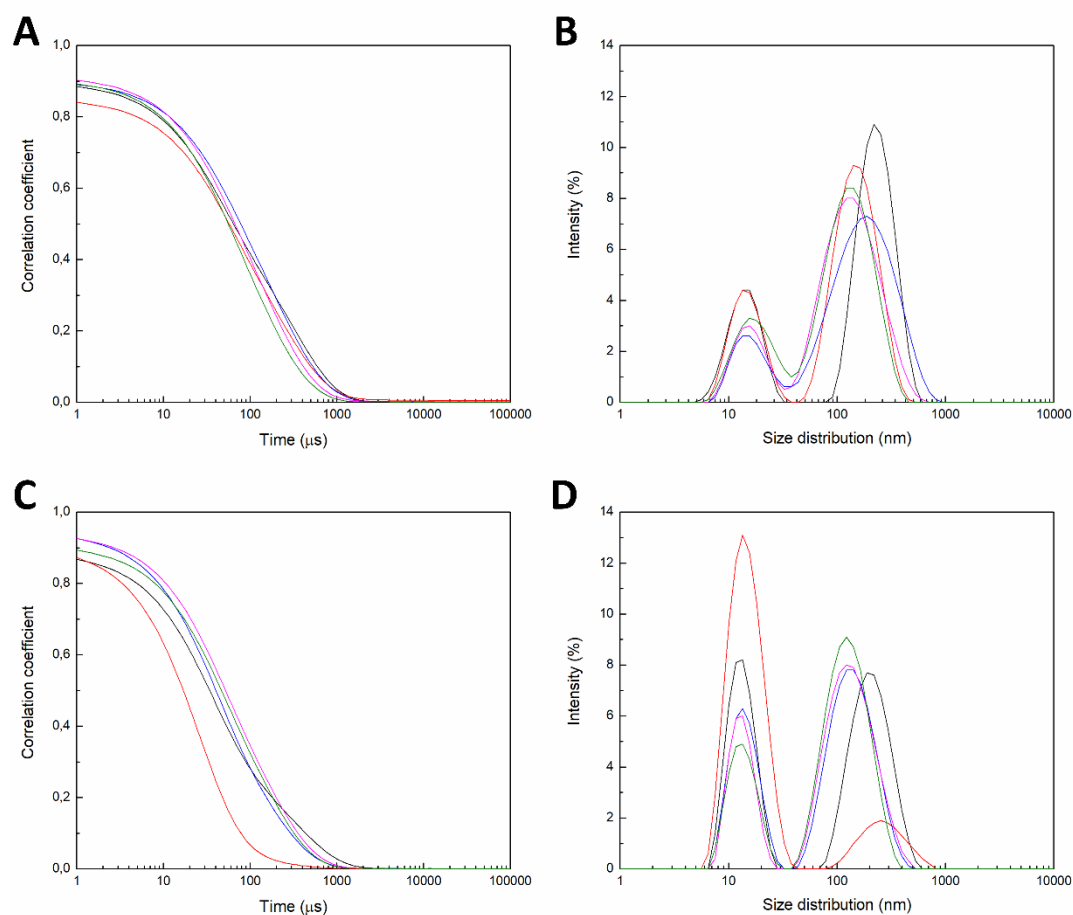


Figure 4.15. Correlation functions (in the left) and size distribution (in the right) obtained by dynamic light scattering (DLS) according to pH for pigeon pea 7S globulin in native (a-b) and partially hydrolyzed (c-d) state. Size distribution curves are the mean of three observations. Solutions at pH 4, 5, 6, 7, and 8 are presented by black, red, blue, pink and green color lines.

4. Conclusion

The characterization of the physicochemical properties of purified proteins provides information to better understand how individual groups of proteins perform when used as food ingredients. In the present work, the physicochemical properties of native and partially hydrolyzed 7S globulin from pigeon pea were characterized. In general, the values predicted by bioinformatics showed a fair estimate of the values obtained from experimental data, mainly for 7S globulin in its native state. Under some conditions, the values for these properties were higher than the predicted values for 7S globulin, suggesting the presence of non-protein compounds. The extinction coefficients and refractive index increment of the purified proteins were affected by pH and partial hydrolysis of the proteins, which resulted in an increase in these values. A solubility above 90% was obtained for native 7S globulin in the presence of high ionic strength. However, partial hydrolysis of 7S globulin reduced its solubility in relation to its native state, in all conditions evaluated. The change in several physicochemical properties caused by the partial hydrolysis of 7S globulin can lead to distinct functional properties, so that a more in-depth study of its functionality in protein ingredients should be further clarified.

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Chapter 5. Air-water interfacial and foaming properties of crude and purified globulin from pigeon pea seeds

Preamble

Preliminary studies in Chapter 4 showed that maintaining the native state of 7S globulin is important for a good performance of pigeon pea protein fractions as a foaming agent. However, the role of non-protein compounds in the functionality of these fractions must be also taken into consideration. The extensive characterization of the composition of pigeon pea ingredients in the previous chapters shows that non-protein compounds have an important role on their functional properties. The elimination of minor compounds by leaching during seed soaking, for example, reduced the foaming properties of protein concentrates (Chapter 3). However, the relationship between the behavior of the surface-active molecules presented in pigeon pea fractions at the air-water interface and the formation and stabilization of foams needs to be further investigated. In this chapter, crude and purified globulin fractions were investigated regarding their air-water interfacial and foaming properties.

Main questions

- How do pigeon pea protein fractions behave at the air/water interface?
- How pigeon pea fractions perform as foaming agents compared to proteins of animal origin?
- Are there differences between the air/water interfacial and foaming properties of protein fractions obtained from seeds of pure and not-pure lines?

Crude protein extracts were obtained using commercial seeds (cv. unknown) and pure line seeds (cv. g18-95). The native 7S globulin was used as a reference. For comparison, whey protein isolate, and bovine serum albumin were used as good foaming agents and lysozyme was used as a poor foaming agent.

Main results

- At low concentrations, purified 7S globulin and crude protein extracts have a similar rheological behavior in the air-water interfacial film;
- At high concentration, crude protein extracts behave differently than the purified 7S globulin at the air-water interface, suggesting that with increasing concentration, other compounds begin to act at the interfacial film, making it less rigid;
- The purified 7S globulin showed better foamability and foam stability than crude protein extracts;
- The purified 7S globulin showed superior foaming properties compared to whey protein isolate and bovine serum albumin;
- Crude protein extracts from different seeds have different foaming properties, probably due to a different composition of non-protein compounds;

Air-water interfacial and foaming properties of crude and purified globulins from pigeon pea seeds

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1.Introduction

Proteins are foaming agents commonly used in the food industry to produce aerated products such as mousses, ice creams, cakes and beverages (Damodaran, 2006; Narsimhan & Xiang, 2018; Zhu et al., 2020). Proteins of animal origin, such as those present in egg white or milk, are the most used due to their well-known good performance in the formation and stabilization of foams (Völpl et al., 2021; J. Yang et al., 2023). With the growing incentive to partially shift from the use of animal proteins to plant-based proteins, several studies have investigated alternative sources of foaming agents based on proteins such as mung bean, Bambara bean, soybean, lupin, quinoa, cowpea, and pea (Rangel et al., 2003; Völpl et al., 2021; Vondel et al., 2023; Yang et al., 2023; Yang et al., 2022), encouraging the exploration of new sources that are still little investigated.

Pigeon pea (*Cajanus cajan* L. Millsp.) is an unconventional pulse native to countries in Asia and Africa, with a protein content of around 20% (Fernández Sosa et al., 2021; Sun et al., 2020). globulin is its main protein, belonging to the 7S globulin fraction (Krishnan et al., 2017; Singh et al., 1990). Conventional protein extraction methods have been used to produce pigeon pea globulin extracts to evaluate their functional properties (Adenekan et al., 2018; Fernández Sosa et al., 2021, 2023; Mwasaru et al., 1999). Such methods consist of the solubilization of proteins in alkaline conditions (pH 8.0-12.0) followed by their isoelectric precipitation (pH 4.0-5.0), allowing the recovery of protein extracts rich in globulins (Gravel & Doyen, 2023). Although such methods allow the recovery of a high amount of proteins, steps such as protein precipitation can cause modifications in their native structures, compromising their functionality (Kumar et al., 2021). Thus, milder aqueous fractionation techniques, without the protein precipitation step, enable the

recovery of proteins in their most native state possible. On the other hand, substantial contents in other compounds such as sugars, starches, phospholipids and phenols may be present in protein extracts obtained by these methods, with possible impacts on their functionality (Yang et al., 2023; Yang et al., 2022).

The purification of globulins allows the reduction of these interferents, based on the isolation of the target protein (Gravel & Doyen, 2023). By using pure proteins in studies of functional properties, it is possible to infer their individual behavior and, consequently, better understand their role in the overall functionality of protein extracts, which generally contain other groups of proteins and minor compounds. Krishna & Bhatia (1985) were pioneers in proposing the purification of pigeon pea 7S globulin, using chromatography techniques. The authors obtained native 7S globulins with a molecular weight of 180 kDa and two types of subunits with molecular weights of 72 and 57 kDa. However, due to the difficulty of purifying proteins in sufficient quantities, the authors did not investigate their functional properties. For this, purification on a preparative scale is necessary to obtain purified fractions on the gram scale (Gueguen et al., 1984).

The comparison between crude protein extracts and purified proteins helps in investigating the role that minor compounds present in protein extracts play in the foam formation and stabilization. To the best of our knowledge, no study in the literature has evaluated the foaming properties of pigeon pea 7S globulin, but some studies using protein concentrates or isolates thereof have been reported (Adenekan et al., 2018; Akintayo et al., 1999; Mwasaru et al., 1999). However, there is a gap to be filled in relation to understanding the relationship between the foaming properties of pigeon pea proteins and their behavior at the air-water interface. When suspensions are prepared to produce foams, proteins first migrate from the bulk solution to the air-water interface. When adsorbed at the interface, protein chains are rearranged and can unfold to form the interfacial film (Narsimhan & Xiang, 2018; Wierenga & Gruppen, 2010). At this stage, other compounds present in the suspension can compete with proteins for adsorption at the interface (Bock et al., 2021). The behavior of proteins at the air-water interface can be investigated by measuring the expansion and compression of the interfacial film, allowing inferences on the mechanical properties and the microstructure of the layer formed.

In this work, we evaluated the air-water interfacial and foaming properties of three different ingredients based on pigeon pea: (i) crude protein extract obtained from commercial seeds (cv. unknown) (CPE-C); (ii) crude protein extract obtained from pure line seeds (cv. g18-95) (CPE-E); and (iii) 7S globulin (7S). The adsorption of proteins and the rheological behavior of the films formed at the air-water interface were investigated, as well as the foamability and foam stability. Whey protein isolate (WPI) and bovine serum albumin (BSA) were used as controls for good foaming agents, while lysozyme (LYZ) was used as a control for poor foaming agent.

2. Materials and methods

2.1 Materials

Pigeon pea seeds were purchased from a local supplier (cv. unknown) (São Paulo, Brazil) and donated (cv. g18-95) by the Brazilian Agricultural Research Corporation (EMBRAPA). Sodium phosphate was purchased from Sigma-Aldrich. Protein assay dye reagent and Coomassie blue were purchased from Bio-Rad (California, USA). Tris-glycine SDS running buffer and Tris base reagent were purchased from ThermoFisher (Massachusetts, USA). Bovine serum albumin was supplied by MP Biomedicals (China). Whey protein isolate (Prolacta®95) was supplied by Lactalis BBA (France). Lysozyme was supplied by OVONOR (Tregueux, France).

2.2 Crude protein extraction

Two different seeds were used to extract the proteins: commercial seeds (cv. unknown) and pure line seeds (cv. g18-95). The seeds were ground to produce flour (< 300 µm) using a disk mill (J.R. Araújo & Cia Ltda., Brazil) at 1730 rpm. For protein extraction, 200 g of each flour were dispersed in 1 L of 50 mM Tris buffer solution pH 8.0. The suspension was stirred for 1 h in a mechanical stirrer (RZR 2041, Heidolph) at 225 rpm. After stirring, the suspension was centrifuged (10000 g x 10 min x 4 °C) and the supernatant was collected. Another 1 L of 50 mM pH 8.0 Tris buffer solution was added to the pellet followed by agitation and centrifugation with collection of the supernatant. The two supernatants were pooled, centrifuged again in the same conditions, and filtered (8-µm MCE membrane, Millipore) to remove small particles. To remove the salts and small molecules like polyphenols, the extracts were injected into a desalting column filled with 7.5 L of Matrix cellulose (GH25 cellulose gel, 45-105 µm Amicon-Millipore). The column was equilibrated with pH 8.0 sodium carbonate buffer. The injection was conducted at a flow rate of 50 mL/min. The collected volume was 2.8 L for each extract. The desalted extracts were frozen and freeze-dried for 72 h. The crude extracts were recovered and stored in a desiccator containing K₂CO₃ saturated solution (RH = 43.2%). In the following, the crude extract from commercial seeds is referred to as CPE-C and the crude extract from pure line seeds as CPE-E.

2.3 7S globulin purification

The procedure for the purification of pigeon pea 7S globulin on a preparative scale was developed in Chapter 3, Part I. A brief description of the methodology is presented in this section. To produce the 7S globulin purified fraction, only commercial seeds were used, due to their greater availability. The protein extraction process was conducted in the same way as previously reported to obtain the crude protein extract. Then, the desalted extract (obtained according to section 2.2) was injected into a Q-Sepharose Fast Flow column (GE Healthcare). Then, the 7S fractions were pooled and

injected into a gel filtration column (Cellufine GCL) for further purification. After this latter step, the fractions were pooled, frozen, and freeze-dried for 72 h.

2.4 Composition

The nitrogen content was determined in a Dumas analyser (Thermo Fischer Scientific, USA). For the crude protein extracts, a nitrogen-to-protein conversion factor of 5.83 was used (Alamanou & Doxastakis, 1997) and for the purified 7S globulin a factor of 5.85 was used, calculated from the amino acid composition of the samples. The lipid content was determined using a extraction method described by Bligh & Dyer (1959) and Folch et al.(1957), with adaptations showed in Chapter 2. All samples were measured in triplicate and the protein content was expressed on a dry basis, after determining the moisture content of the samples using the gravimetric method under a nitrogen atmosphere (TGA 2050, TA-Instruments).

2.5 Fluorescence measurements

Three-dimensional fluorescence measurements (excitation-emission fluorescence matrices, 3D-EFEM) were performed in a spectrofluorimeter (FP-8550, Jasco, Japan) using the front-surface mode. Protein powders were placed in a powder cell and gently compacted. Measurements were performed over an excitation wavelength range from 240-480 nm, and an emission wavelength range from 280-520 nm. An acquisition step of 1 nm and a bandwidth of 2.5 nm were fixed for excitation and emission.

2.6 Protein dispersions

Samples were dissolved based on protein content (% w w⁻¹) in a 500 mM phosphate buffer, pH 8.0, stirred for 2 h at room temperature (Vondel et al., 2023). Total protein and soluble protein solutions were used in the experiments. For analysis of soluble proteins, samples were centrifuged (10000 g x 10 min x 20°C) after stirring, with collection of the supernatant. All samples were dissolved in a protein concentration range from 0.1 to 4.0 (g L⁻¹), specified in the sections below.

2.7 SDS-PAGE

The protein composition of the crude protein extracts and purified 7S globulins was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing and reducing conditions with 2-β-mercaptoethanol (5%, v v⁻¹). About 100 µL of each sample (1 mg total proteins mL⁻¹) were diluted in 100 µL of LDS (2x). The tubes were heated for 5 min at 95 °C. After heating, an aliquot of 15 µL of each sample was added to the wells of the electrophoresis gel. The electrophoresis tank was filled with MES SDS running buffer (Novex) and the experiment was conducted under a voltage of 200 V and 100 mA until complete migration of the samples. After migration, the gel was added to a container with fixation solution (2% phosphoric acid, 50% ethanol) and kept under agitation for 30 min. Then, the solution was discarded, and the same procedure

was repeated. After the fixation step, the wash solution (17% ethanol, 15% ammonium sulfate, 2% phosphoric acid) was added and the gel was shaken for 30 min. After washing, the sensitization solution was added under agitation for 20 min with the addition of 100 mg of Coomassie Brilliant Blue G 250 dye (Serva) for proteins coloration. Then, the gel was kept under agitation overnight and scanned.

2.8 Particle size distribution

The size distribution of protein suspensions was analyzed using dynamic light scattering (DLS) in a Zetasizer Nano ZS (Malvern Instruments Ltd., UK). Solutions with 1% (w/w) soluble protein were filtered over a 0.2- μm syringe filter. The size distribution was determined by adding 0.7-mL aliquots into cuvettes and equilibrating the samples for 120 s at 20 °C before analysis. The refraction index used was 1.45 for protein dispersions. The size distribution measurements were performed in automatic mode in 10 scans. All measurements were at least performed in triplicate at 20 °C.

2.9 Surface tension and surface dilatational properties

The mechanical properties of the air-water interface were studied by performing surface dilatational rheology in a drop tensiometer (Traker, Teclis Scientific, France). Soluble protein solutions were evaluated at 0.1 and 1 g L⁻¹. The solutions were loaded in a syringe connected to a straight needle. A pendant droplet with a surface area of 10 mm² was formed at the tip of the needle and the surface tension was calculated by fitting the Young-Laplace equation to the shape of the droplet. Before analyses, the droplets were equilibrated for 1800 s, to allow proteins to migrate to and adsorb at the interface. Amplitude sweeps were performed at a constant frequency of 0.02 Hz and an amplitude of 10, 20, and 30% deformation. In these oscillatory deformations, five cycles were performed for each amplitude step. All measurements were performed at least in triplicate at 20 °C. The raw data were used to calculate the surface pressure (π) and area deformation (ΔA), according to equations 1 and 2 below.

$$\pi = \gamma - \gamma_0 \quad (1)$$

$$\Delta A = \frac{(A - A_0)}{A_0} \quad (2)$$

where γ and γ_0 are the surface tension of deformed and non-deformed interface, respectively, and A and A_0 are the area of deformed and non-deformed interface, respectively. The data were shown as Lissajous plot by plotting the variation in surface pressure against the applied deformation. The elastic (E') and the storage (E) moduli were calculated using the equipment's software. The loss tangent was calculated as the ratio of E''/E' . All plots and calculations were generated using the middle three oscillations.

2.10 Foaming properties

Foaming properties were evaluated using total and soluble protein solutions (4 g L⁻¹). Whey protein isolate (WPI) and bovine serum albumin (BSA) were used as controls for good foaming properties and lysozyme (LYZ) was used as control for poor foaming properties. A 2-mL aliquot of each sample was added to 5-mL syringes. The syringe containing the protein solution was connected to another syringe containing 4 mL of air through a tube of 1 mm diameter. The foam was formed inside the syringes by making back and forth movements manually for 15 s. Then, the syringe containing the foam was disconnected, capped, and inverted onto a holding support in front of a black background. Images were captured every 1 min by a camera (EOS, Canon, Tokyo, Japan). The total experiment time was 120 min.

The ImageJ software (NIH Image, Maryland, USA) was used to process raw foam images and determine the foamability, foam stability and volume of drained liquid. The foam (white) was distinguished from the empty spaces (black) of the syringe by tuning the threshold of the gray values of pixels. The foamability (%) was calculated as the ratio between the volume occupied by the foam immediately after production ($t = 0$ min) and the total volume of the syringe. The foam stability and the volume of drained liquid were evaluated in a similar way, considering the following times: 1, 3, 5, 10, 15, 30, 45, 60, 90 and 120 min. All measurements were done in triplicate.

2.11 Statistical analysis

Data were analyzed using the OriginPro 2016 software. Variance analysis (ANOVA) followed by Tukey post hoc test was applied to determine the difference between samples at a 5% significance level. The experiments were carried out in three independent replicates.

3. Results and discussion

3.1 Characterization of crude protein extracts and purified fractions

3.1.1 Composition

Both crude protein extracts had a protein content of around 82 wt.%, while the 7S globulin fraction had a protein content of around 89 wt.% (Table 5.1). Kornet et al. (2021) obtained a pea protein isolate with a protein content of around 88% using a mild extraction process combined with diafiltration. Regarding non-proteinaceous compounds, the crude protein extracts showed substantial lipid content, around 8.2-8.5 wt.% (Table 5.1). It was not possible to quantify the lipid content of purified 7S globulin, given the limiting amount of this sample and the high sample weight required for analysis (around 1 g per replicate). The lipid content found in crude protein extracts from pigeon pea is similar to the values reported in study of Keuleyan et al. (2023) for pea and lupin protein concentrates, which had levels around 5 and 9%, respectively. High lipid contents were also reported in Chapter 3 of this thesis for pigeon pea protein concentrates using an extensive extraction method. Our previous results also showed around 13 wt.% of phospholipids in relation to the total lipid content of pigeon pea protein concentrates, which must be taken into consideration when determining interfacial/foaming properties of these samples, since phospholipids may also adsorb at the air-water interface. The presence of phospholipids can hinder protein-protein lateral interactions at the interface. A high lipid content in protein concentrates has been related to decreases in surface dilatational and shear moduli.

Table 5.1. Protein and lipid content of crude protein extracts and purified 7S globulin from pigeon pea seeds.

Sample	Protein content (% d.b)	Lipid content (% d.b)
CPE-C	81.9 ± 0.9 ^b	8.2 ± 0.3 ^a
CPE-E	82.2 ± 0.5 ^b	8.5 ± 0.2 ^a
7S	89.3 ± 0.2 ^a	-

Different letters indicate difference between samples ($p < 0.05$). Crude protein extract obtained from commercial seeds (cv. unknown), crude protein extract obtained from pure line seeds (cv. g18-95), and pigeon pea 7S globulin are indicated by CPE-C, CPE-E, and 7S, respectively.

3.1.2 Fluorescence measurements

The occurrence, fluorescence patterns and intensity of endogenous fluorophores present in the flours, crude protein extracts, and purified 7S globulins were evaluated by three-dimensional excitation-emission fluorescence matrices (3D-EEFM) in front-surface mode on the dry powders (Figure 5.1). The 3D-EEFM showed that both pigeon pea flours presented two different regions of fluorescence: one referring to the protein compounds ($\lambda_{Ex} = 270-290$ nm, $\lambda_{Em} = 300-360$ nm corresponding mainly to the tryptophan/tyrosine emission), and another region referring to the fluorescence emission

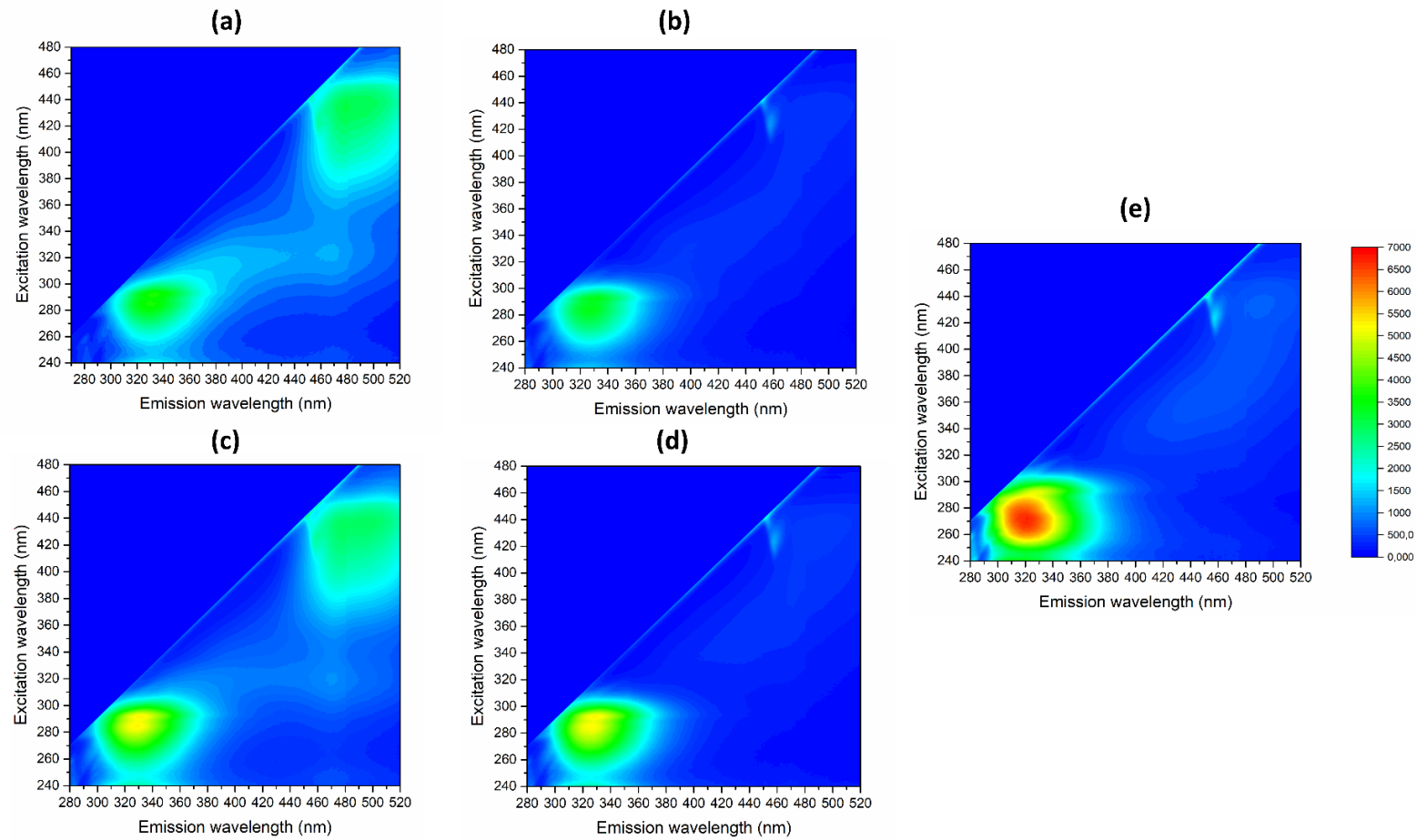


Figure 5.1. Three-dimensional excitation-emission fluorescence matrices of flours, crude protein extracts and purified 7S globulin from pigeon pea seeds. **(a)** pigeon pea flour from commercial seeds (cv. unknown), **(b)** crude protein extract from commercial seeds, **(c)** pigeon pea flour from pure line seeds (cv. g18-95), **(d)** crude protein extract from pure line seeds, **(e)** pigeon pea 7S globulin. The color tone corresponds to the fluorescence intensity (AU), see legend on the right-hand side.

of non-protein compounds ($\lambda_{\text{Ex}} \approx 400\text{-}440\text{ nm}$, $\lambda_{\text{Em}} = 450\text{-}500\text{ nm}$). While the region of non-protein compounds showed similar fluorescence intensity for both flours, the protein region showed a significant difference, with pure line seeds flour showing a higher fluorescence intensity than commercial seeds flour. Considering that the flours have similar protein content ($\approx 20\%$), this result suggests a possible difference in the content, exposure and chemical environment of tryptophanyl residues between the samples. In crude protein extracts, an effective elimination of non-protein endogenous fluorophores ($\lambda_{\text{Ex}} \approx 400\text{-}440\text{ nm}$, $\lambda_{\text{Em}} = 450\text{-}500\text{ nm}$) was noted in both samples compared to their respective flours. Such compounds were removed in the desalting step of the protein extracts.

As previously discussed in Chapters 3 and 4, the second region of fluorescence is attributed to the emission region of several phenolic compounds. Some phenolic compounds can affect the chemical state of protein that could in turn affect their interfacial properties. However, with the process used in this work and as evidenced by the fluorescence measurements, the significant presence of phenolic compounds in the protein fractions used is not expected.

3.1.3 Protein composition and size distribution

The protein composition assessed by SDS-PAGE showed different bands, depending on the sample (Figure 4.2). SDS-PAGE showed several bands in the CPE-C and CPE-E samples, with four main bands being identified: two bands between 50 and 75 kDa and two bands between 37 and 50 kDa. Two bands with molecular weight between 100 and 150 kDa were also visible in both samples, with the band closer to 100 kDa being more visible in reducing conditions. Several bands were identified with molecular weight $< 37\text{ kDa}$, mainly in the presence of a reducing agent. The 7S globulin showed a band between 50 and 75 kDa and two bands close to 50 kDa, characteristic of the α - and β -chains of pigeon pea 7S globulin, respectively. The protein band close to 100 kDa was absent in the 7S globulin without the presence of reducing agent and barely visible when the reducing agent was added. Similarly, bands of molecular weight $< 37\text{ kDa}$ were also not visualized in non-reducing conditions and visualized at low intensity in reducing conditions. The results show the absence of aggregates around 100 kDa and of low molecular weight compounds ($< 37\text{ kDa}$), in the 7S globulin. Although crude extracts consist mainly of 7S globulins, other proteins are present in the samples, such as albumins, protease inhibitors, and 11S globulin, based on the proteomic analysis of Chapter 4.

The hydrodynamic radius of protein molecules affects their diffusion towards the interface, being an important parameter to be considered for studies of interfacial properties (Shrestha et al., 2008). The size distribution of the samples showed a bimodal distribution for the crude protein extracts and the 7S globulin purified fraction with a smaller population around 15 nm and another larger population around 175 nm (Figure 4.3). The smaller population was around 17 nm in size for both crude protein extracts. On

the other hand, the purified 7S globulin showed values around 15 nm. A greater contribution of the smaller population is expected for the purified 7S globulin fraction relative to the crude extracts, as a size of 10–12 nm is characteristic of 7S globulin (Ruíz-Henestrosa et al., 2007). The largest population presented a size of around 175 nm for CPE-C and CPE-E, while the 7S globulin presented value around 153 nm, highlighting a certain degree of aggregation of the proteins in the suspensions. However, there is no significant change in the distribution of the hydrodynamic radius, so that the difference in diffusion at the air-water interface should not be a discriminant factor.

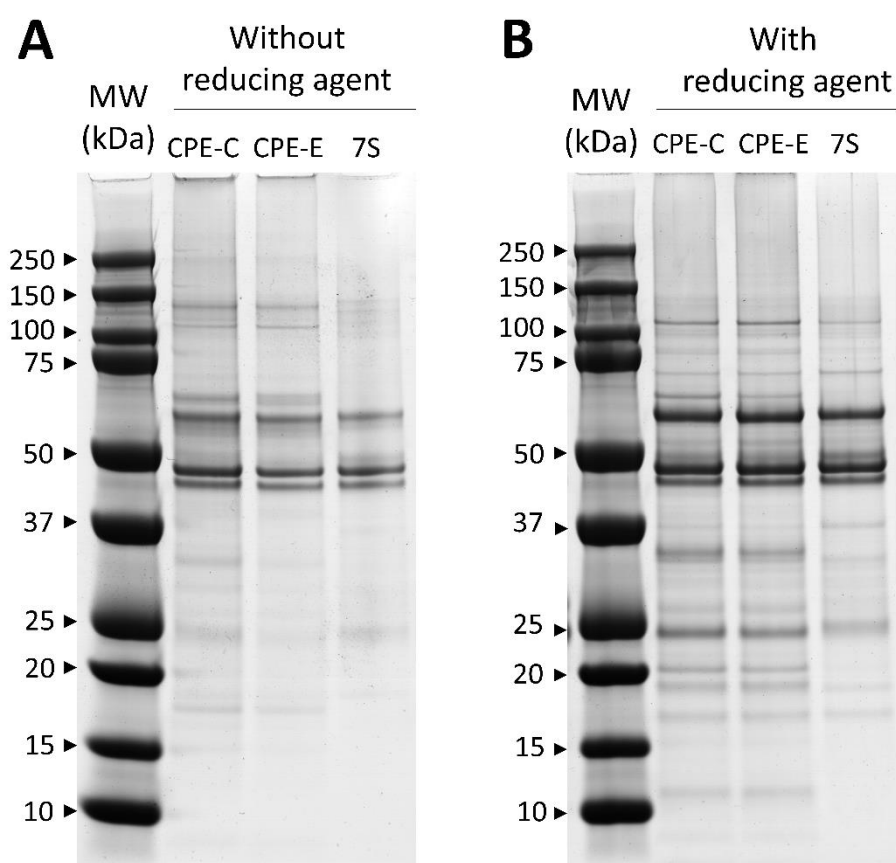


Figure 5.2. SDS-PAGE of crude protein extracts and purified 7S globulin from pigeon pea with (a) and without (b) reducing agent 2- β -mercaptoethanol. The crude protein extract from commercial seeds (cv. unknown), crude protein extract from pure line seeds (cv. g18-95), 7S globulin are indicated by CPE-C, CPE-E, and 7S, respectively.

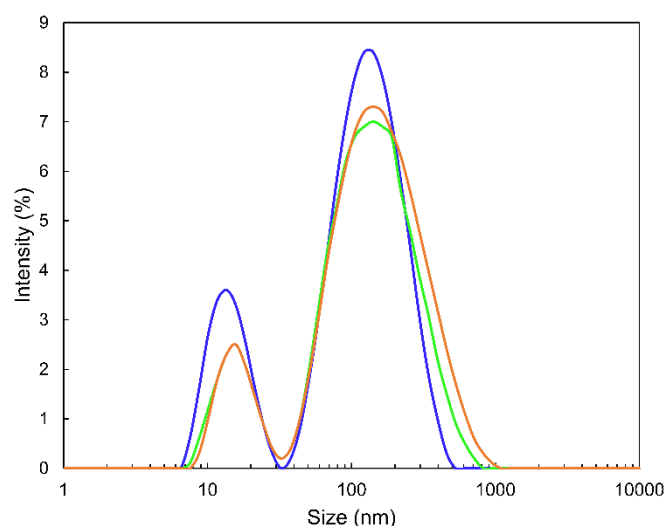


Figure 5.3. Size distribution of crude protein extracts and purified 7S globulin from pigeon pea. The crude protein extract from commercial seeds (cv. unknown), crude protein extract from pure line seeds (cv. g18-95), and 7S globulin are indicated by the green, orange, and blue lines.

3.2 Air-water interfacial properties

3.2.1 Adsorption behavior

The air-water interfacial properties of crude protein extracts and purified 7S globulin fraction were evaluated at concentrations of 0.1 and 1.0 g L⁻¹. To form stable foams, dissolved proteins must diffuse from the bulk solution to the interface and switch to an absorbed state, orienting their hydrophobic parts towards the air phase and their hydrophilic parts towards the water phase (Graham & Phillips, 1979). Figure 4.4 shows the adsorption of proteins at the air/water interface over 10800 s of equilibrium. The adsorption of CPE-E was faster among all samples, followed by CEC-C and the 7S globulin. Working at lower concentrations (0.1 g L⁻¹) (Fig. 5.4a) it is possible to see the effect of the slower migration of surfactant agents to the interface, in relation to dispersions at higher concentrations (1.0 g L⁻¹) (Fig. 5.4b). Furthermore, from 100s onwards it is possible to see a clearer difference in the reduction in the surface tension of both crude protein extracts at low concentration, with CPE-E leading to a faster reduction in surface tension than CPE-C. At high concentration, it takes around 1000s for the purified 7S globulin to reduce the surface tension to levels similar to those of crude protein extracts. The faster adsorption of crude protein extracts at the air/water interface compared to purified 7S globulin, could be due to the presence of proteins of low molecular weight and/or small non-proteinaceous compounds, which consequently tend to migrate to the interface more quickly. Also, compounds such as small sugar molecules and phospholipids may be present in protein extracts and it can compete and/or interact with proteins at the interface, modifying their surface properties (Bock et al., 2021).

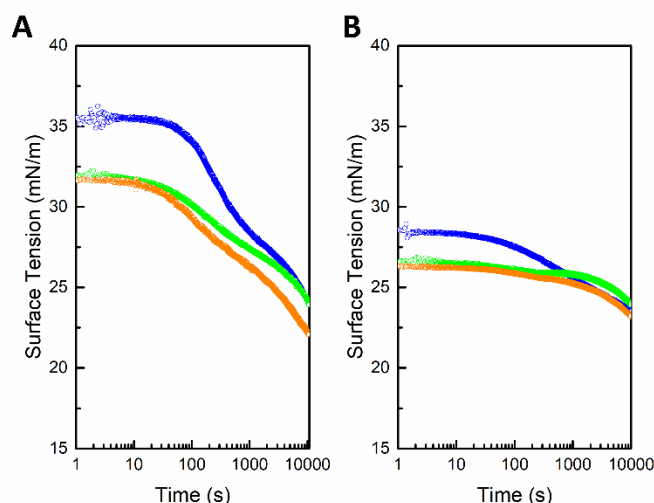


Figure 5.4. Air-water surface tension over time of pigeon pea protein suspensions at 0.1 g/L **(a)** and 1.0 g/L **(b)**. The crude protein extract from commercial seeds (cv. unknown), crude protein extract from pure line seeds (cv. g18-95), and native 7S globulin from pigeon pea are indicated by the colors green, orange, and blue.

3.2.2 Surface dilatational rheology

In all conditions evaluated, a decrease in the storage modulus E' values was observed with increasing the deformation amplitude (Figure 5.5). This indicates that the microstructure of the interfacial film was impacted by high deformations, being classified as a non-linear viscoelastic behavior. For protein suspensions at 0.1 g L⁻¹, purified 7S globulin led to the highest reduction in moduli from 21 to 15 mN/m, while the films formed from crude protein extracts CPE-C and CPE-E showed similar reductions of 18 to 13 mN/m (Figure 5.5a). These results imply a more rigid layer produced with 7S globulin and, consequently, the lower values obtained for the crude protein extracts suggest that there is less perturbation of the surface microstructure compared to purified 7S globulin. Similarly, for protein suspensions at 1 g L⁻¹, the 7S globulin-based films had the highest reduction in moduli from 20 to 14 mN/m, followed by the two crude protein extracts, CPE-C and CPE-E, which showed similar values of decrease from 9 to 7 mN/m (Figure 5.5c). Comparatively, pea and bambara groundnut protein concentrates showed elastic modulus in the range of 15-30 mN/m (Yang et al., 2022; Yang et al., 2021; Hinderink et al., 2020). In relation to animal proteins, the elastic moduli for WPI and egg white proteins reported by Shen et al. (2024) were considerably higher than systems stabilized by mildly extracted lentil protein isolate, with values around 110 and 80 mN/m, respectively. According to the authors, the elastic moduli of animal proteins showed higher dependence on deformation, compared to plant-based proteins, with the WPI-stabilized interface being more disrupted by large dilatational deformations than lentil protein-stabilized interface.

Regarding the loss tangent (E''/E'), all samples presented values < 1.0 , which indicates a predominantly elastic behavior. In the case of suspensions at 0.1 g L^{-1} , all samples presented very low loss tangent values, ranging from 0.08 to 0.27, indicating that all interfaces presented highly elastic responses to deformations (Sagis & Fischer, 2014) (Figure 5.5b). In the case of suspensions at 1.0 g L^{-1} , the purified 7S globulin showed a low loss tangent range as well (0.19-0.32), but the crude protein extracts showed higher loss tangents, with values of 0.56 for CPE-C and 0.73 for CPE-E, regardless of the applied deformation (Figure 4.5d). The fact that the loss tangent observed for the crude protein extracts was fairly constant indicates that the elastic contribution is independent of the applied deformation. The fact that crude protein extracts have a higher loss tangent value indicates that the interface formed has a lower tendency to relax to its initial organization when the deformation stops, which can be, for instance, an indication of a lower connectivity of the interfacial network.

The amplitude sweeps studies carried out show that at low concentrations, purified 7S globulin and crude protein extracts have a similar rheological behavior in the air-water interfacial film, probably due to the dominance of the action of 7S globulins at the interface formed by all samples. However, with increasing concentration, crude protein extracts begin to behave at the air-water interface differently than the purified fractions, suggesting that with increasing concentration, other compounds begin to act at the interface (other groups of proteins or non-protein compounds) making it less rigid. In turn, the purified 7S globulin showed a more rigid interface, independently of the bulk concentration.

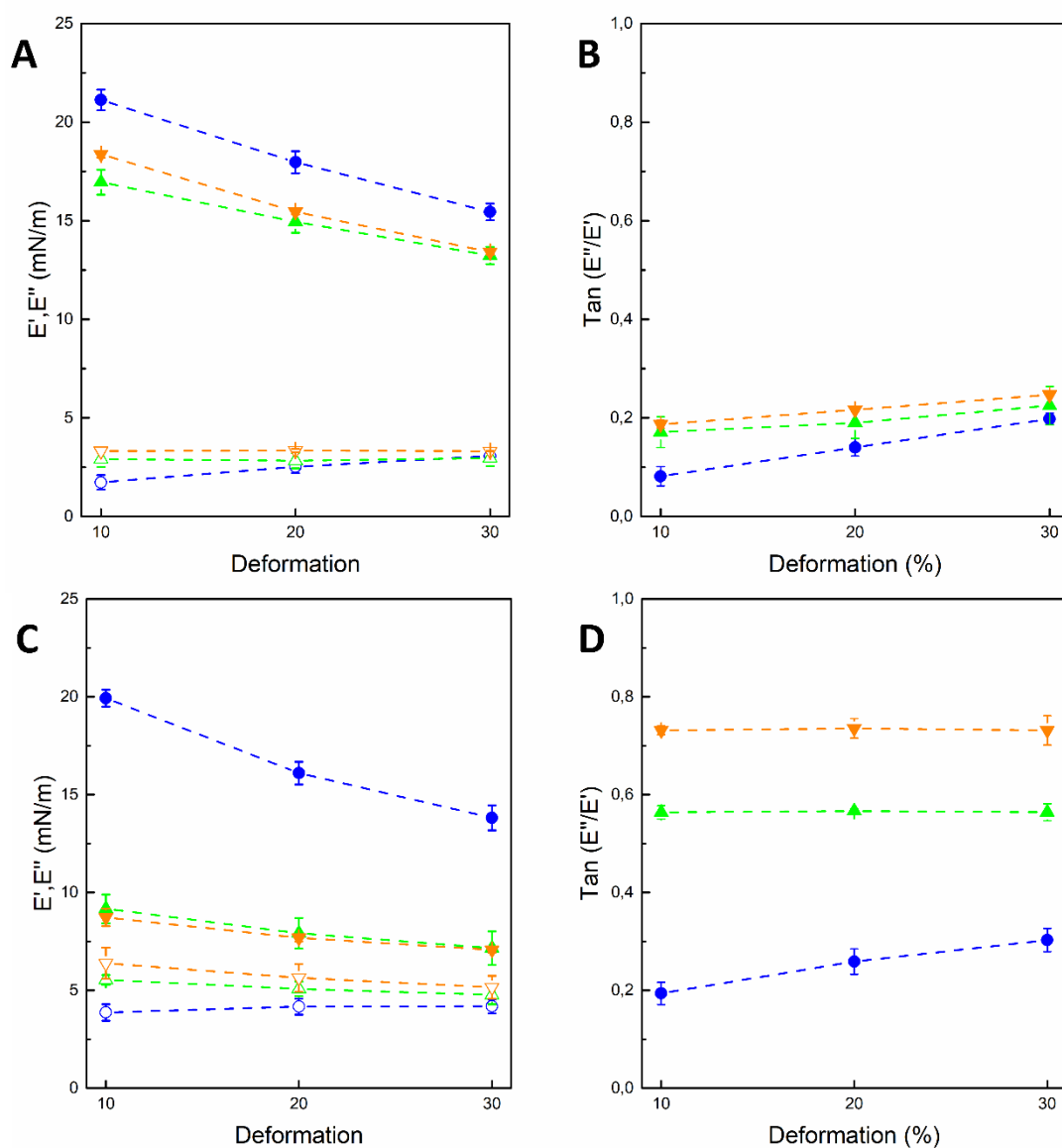


Figure 5.5. Surface dilatational moduli versus deformation amplitude (**a** and **c**), and the loss tangent (**b** and **d**) of pigeon pea protein suspensions at 0.1 g/L (in the top) and 1.0 g/L (in the bottom). The storage modulus (E') and the loss modulus (E'') are represented by full symbols and empty symbols respectively. Symbols indicate different samples: crude protein extract from commercial seeds (cv. unknown) ($\blacktriangle$), crude protein extract from pure line seeds (cv. g18-95) ($\blacktriangledown$), and pigeon pea 7S globulin ($\bullet$).

3.2.3 Lissajous plots

The Lissajous curves represent the response in surface pressure change upon deformation of the formed interfacial film during the expansion (top of the curve) and compression (bottom of the curve) cycles (Supplementary Figure 5.1). A completely viscous, completely elastic and viscoelastic response are indicated by Lissajous plots that have the shape of a circle, a straight line and an ellipse, respectively. The angle formed between the overall slope of the plot and the horizontal axis corresponds to the interfacial stiffness and an axis more inclined to the vertical suggests a stiffer interface (Sagis & Scholten, 2014; Yang et al., 2023). This representation is therefore complementary to the calculation of the moduli and can be helpful to capture interfacial features leading to a non-sinusoidal response upon dilatational deformation, as this then results in asymmetric Lissajous curves.

For suspensions with a concentration of 0.1 g L^{-1} , the Lissajous plot showed similar widths for all samples except CPE-E, indicating a similar dissipation energy after deformation for CPE-C and the 7S globulin. For suspensions with a concentration equal to 1.0 g L^{-1} , the Lissajous plots also showed similar widths for all samples except for CPE-E, in a similar way to what was observed at low concentration. At 30% deformation, purified 7S globulin showed slight asymmetry, while samples CPE-C and CPE-E showed more symmetrical behavior even at high deformation.

The purified 7S globulin also led to a plot with a greater inclination towards the vertical axis compared to the plots for crude protein extracts, which presented an inclination more towards the horizontal axis. The presence of more horizontal curves for crude protein extracts when evaluated at high protein concentration indicates that the changes in surface tension is moderate/low. This means that the changes in surface tension induced by the change in area can be rapidly compensated for. The fact that the viscoelastic modulus is lower at 1 g L^{-1} than at 0.1 g L^{-1} suggests that the composition of the interfacial film is bulk concentration-dependent. This could be linked to there are small surface-active molecules that can rapidly adsorb to and desorb from the interface during oscillations (Yang et al., 2021). Thus, at higher concentration it is observed a higher relative importance of these molecules compared to large and slower proteins. Figure 4.6 presents a schematic overview that summarizes the effect of concentration on the behavior of dispersions of crude protein extract and purified 7S globulin.

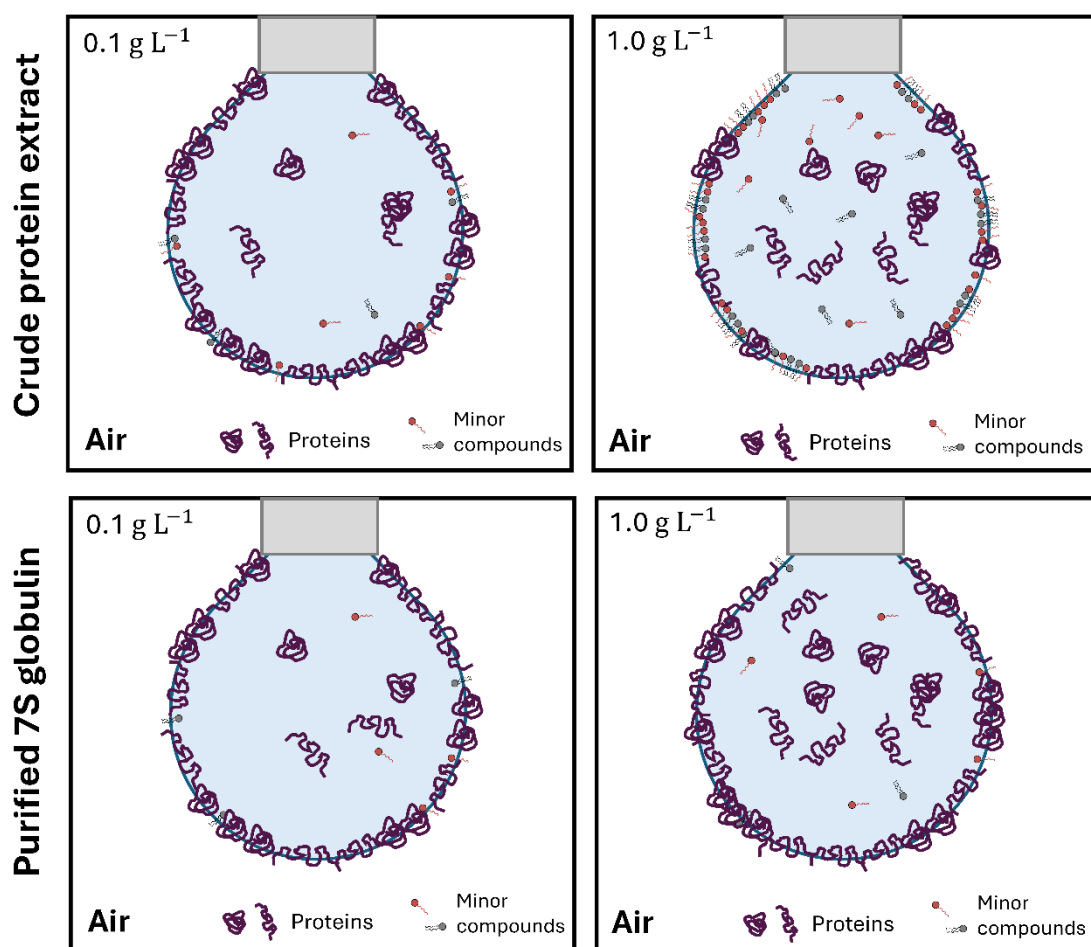


Figure 5.6. Schematic overview of the behavior at the air-water interface of dispersions of crude protein extract and purified 7S globulin at low (0.1 g L^{-1}) and high (1.0 g L^{-1}) concentrations.

3.3 Foaming properties

3.3.1 Foamability

The foamability of crude protein extracts and purified 7S globulin were evaluated using total and soluble protein suspensions. Using only soluble proteins increased the foamability of the two crude protein extracts (Figure 5.7) and LYZ, compared to the total protein suspension. No difference in foamability was observed between the use of total or soluble protein suspensions for purified 7S globulin, BSA and WPI ($p > 0.05$). This dichotomy may be related to the presence of aggregates in the total protein suspensions of the former samples, which can reduce the adsorption of proteins at the interface and, consequently, their foamability. The similar performance of the purified 7S globulin with the control proteins BSA and WPI stands out, indicating a foamability similar to the proteins of animal origin evaluated. There was no difference between the foaming capacity of the crude protein extracts ($p > 0.05$), which presented values slightly below the purified 7S globulin. LYZ used as a negative control, was the sample that showed the lowest foamability.

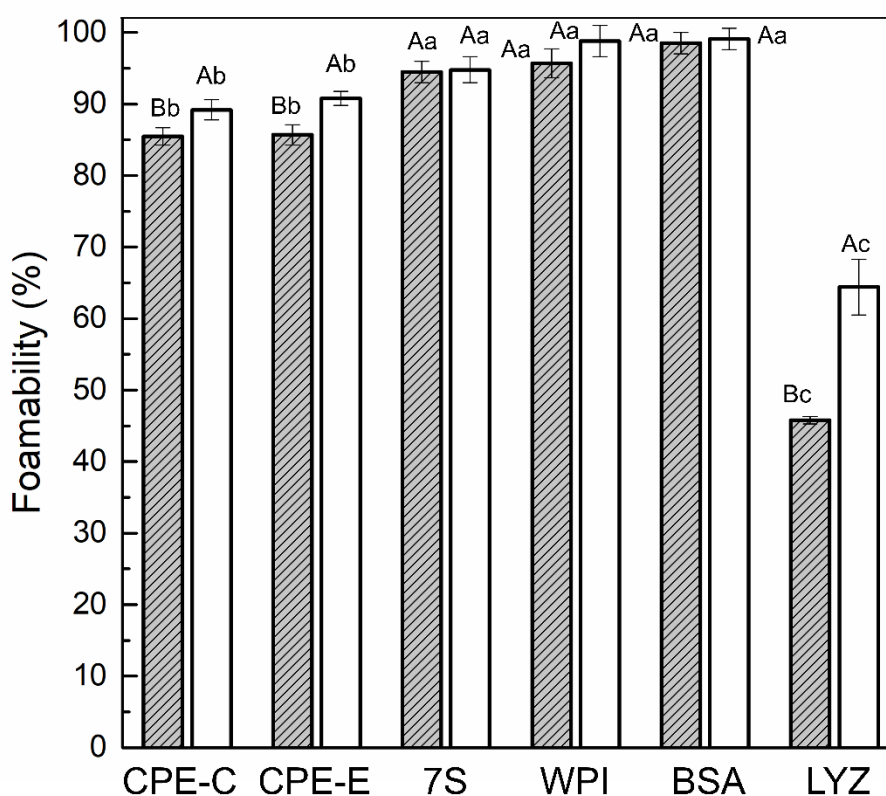


Figure 5.7. Foamability of crude protein extracts and purified 7S globulin from pigeon pea. Gray bars indicate total protein suspensions (4 g L^{-1}) and white bars indicate soluble protein suspensions (4 g L^{-1}). Uppercase letters indicate differences between total protein suspensions and soluble protein suspensions for a same sample and lower case letters indicate differences between samples for total protein suspensions or soluble protein suspensions ($p < 0.05$).

3.3.2 Foam stability

3.3.2.1 Appearance of foams

The physical appearance of the foams was evaluated in terms of the macroscopic homogeneity or heterogeneity of the bubbles immediately after production and after 120 min of observation (Figure 5.8). After production, the physical appearance of the foams produced with soluble protein suspensions was more homogeneous than that produced with total protein suspensions, which presented some larger bubbles that could be observed mainly in the crude protein extracts (Figure 5.8a). The BSA and WPI controls showed very small bubble sizes immediately after production in both types of suspension, having the highest homogeneity among the samples. On the other hand, LYZ led to very heterogeneous bubble sizes since its production, in both cases. After 120 min of storage, the ingredients based on pigeon pea proteins led to foams with greater homogeneity in relation to the shape and size of the bubbles for soluble protein suspensions, while the foams produced with total protein suspensions looked more heterogeneous (Figure 5.8b). It is also noted that for soluble protein suspensions, pigeon pea crude extract had high similarity with the appearance of WPI foam after 120 min of storage.

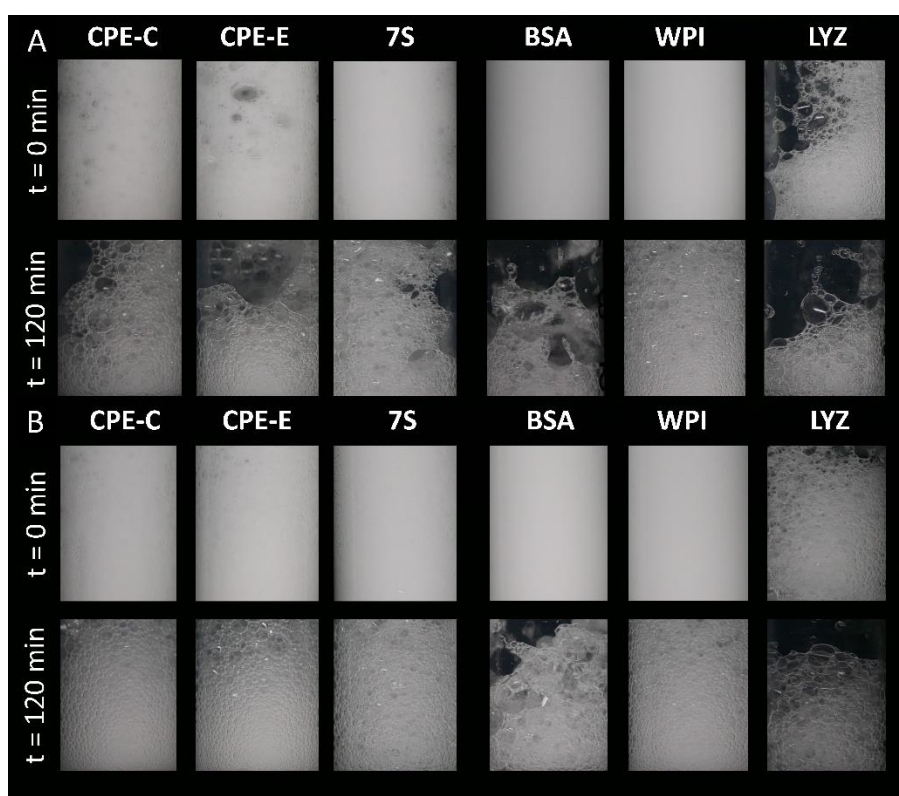


Figure 5.8. Physical appearance of the foams after production ($t = 0$ min) and after 120 min of storage for total (**a**) and soluble (**b**) protein suspensions. Crude protein extract from commercial seeds (cv. unknown), crude protein extract from pure line seeds (cv. g18-95), pigeon pea 7S globulin, bovine serum albumin, whey protein isolate, and lysozyme are indicated by CPE-C, CPE-E, 7S, BSA, WPI and LYZ, respectively.

3.3.2.3 Foam stability and liquid drainage

Different destabilization mechanisms can occur over time, such as: liquid drainage, flocculation and creaming (Narsimhan & Xiang, 2018). The stability of the formed foams was evaluated by monitoring the foam layer and the volume of liquid drained over time (Figure 5.9). For foams formed with total protein suspensions, the purified 7S globulin was the most stable after 120 min of experiment (65%), followed by WPI (61%), CPE-E (51%), BSA (40%), CPE-C (36%), and LYZ (18%) (Figure 5a). On the other hand, foams formed using soluble protein suspensions showed a stability of 69% for N-7S, 59% for WPI, 48% for CPE-E, 39% for CPE-C, 30% for BSA, and 20% for LYZ (Figure 5.9b). Akintayo et al. (1999) and Adenekan et al. (2018) observed a foam stability after 120 min of about 50-54% and 55% for pigeon pea protein concentrate and isolate, respectively.

The type of protein suspension did not significantly impact the observed stability ($p > 0.05$), except for BSA, which had stability reduced by 10% when using soluble protein suspensions. This difference can be explained by the loss of aggregates that contribute positively to the foam stability formed by BSA. The volume of liquid drained over time showed a similar trend for all samples, which at the end of 120 min presented around 60-70% of their initial volume of liquid drained, both for total and soluble protein suspensions (Figure 5.9c-d). Around 50% of the liquid volume was drained for all samples after 40-45 min, except for LYZ, which since the formation of the foam has already shown liquid drainage of around 60%. Conversely, BSA was the sample whose liquid drainage was more gradual compared to the other samples.

The better performance observed for the foaming properties of purified 7S globulin as compared to crude protein extracts may be related to the interfacial properties. For instance, at high concentrations the behavior at the air-water interface differs between crude protein extracts and purified 7S globulin, with the latter presenting more rigid interfacial films. More rigid interfacial films tend to have greater resistance against foam destabilization phenomena. On the other hand, less rigid films favor gas loss over time.

Furthermore, the better foam stability presented by the CPE-E in relation to CPE-C suggests a different composition of stabilizing agents present in the samples. The SDS-PAGE analysis indicates a similar protein composition for the protein extracts of the two seed types (Figure 5.2), but the composition of non-protein compounds is not well known and can differ significantly depending on cultivars, soil, climatic conditions, etc. Given the already discussed influence that these minor compounds may have on the air-water interface, their identification, quantification and possible implications in different pigeon pea cultivars should be clarified in future work.

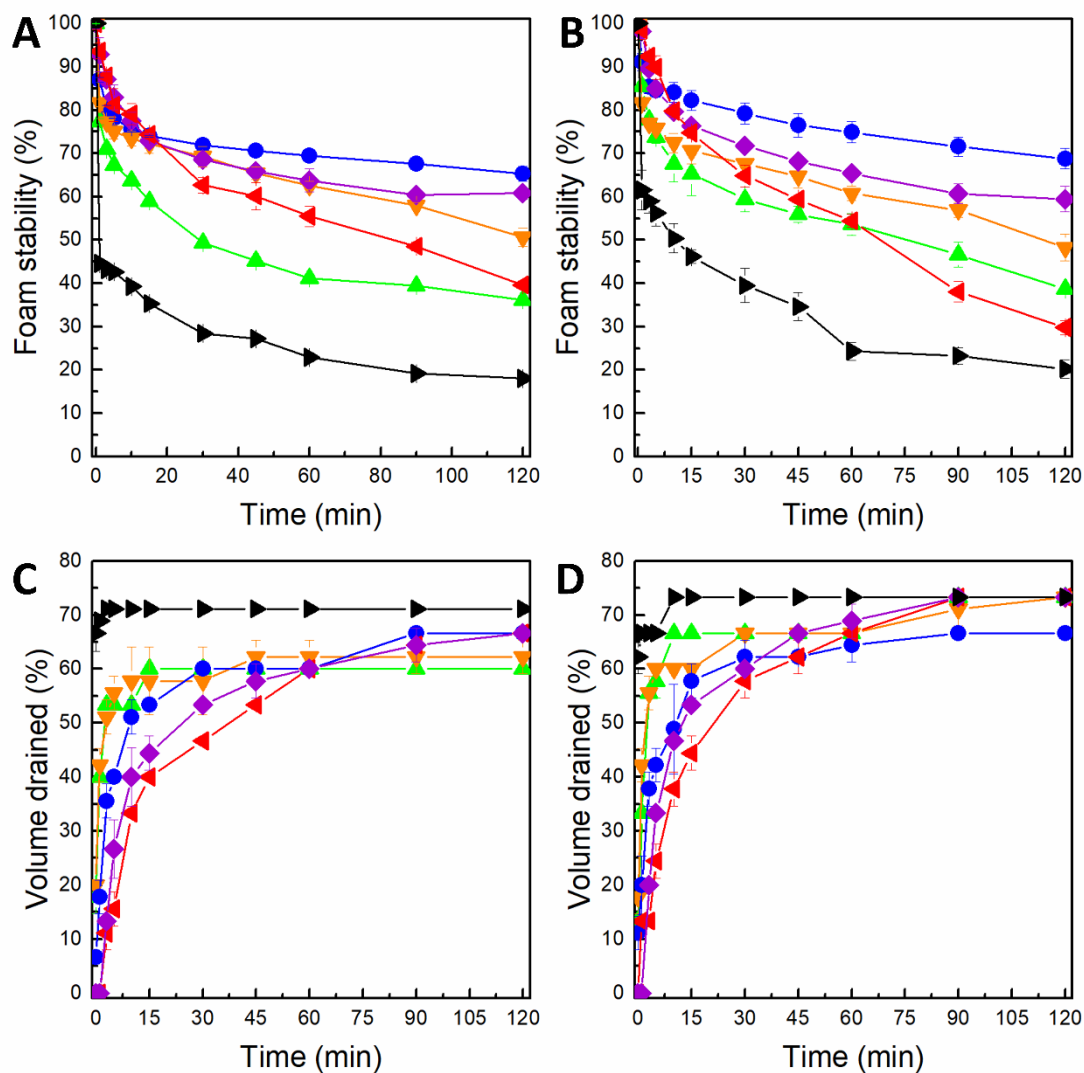


Figure 5.9. Foam stability over time (a and b) and volume of liquid drained (c and d) of total (in the right) and soluble (in the left) protein suspensions. Symbols indicate different samples: crude protein extract from commercial seeds (cv. unknown) (▲), crude protein extract from pure line seeds (cv. g18-95) (▼), pigeon pea 7S globulin (●), bovine serum albumin (◄), whey protein isolate (◆), and lysozyme (►).

4. Conclusion

In this work, the air-water interfacial and foaming properties of crude protein extracts and purified 7S globulin of pigeon pea were evaluated. In low protein concentration suspensions, no difference was observed in the behavior at the interface of crude protein extracts and purified 7S globulin, which presented the same dissipation energy after deformation. With the increase in the concentration of protein suspensions, it was observed that the crude extracts formed less rigid interfacial films when compared to the purified 7S globulin, which presented viscoelastic solid-like interfacial layers. It is suggested that in more concentrated suspensions, the action of other compounds occurs at the interface formed by the crude protein extracts, causing the globulins to no longer have full control over the formation of the interfacial film formed, resulting in a reduction in its rigidity. These results highlight a possible impact of minor compounds present in crude protein extracts that may compete with 7S globulin for interface stabilization. The purified 7S globulin also presented foam stability superior to that of whey protein and bovine serum albumin, highlighting the potential of 7S globulin from pigeon pea for applications as a foaming agent in the food industry as an alternative source to proteins of animal origin.

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Supplementary material

Air-water interfacial and foaming properties of crude and purified globulins from pigeon pea seeds

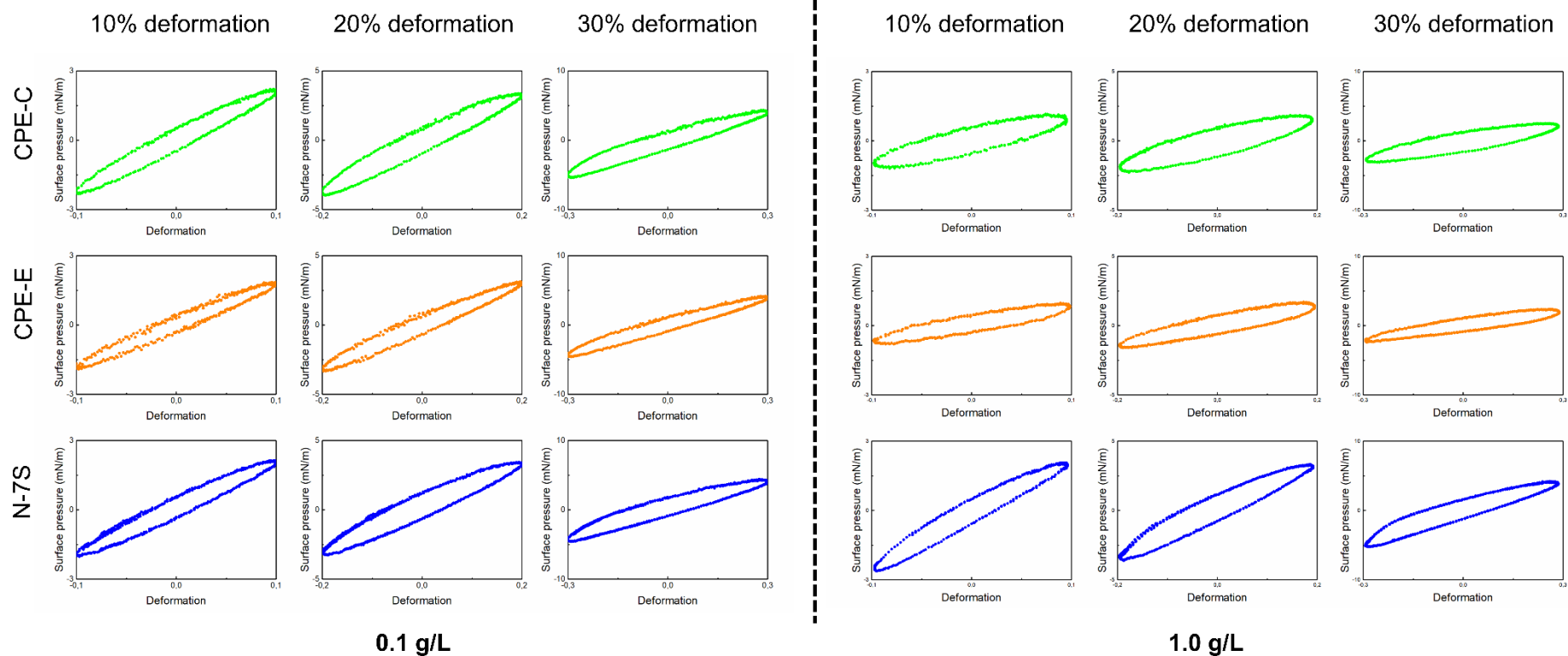
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Supplementary Figure 5.1. Lissajous plots showing the change in surface pressure as a function of 10, 20 and 30% deformation of the drop area with pigeon pea protein suspensions at 0.1 g/L and 1.0 g/L. Symbols indicate different samples: crude protein extract from commercial seeds (cv. unknown) ($\blacktriangle$), crude protein extract from pure line seeds (cv. g18-95) ($\blacktriangledown$), and pigeon pea 7S globulin ($\bullet$).

Chapter 6. Discussion, Conclusions and Perspectives

The objective of this thesis was to extract, characterize and evaluate the functional properties of pigeon pea protein fractions. In this study we used a set of biochemical, spectrophotometric and biophysical techniques to obtain and characterize the protein fractions of pigeon pea. Chapters 3 to 5 discuss the results obtained in this research regarding the literature. The purpose of this chapter is to bring together the main findings of this thesis and discuss the results in an integrated way. Finally, a general conclusion and perspectives for future work are presented.

Part I. General discussion

1. Pigeon pea protein fractions obtained by different fractionation processes

Pigeon pea protein fractions were obtained using different methods: (i) protein extraction by alkaline solubilization (pH 12.0) followed by isoelectric precipitation (pH 4.5); (ii) mild extraction at pH 8.0 followed by desalting, called crude protein extract (ii) and mild extraction at pH 8.0 followed by extract desalting and purification of 7S globulin by liquid chromatography (iii) (Figure 5.1). The 7S globulin purification followed two different routes: dialysis of protein fractions against ammonium carbonate buffer (process I) or replacement of dialysis by injection into a gel filtration column equilibrated with ammonium carbonate buffer (process II). While the process containing dialysis resulted in an unexpected partial hydrolysis of 7S globulin, the process with salt removal during gel filtration was efficient in maintaining 7S globulin in its native state. The mechanisms of the observed hydrolysis are not clear, some hypotheses for this observed phenomenon are discussed in the following section.

All fractionation methods used have advantages and disadvantages that must be taken into consideration when obtaining protein-rich fractions. The main advantages of precipitation-based extraction are that it is a method widely used by the food industry and guarantees a higher process yield. On the other hand, the solubilization steps at more alkaline pHs and the precipitation of proteins by acidification can cause changes in the conformation of proteins, which can affect their functionality. The mild extraction method has the advantages of maintaining proteins in their most native state due to the absence of protein precipitation steps, in addition to a yield similar to the conventional process that uses precipitation. A disadvantage of this process, however, is less elimination of proteins from other groups, such as albumins, compared to the process that relies on the precipitation step. The main advantage of purifying proteins by liquid chromatography is the elimination of the small molecules that may affect the chemical state of proteins (ex. polyphenols), allowing the isolation of the target protein. This allows the purified protein to be used as a reference to evaluate functional properties of interest, for example. A disadvantage, however, is the process time and higher cost. In relation to this, in this thesis a faster purification method with less water consumption was proposed, by replacing the dialysis of protein fractions with direct injection of the fractions into the gel filtration column. The method was efficient in maintaining the proteins in their native state throughout the purification process, but compared to the purified fraction that was subjected to dialysis, it showed more contaminants according to SDS-PAGE analysis, in addition to a higher trypsin inhibitors activity (TIA).

In terms of process yield, the first two methods used showed a similar yield of around 12-13% w w⁻¹, while the last method showed a yield of around 2% w w⁻¹. Although the purified fractions had a low mass yield, it was recovered around 23-29% of the soluble 7S globulin from pigeon pea.

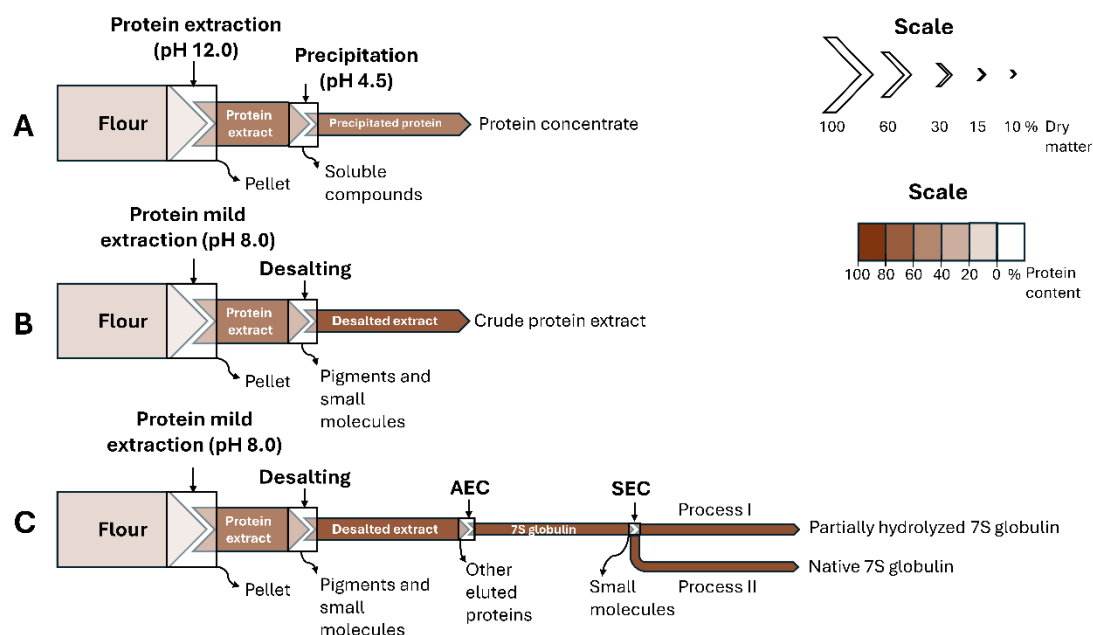


Figure 6.1. Fractionation of pigeon pea proteins by different methods. **(a)** Precipitation-based extraction **(b)** Mild protein extraction and desalting. **(c)** Mild protein extraction followed by desalting and purification of pigeon pea 7S globulins by liquid chromatography.

The darker brown color of protein concentrates obtained by precipitation-based extraction method differed from the light brown color obtained by the mild extraction method, while the purified fraction presented a white color (Figure 6.2). The lighter color presented by the protein fractions obtained by processing under mild conditions and chromatography separation is due to the removal of pigments during extract desalting and mainly at the less alkaline pH (8.0) used in the mild extraction process. The higher pH (12.0) used in the precipitation-based extraction can cause rapid oxidation of phenolic compounds, which bond with protein side chains via hydrogen bonds. The darker color of the concentrates resulting from this process suggests the presence and/or modification of phenolic compounds throughout the extraction (POTIN et al., 2019; RASHWAN et al., 2023). The dark brown color of the protein fractions obtained by the first process still showed to influence some analyzes that were performed, such as the fluorescence measurements, where an extinction of the fluorescence (very low emission fluorescence intensity) of the samples was observed. To avoid this effect, the evaluation of samples in the form of dispersions showed to be more appropriate than in the form of powders. Furthermore, greater turbidity in these samples is expected, which may compromise UV-Vis absorbance measurements. In this sense, the protein fractions obtained by the mild extraction method are more suitable for carrying out these experiments.

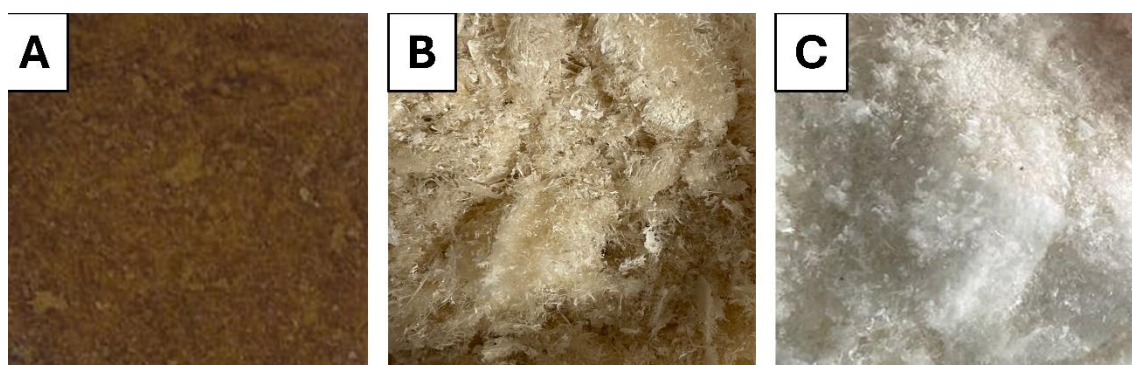


Figure 6.2. Physical appearance of pigeon pea protein fractions obtained by the precipitation-based extraction (a), mild extraction (b) and mild extraction and purification of pigeon pea 7S globulin by liquid chromatography (c).

2. Composition of the pigeon pea protein fractions

The different fractionation methods investigated in this thesis resulted in protein fractions with protein content ranging from 64 to 89% (Table 6.1). The other non-protein compounds, such as carbohydrates and lipids, were also identified and presented a significant contribution to the composition of the protein fractions. It is important to highlight that few studies in the literature had characterized these macronutrients in depth, using chromatographic techniques for their identification and quantification. In this sense, this thesis brought new insights into the composition of pigeon pea protein fractions.

2.1 Possible glycosylation of pigeon pea proteins

The pigeon pea 7S globulin has been reported as a glycoprotein previously in the literature (KRISHAN et al., 1985; KRISHAN et al., 2017). The composition of the protein fractions obtained in this thesis reinforces the glycosylation of pigeon pea proteins, as was observed by the high mannose content in the protein concentrates and in the purified 7S globulin fractions. For native 7S globulin, for example, a mannose content of approximately 16 mg/g of protein was found. It is important to highlight that not even soaking and boiling pre-treatments, fractionation processes or the extensive dialysis steps during protein purification reduced mannose levels in protein fractions, unlike what happened with other neutral sugars. This may be an indication that mannose is strongly bound to the extracted proteins. In fact, mannose has been reported as the main sugar involved in the glycosylation of globulins (DURANTI et al., 1981).

Glycosylation is dependent on the structure of the protein and the carbohydrate attachment site. The phenomenon of glycosylation is species-specific, so polypeptides encode information that directs their own glycosylation pattern. Generally, for an individual glycoprotein, more than one carbohydrate unit is present, fixed at different positions by O-linkage, N-linkage, or both (LIS & SHARON, 1993). Glycosylation can alter different physicochemical properties of glycoproteins, such as solubility, mass, size, viscosity and electrical charge, in addition to providing higher thermal stability and protection against proteolysis (LIS & SHARON, 1993).

Glycosylation also modifies the functional properties of proteins. It was demonstrated by Liu et al. (2022) that as the degree of glycosylation increases, the foaming capacity and foam stability of egg white proteins also increase. The authors also observed that in the presence of sugars, the particle size of the egg white protein reduced, and its solubility increased. The higher mannose content found in the purified pigeon pea 7S globulin fractions perhaps indicates a higher degree of protein glycosylation, explaining the better performance of the purified fractions in relation to the non-purified fractions. However, there is a lack of studies in the literature regarding the effects of glycosylation on the functionality of plant-based proteins. The results of this thesis show that this is a potential topic for future investigations. Fluorescein-phenylboronate gel electrophoresis (Flu-PAGE) can be a rapid technique to experimentally probe the glycation of these proteins. Another method would be through cesium chloride centrifugation, as conducted in a study by Krishna et al. (1977) to characterize glycoproteins in pigeon pea fractions.

Finally, another topic that deserves attention is the potential of glycoproteins as allergenic agents in foods. Many foods rich in glycoproteins, such as soy, peanuts, potatoes and olives, can cause allergic reactions. This is mainly due to modifications of *N*-glycans such as α 1,3-fucosylation and β 1,2-xylosylation, which are common in plants but absent in mammals, which makes plant glycoproteins have antigenic activities against mammalian cells (LI et al., 2016). Possible allergens present in pigeon pea have not yet been thoroughly investigated in the literature. However, given the high homology of pigeon pea 7S globulin with soybean 7S globulin, already previously identified as a potential food proallergic, the effects of ingesting pigeon pea glycoproteins should be better understood.

2.2 Accumulation of lipids in protein fractions

Lipids showed significant levels in all protein fractions evaluated, ranging from approximately 8.2% using the mild extraction method and reaching approximately 12% for the alkaline extraction followed by isoelectric precipitation method (Table 6.1). Due to the limited amount of sample, it was not possible to determine the lipid content for the purified 7S globulin. These results draw attention to the accumulation of lipids in protein fractions, with a significant impact on the fractionation method used, although the mechanisms behind the accumulation of lipids in protein fractions are still unclear.

It is important to highlight that although pigeon pea seeds have a low lipid content ($< 5\%$, w w⁻¹), the contribution of this macronutrient to the protein fractions should not be disregarded. Around 13% of the total lipids identified in pigeon pea protein concentrate obtained from raw seeds are phospholipids, a class of lipids that can contribute to the functionality of food ingredients.

This accumulation has not only been observed in this work, but also in many other pulse protein ingredients studied in our laboratory, like pea and lupin protein concentrates (KEULEYAN et al., 2023) and other ongoing research on faba bean. This points to the fact that this effect seems quite generic, yet still largely overlooked in literature.

Table 6.1. Composition of flour and different protein fractions from pigeon pea seeds.

	Composition (g 100 g ⁻¹ , d.b)										
	FC	FE	FS	FB	PPC-C	PPC-S	PPC-B	CPE-C	CPE-E	N-7S	H-7S
Moisture	9.0	9.1	10.0	7.8	8.3	10.1	9.3	10.0	9.9	9.0	9.1
Protein	19.5	19.7	21.5	20.2	63.7	68.3	62.2	81.9	82.2	89.3	88.1
Total Carbohydrate	64.6	n.a	61.1	64.5	11.1	9.1	15.4	n.a	n.a	n.a	n.a
<i>Mannose</i>	0.4	n.a	0.4	0.4	1.0	0.9	1.1	n.a	n.a	1.4	1.7
<i>NSP</i>	18.7	n.a	18.0	17.3	3.6	2.8	3.4	n.a	n.a	2.9	2.3
<i>RFO</i>	7.3	n.a	5.5	4.6	2.8	2.1	1.7	n.a	n.a	n.a	n.a
<i>Starch</i>	34.7	n.a	35.6	40.3	2.8	2.8	9.4	n.a	n.a	n.a	n.a
Total Lipids	4.0	4.1	3.1	2.8	12.6	9.3	4.0	8.2	8.5	n.a	n.a
<i>Neutral Lipids</i>	2.0	n.a	1.4	2.0	6.4	4.0	1.7	n.a	n.a	n.a	n.a
<i>Phospholipids</i>	0.3	n.a	0.3	0.6	1.7	1.3	n.d	n.a	n.a	n.a	n.a
Ash	4.3	4.0	4.1	2.9	7.5	7.2	7.5	n.a	n.a	n.a	n.a
Total amount of characterized compounds	92.4	27.8	90.3	90.4	94.9	93.9	89.1	90.1	90.7	92.2	90.4

FC indicates commercial seed flour (cv. unknown), FE indicates pure line seed flour (cv. 18g-95), FC indicates soaked seed flour, FB indicates soaked-boiled seed flour, PPC-C indicates protein concentrate produced with raw seeds, PPC-S indicates protein concentrate produced with soaked seeds, PPC-B indicates protein concentrate produced with soaked-boiled seeds, CPE-C indicates crude protein extract obtained from commercial seeds, CPE-E indicates crude protein extract obtained from pure line seeds (cv. 18g-95), N-7S indicates native 7S globulin, H-7S indicates partially hydrolyzed 7S globulin, NSP indicates non-starch polysaccharides and RFO indicates raffinose family oligosaccharides. Values below the detection limit are expressed as n.d and measures not performed are expressed as n.a.

2.3 Modifications in endogenous fluorophores

In this thesis, fluorescence measurements were used to monitor the occurrence, fluorescence patterns and intensity of the fluorophores endogenously present in pigeon pea protein fractions. The three-dimensional excitation-emission fluorescence matrices (3D-EEFM) showed two regions of fluorescence for pigeon pea samples: one corresponding to emission of proteins ($\lambda_{\text{Ex}} = 270\text{-}290\text{ nm}$, $\lambda_{\text{Em}} = 300\text{-}360\text{ nm}$ corresponding mainly to the tryptophan/ tyrosine emission), and another region referring to the fluorescence emission of non-protein compounds ($\lambda_{\text{Ex}} \approx 400\text{-}440\text{ nm}$, $\lambda_{\text{Em}} = 450\text{-}500\text{ nm}$ for solid state and $\lambda_{\text{Ex}} \approx 300\text{-}340\text{ nm}$ $\lambda_{\text{Em}} = 440\text{-}480\text{ nm}$ for liquid state) (Figure 6.3). Also, a pH-dependence response to the fluorescence was observed for these non-protein compounds when evaluated in the liquid state, with a lower fluorescence intensity being observed at more alkaline pHs (8.0-10.0) compared to acidic pHs (4.0-6.0) (Chapter 4).

Considering that the second region of fluorescence observed refers to non-protein compounds, its elimination was one of the focuses of the studies carried out in this thesis with the aim of improving the extraction and isolation of target proteins. We did not identify such compounds in this work, but based on their fluorescence patterns, they are expected to be free phenolic compounds and/or complexed with other compounds (LOCQUET et al., 2018). Membrane ultrafiltration showed to be a fast technique for separating and concentrating these compounds and could be a means of possibly obtaining extracts for identification and quantification of the phenolic profile by liquid chromatography, for example. The identification and quantification of these individual compounds can help to quickly monitor their presence in different pulses through fluorescence measurements. The desalting of protein extracts also showed to be efficient in eliminating these compounds before the purification process, as was observed by the total elimination of this second region in the purified 7S globulin. On an industrial scale, this result could be obtained by adding an ultrafiltration step.

Regarding the protein fluorescence emission region, the fluorescence intensity observed in pigeon pea flour (maximum intensity around 3000) increased by more than half for the purified 7S globulin fractions (maximum intensity around 7000). Although the protein contents were different between the samples, the amino acid composition showed a similar tyrosine and tryptophan content between the flours and the purified protein fractions. The significant increase in fluorescence intensity in samples more concentrated in proteins can be explained by an increase in the fluorescence quantum yield of tryptophan.

An important point to be highlighted is the presence of this region in protein concentrates obtained by precipitation-based extraction (Chapter 3), but with a very reduced fluorescence intensity. This reduction in fluorescence intensity, which was also observed for the protein region, can be explained by the brown color of these protein concentrates. The presence of dark and/or highly light-absorbing compounds can lead to fluorescence extinction (MULLER et al., 2011).

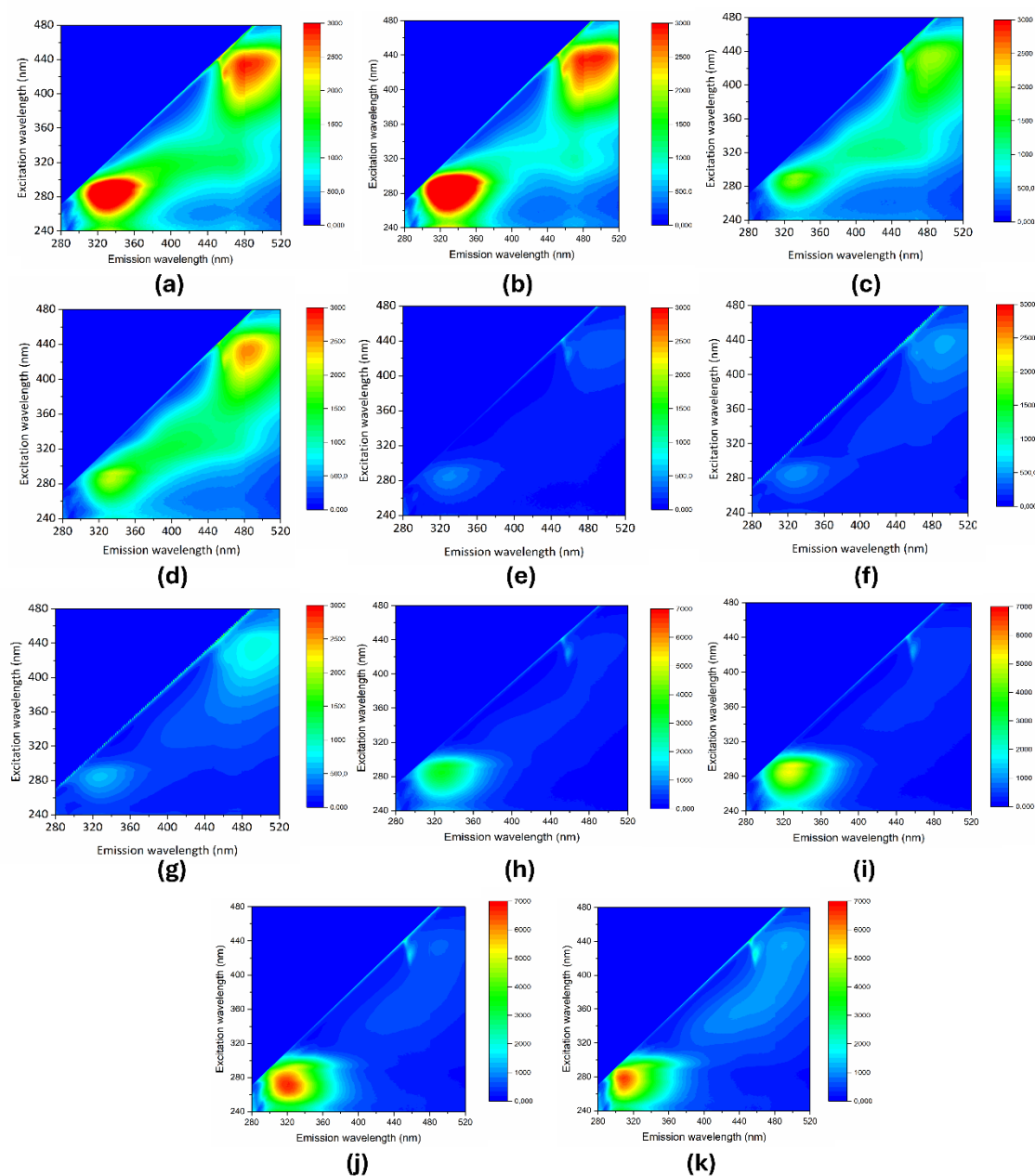


Figure 6.3. Three-dimensional excitation-emission fluorescence matrices (3D-EEFM) of pigeon pea flours and protein fractions analyzed by front-surface mode. **(a)** commercial seed flour. **(b)** pure line seed flour (cv. 18g-95). **(c)** soaked seed flour. **(d)** soaked-boiled seed flour. **(e)** protein concentrate produced from raw seeds. **(f)** protein concentrate produced from soaked seeds. **(g)** protein concentrate produced from soaked-boiled seeds. **(h)** crude protein extract obtained from commercial seeds. **(i)** crude protein extract obtained from pure line seeds (cv. 18g-95). **(j)** Native 7S globulin. **(k)** Partially hydrolyzed 7S globulin.

2.4 Reduction in trypsin inhibitors activity (TIA)

The reduction in the trypsin inhibitors activity (TIA) in pulse seed-based ingredients is of great importance for their greater digestibility. Legume trypsin inhibitors have two families, classified according to their molecular weight: Bowman-Birk (BBTIs) of 8 kDa and Kunitz (KTIs) of 20 kDa (AVILÉS-GAXIOLA, CHUCK-HERNÁNDEZ, & SERNA SALDÍVAR, 2018). In pigeon pea flour, accessions for trypsin inhibitor 1A (AOA151SYZ1), Kunitz-type trypsin inhibitor KT11 (AOA151SZ51) and Bowman-Birk type proteinase inhibitor 2 (AOA151RI37) with molecular weights around 21, 17 and 12 kDa, respectively, were identified by proteomic analysis. In SDS-PAGE analysis, it was possible to identify bands between 15-20 kDa that may be related to the presence of KTIs in flours, protein concentrates and purified fractions of pigeon pea 7S globulin. Bands around 12 kDa, related to the presence of BBTIs, were identified by SDS-PAGE only in pigeon pea flour and crude protein extracts, being more visible in the presence of a reducing agent. With this preliminary identification, the TIA in the flours and in the different protein fractions obtained was evaluated.

In this thesis, some processes used can contribute positively to the elimination of trypsin inhibitors and, consequently, a reduction in their activity. Among them are seed pre-treatments and different protein fractionation processes. The combination of soaking and boiling pre-treatments reduced the TIA in pigeon pea flour by around 86%. The use of flours produced from soaked-boiled seeds for protein fractionation resulted in protein concentrates with approximately 92% reduction in TIA, in relation to flour produced with seeds without pre-treatments. These results show that seed pre-treatments combined with the fractionation process can reduce the TIA to very low levels. On the other hand, when the seeds are not subjected to pre-treatment, the opposite occurs: there is an increase in the TIA in the protein concentrate by approximately 39% of the value presented by the flour counterpart.

However, the purification process of pigeon pea 7S globulin showed reductions of approximately 57 and 83% in the TIA levels for native and partially hydrolyzed 7S globulin, respectively, in relation to the initial flour. This result, different from the increase in TIA observed for protein concentrates, can be explained by the different separation steps that the protein extract goes through the purification process. In fact, the protein fraction not retained in the anion exchange column showed a predominance of trypsin inhibitor 1A (AOA151SYZ1) in its composition, indicating that during this step the elimination of these trypsin inhibitors occurs. Furthermore, although the purification process without dialysis steps was positive in maintaining the 7S globulin in its native state, there was a significant increase in TIA, of approximately 61% in relation to the process that used dialysis. This implies that the dialysis step is more efficient than gel filtration for the elimination of trypsin inhibitors. Using the appropriate cutoff, it would also be possible to eliminate these trypsin inhibitors by ultrafiltration.

In general, these results show that with soaking and boiling pre-treatments, processes that are commonly used at home or industrially, it is possible to significantly

reduce the TIA in pigeon pea ingredients. On the other hand, the anion exchange separation and dialysis steps play an important role in eliminating these compounds throughout protein purification processes.

3. Impact of seeds pre-treatment on the composition and nutritional quality of pigeon pea-based ingredients

Two seed pre-treatments were used in this thesis, soaking and boiling, due to the advantages from the point of view of eliminating anti-nutritional compounds present in pigeon pea and consequent improvement of their digestibility and nutritional quality (DEVINDRA et al., 2012; ONIMAWO & AKPOJOVWOK, 2006). Although such techniques have already been evaluated for pigeon pea and other species, there are not many studies that have used soaked and soaked-boiled seeds for protein fractionation processes. With this approach, a significant impact of pre-treatments on the composition of the ingredients obtained was demonstrated (Table 6.2).

From a nutritional point of view, the reduction in the levels of raffinose family oligosaccharides (RFOs), trypsin inhibitors activity (TIA) and total soluble phenolic compounds (TSCP) indicate an improvement in the digestibility of seeds subjected to soaking and boiling, since oligosaccharides can cause flatulence problems (DEVINDRA et al., 2012; RAKSHIT et al., 2015), while trypsin inhibitors hinder the action of digestive enzymes on proteins and some phenolic compounds can also act to inhibit enzymatic action and reduce nutrient absorption (CHIGWEDERE et al., 2019). On the other hand, soaking and boiling pre-treatments also resulted in the loss of compounds beneficial to health, such as minerals and tocopherols, the latter being a natural antioxidant that can easily degrade during food processing.

In addition to this impact from a nutritional point of view, changes in some compounds can also modulate the functional properties of protein concentrates. In this sense, two compounds that were affected by the soaking and boiling treatments were starch and phospholipids. While the starch content increased 3-fold in pigeon pea protein concentrate after pre-treatments, the phospholipid content was completely depleted. Both compounds can play important roles in the functionality of food ingredients, since starches can help stabilize foams, gels and emulsions, while phospholipids have a high absorption capacity at the air/water and oil/water interface.

Table 6.2. Impacts of soaking and boiling pre-treatments on the composition of pigeon pea ingredients and their possible causes.

Impact	Possible causes	Reference
Reduction in raffinose family oligosaccharides (RFOs) levels in flours and protein concentrates from pre-treated seeds	Leaching of raffinose, stachyose and verbascose into the soaking water; Heat-induced hydrolysis of oligosaccharides to disaccharides and monosaccharides	(ONIGBINDE & AKINYELE, 1983)
Mannose accumulation in protein concentrates	Potential existence of (galacto)(gluco)mannanes in protein concentrates; High mannose <i>N</i> -glycans of <i>N</i> -glycoproteins; Glycosylation of pigeon pea proteins	(THANH & SHIBASAKI, 1976; KRISHNAN et al, 2017)
Higher starch content in flour and protein concentrates from soaked-boiled seeds	Starch gelatinization, leading to greater release and solubilization of amylose and amylopectin	(WATSON & JOHNSON, 1965)
Presence of monoglycerides in pre-treated seeds	Triglyceride hydrolysis during pre-treatments	(ABDEL-RAHMAN, 2007)
Degradation of tocopherols in soaked and soaked-boiled seed ingredients	Capacity of tocopherols to act as chain-breaking antioxidants in the presence of free radicals, notably when heat treatments are applied	(HIDALGO et al., 2008; OTTAWAY et al., 2010; BALAKRISHNAN & SCHNEIDER, 2023)
Loss of low molecular weight proteins (< 37 kDa)	Leaching of small proteins into the soaking water.	(SIDDIQUE et al,1995)
Reduction of trypsin inhibitors activity (TIA) in flours and protein concentrates from pre-treated seeds	Leaching and degradation of trypsin inhibitors.	(CHIGWEREDE et al., 2019)
Reduction of the total soluble phenolic compounds in flours from pre-treated seeds	Leaching of phenolic compounds during soaking; Oxidation of phenolic acids during boiling	(CHIGWEREDE et al., 2019)
Reduction in ash content in flours from pre-treated seeds	Leaching of minerals during pre-treatments	(CHIGWEREDE et al., 2019)

4. Functional properties of pigeon pea protein fractions

The effect of different protein fractionation processes on the functional properties of protein fractions was one of the main objectives of this thesis. Firstly, the effect of using pre-treated seeds on the functionality of pigeon pea protein concentrates was performed, investigating their emulsifying, foaming and gelling properties. After this screening into different functional properties, the foaming property was chosen for further studies using purified 7S globulin as a reference, in comparison with crude protein extracts. The air/water interfacial properties were also performed to better understand the rheological behavior of pigeon pea 7S globulin after adsorption at the interface.

4.1 Solubility of different protein fractions

Solubility of protein fractions plays an important role in their functionality. The properties of the pigeon pea protein concentrates obtained in Chapter 2 were evaluated at pH 7, where the protein concentrate obtained from raw seeds showed a solubility of around 55% and the protein concentrates obtained from soaked and soaked-boiled seeds showed a solubility around 30-35%. This difference in solubility may contribute to the better performance in functionality of the protein concentrate obtained from raw seeds in relation to protein concentrates obtained from pre-treated seeds. In turn, the purified fractions of 7S globulin showed solubility of around 55-60% at pH 7 and 50mM of ionic strength (Chapter 4), a condition close to that evaluated for protein concentrates produced from pre-treated seeds. Although the methods used to evaluate solubility in the two chapters are not the same, a similarity can be seen between the values of the protein concentrate obtained from raw seeds and the purified fractions. The fact that protein concentrates obtained from pre-treated seeds have lower solubility is probably due to changes in the conformation of proteins resulting from the boiling treatment, for example, and the greater occurrence of insoluble aggregates.

4.2 Effect of seed pre-treatments on functionality

The soaking pre-treatment was shown to have an effect only on the foaming capacity of the protein concentrates, reducing the values obtained by the protein concentrate produced with raw seeds by around 6%. This difference can be explained by the loss through leaching of compounds that can also adsorb to the air/water interface, acting as foaming agents. Saponins, for example, are well-known foaming agents that are present in pigeon pea seeds at values that can vary from 2164 to 3494 mg 100g⁻¹ (SINGH et al., 2017). Apart from the foaming properties, however, no other functional properties were affected by the soaking pre-treatment only.

However, the soaking + boiling pre-treatment significantly affected all functional properties evaluated. While it decreased the emulsifying capacity, foaming capacity and foam stability, in relation to the protein concentrate produced with raw seeds, it also increased the emulsion stability and the least gelation concentration. These results can be explained by changes in the conformation of proteins due to heat treatment, in

addition to the intensification of leaching of minor compounds and the greater accumulation of starch in these concentrates, which can have a significant effect on the emulsion stability, for example. On the other hand, the elimination of phospholipids in protein concentrates produced with soaked-boiled seeds can also contribute to the reduction of emulsifying and foaming activity, for example.

When the results obtained for the functional properties of protein concentrates are interpreted together with the extensive characterization of the composition of these ingredients, a more multifaceted view of the impacts that seed pre-treatments have on functionality can be obtained. Highlighting this is important considering that often such results, such as a decrease in functionality, for example, are interpreted solely as a result of changes in the solubility, state of aggregation and conformation of proteins. However, other components also play a significant role in the composition and functionality of these ingredients and must be taken into consideration to interpret the results obtained.

4.3 Foaming properties

In Chapters 4/5, the foaming properties of crude protein extracts and purified 7S globulin fractions were evaluated using a different methodology from that used in Chapter 3, where the foam is produced and evaluated using a system of syringes connected by a small tube. This method is more suitable for evaluating samples that are available in small quantities. However, even with the methodological difference, it is possible to make some observations regarding the results.

The protein concentrates obtained by alkaline solubilization followed by isoelectric precipitation showed a foam stability after 120 min of around 70-83%, while the purified 7S globulin fractions showed values around 50-65% and the crude protein extracts showed values around 36-51%. An important aspect about these results is that the protein concentrates showed almost no visible bands < 37 kDa in SDS-PAGE analysis, while more visible bands in this region were seen in the purified fractions and, mainly, in the crude protein extracts. The greater incidence of other groups of low molecular weight proteins may result in greater competition of these proteins with 7S globulin for the air/water interface. In addition to the presence of other groups of proteins, minor compounds such as sugars and phospholipids also act as foaming agents. Among the fractionation processes used, it is expected that these compounds are more present in protein concentrates obtained with the method of alkaline solubilization followed by isoelectric precipitation and less present in purified 7S globulin fractions.

In relation to pigeon pea 7S globulin, a significant difference was observed between the foam stability of native 7S globulin in relation to partially hydrolyzed 7S globulin, which presented lower foam stability. This result highlights potential negative effects of partial hydrolysis of 7S globulin on its functionality, although the changes in protein structure that lead to this decrease in the foam stability are not clear.

Also, native 7S globulin showed greater foaming capacity and foam stability than crude protein extracts. At higher protein concentrations, the contribution from other

components presents in the crude protein extracts (albumins, sugars, phospholipids, etc.) is expected, resulting in the loss of the 7S globulin domain at the air/water interface and a decrease in stiffness of the interfacial film formed, according to analysis on the drop tensiometer.

Furthermore, the crude protein extract produced with pure line seeds (cv. g18-95) showed better performance than the crude protein extract produced from commercial seeds (cv. unknown), which is probably linked to a different composition of minor compounds that adsorb at the air-water interface.

In relation to bovine serum albumin, the pigeon pea protein fractions showed better foaming stability after 120 min. Purified pigeon pea 7S globulin performed even better than whey protein isolate in terms of foam stability, highlighting its potential as an alternative to foam agents of animal origin.

5. Protein composition of pigeon pea

Previous studies used Osborne-like procedure based on the different solubilities of protein groups to estimate the fractions of globulins (60-66%), glutelins (19-25%), albumins (7-9%) and prolamins (2-5%) in pigeon pea seeds. However, these methods can lead to accuracy errors, since a significant amount of albumins may be present in globulin extracts, for example. This is reinforced by SDS-PAGE analyzes, which have been used to identify low molecular weight contaminants in globulin-rich extracts. Until the beginning of this thesis, few studies had been published using liquid chromatographic techniques to separate, identify and quantify the groups of proteins present in the pigeon pea seed. The pioneering work of Krishna et al. (1985) proposed methods for purification on an analytical scale of pigeon pea 7S globulin, which brought the first basic knowledge about this protein. Furthermore, it was identified by the authors that pigeon pea 7S globulin is a glycoprotein. More recently, Krishnan et al. (2017) reported high homology of pigeon pea 7S globulin with soybean 7S globulin.

It is important to highlight that the genetic resources of pigeon pea have not been studied as deeply as other species such as wheat, peas, soybeans or maize. Consequently, protein sequence resources in databases such as UniprotKB have not yet been validated. This leads to remaining questions regarding the formal identification of pigeon pea proteins in the evaluated samples. Proteomic analysis performed in this work showed the presence of around 125 protein groups in pigeon pea flour. The α chain of 7S β -conglycinin was the protein with the most recognized spectra, corresponding to the main protein reported in the literature for pigeon pea (KRISHNAN et al., 1977). Basic 7S globulin and a sucrose-binding protein were also identified among the main proteins, although it is not clear what these proteins are based on the available accessions. According to proteomic analysis, the basic 7S globulin shows homology with lupin gamma-conglutinins and with peptidase (of aspartic type). On the other hand, sucrose-binding proteins shows homology with cupin type-1 domain-containing protein (Uniprot accession I1L860). Since cupin domains are found in 7S-11S globulin family (DUNWELL, 1998), this may indicate that this sucrose binding protein is indeed a globulin-type protein. The identification of

glycinin in flour indicates the presence of the 11S fraction in pigeon pea seeds, although its quantity is considerably lower than that of the 7S fraction (KRISHNAN et al., 2017). The 2S albumin and oleosins were also identified in flour. Different protease inhibitors such as cysteine inhibitor, trypsin inhibitor 1A, Kunitz-type trypsin inhibitor KTI1, Bowman-Birk type proteinase inhibitor and subtilisin inhibitor are also present in pigeon pea flour, as well as endogenous enzymes, including lipoxygenase, peroxidase, peroxygenase and aspartic proteinase.

As shown by SE-HPLC analysis under denaturing conditions, around 87% of pigeon pea proteins are extractable, with 13% proteins being non-extractable. Of the total extractable proteins, 7S globulin accounts for 48%. The unextracted fraction may be due to the aggregation state of the proteins, identified by peaks between 100-1000 kDa in the SE-HPLC analysis. Proteins with molecular weights around 52 and 38 kDa were identified by SE-HPLC, probably referring to 7S globulin. However, in SDS-PAGE analysis, the protein bands identified had subunits around approximately 64 and 49 kDa. It is not clear why this difference in molecular weight is observed between the different techniques. These values are quite different from the 72 and 57 kDa subunits identified by Krishna & Bratia (1985) for pigeon pea 7S globulin, or from the 65, 52 and 47 kDa subunits reported by Fernández Sosa et al. (2021) and Krishna et al. (2017). Furthermore, the protein bands were not sensitive to the reducing agent 2- β -mercaptoethanol, being compatible with 7S globulins. The same protein bands were seen at a lower intensity in SDS-insoluble proteins, suggesting that there is a fraction of 7S globulin that is not extractable. Finally, peaks with 21 and 16 kDa were also identified, which are consistent with the presence of albumins and protease inhibitors present in the flour.

This characterization of the protein composition of pigeon pea flour allowed to identify 7S globulin as its main protein, corresponding to approximately half of the soluble proteins. In parallel, several trypsin inhibitors were identified, highlighting the need for processes that remove them to ensure greater digestibility of pigeon pea-based ingredients. The presence of endogenous proteases in flour is also relevant for obtaining protein ingredients from pigeon pea and will be discussed in more detail in section 4 of this chapter.

6. Possible mechanisms of pigeon pea 7S globulin proteolysis

The proteolysis of pigeon pea 7S globulin that was identified in this work is unprecedented in the literature. However, proteolysis of 7S globulin from other species has been reported, including soybean, which has high homology with pigeon pea. Morita et al. (1994) observed proteolysis of the α chain of 7S globulin in mature soybean seeds, while the β chain was resistant to hydrolysis, in agreement with the phenomenon observed in our study. According to the authors, hydrolysis possibly occurred due to the action of a neutral/alkaline serine protease. Although no serine protease was identified in pigeon pea protein fractions, other endogenous proteases may have caused partial hydrolysis.

From the data obtained in the proteomic analysis, accessions exhibiting enzymatic activity were investigated. The identification of the aspartic protease enzyme was

evidenced by the presence of a basic 7S globulin (A0A151TW66) and aspartic proteinase (A0A151SU18) in the flour, total protein extract and in the hydrolysis product of approximately 25 kDa. Also, the identification of the leucine aminopeptidase enzyme was evidenced by the accessions of an aminopeptidase (A0A151R7C2) and leucine aminopeptidase 1 (A0A151RXM3) in the flour and total protein extract. Aspartic proteases have an optimum pH of 3.0 to 5.5 and are inhibited by pepstatin (1 μ M) (SZECL, 1992). Given the alkaline pH conditions used in our extraction method, it is not expected that aspartic proteases would find optimal conditions of action. On the other hand, leucine aminopeptidase has an optimum pH of around 8, and its action is inhibited by bestatin (1 μ M) and EDTA (5 mM). Although leucine aminopeptidase may have an action on pigeon pea protein extract, the addition of EDTA in our process weakens this hypothesis.

It is important to highlight that this partial hydrolysis was not observed during the optimization stage of the purification process, which was performed on an analytical scale, which indicates that the proteolysis of 7S globulin is related to the increase in the process scale. One hypothesis would be that throughout the purification process the protease responsible for the cleavage is also concentrated and reaches an optimal concentration for action. The fact that the process with injection of the extract directly into the gel filtration column prevents hydrolysis from occurring may be due to the shorter time of this process in relation to dialysis.

Our results highlight the difficulty in maintaining proteins in their native states throughout purification processes, especially at larger scales. We proposed a faster purification protocol, without the traditional dialysis step, which avoided the occurrence of hydrolysis of the 7S β globulin. Consequently, the salt removal process during gel filtration helps preserve proteins in their most native state. We also identified that this unexpected hydrolysis that was observed throughout the purification process affects the functionality of 7S globulin, at least in relation to its foaming properties.

Part II. General conclusions and perspectives

This PhD thesis had as its main goal the extraction, characterization and evaluation of the functional properties of protein fractions from pigeon pea. The study carried out was divided into three parts:

- Evaluation of the effect of seed pre-treatment on the composition and functional properties of pigeon pea seeds ingredients;
- Purification on a preparative scale of 7S globulin from pigeon pea using a mild protein extraction method;
- Assessment of air/water interfacial and foaming properties of pigeon pea protein fractions;

In the first part of the study, soaking and boiling pre-treatments modified the composition of pigeon pea flour and protein concentrates. From a nutritional point of view, pre-treatments reduced the levels of trypsin inhibitors, raffinose family oligosaccharides and soluble phenolic compounds in flours and protein concentrates, making a positive contribution to the better digestibility of these ingredients. In terms of functionality, protein concentrates obtained from soaked-boiled seeds had greater water holding capacity, least gelation concentration and emulsion stability than protein concentrate obtained from seeds without any treatment. We further demonstrated that lipids and carbohydrates, together, account for around a fifth of the composition of protein concentrates, playing a significant role in the functionality of these ingredients.

The second part of this thesis is focused on the first study on the preparative-scale purification of 7S globulin from pigeon pea. Only purification studies on an analytical scale had been conducted for the purification of 7S globulin from pigeon pea and in this work purification on a larger scale was proposed, optimized and performed, allowing the recovery of this protein in sufficient quantities for also probe its functional properties. Throughout the large-scale process, however, we observed a partial hydrolysis of 7S globulin, probably caused by the action of some endogenous protease. Replacing the dialysis step with injection into a gel filtration column to eliminate salts and minor compounds showed to be a faster and more efficient alternative to preserve the native state of 7S globulin. We demonstrated that partially hydrolyzed 7S globulin has lower foaming capacity and lower foam stability when compared to native 7S globulin. In terms of physicochemical parameters, native 7S globulin presented values similar to those obtained in predictive studies using pigeon pea sequences and their amino acid composition. On the other hand, partially hydrolyzed 7S globulin presented unconventional values, reiterating the importance of the native state of proteins to accurately determine their physicochemical properties.

In the third part of this thesis, we demonstrate the different behavior that a crude protein extract and a purified 7S globulin present at the air/water interface and its implications for foaming properties. Purified 7S globulin exhibits a stiffer interfacial film and better foaming properties than crude protein extracts. A decrease in the rigidity of interfacial films stabilized by crude protein extracts can be explained by the loss of the 7S

globulin domain at the interface and the greater contribution of other adsorbent compounds, such as sugars, phospholipids, phenols, among others. The type of seed used in the production of protein extracts also had an impact on the properties evaluated, probably due to different levels of minor compounds present. Finally, we also demonstrated that fractions rich in 7S globulin from pigeon pea perform similarly or even better than foaming agents of animal origin, such as whey protein isolate and bovine serum albumin.

The perspectives for the work carried out in this thesis involve investigating other functional properties of pigeon pea protein fractions, such as emulsifying and gel-forming properties. The identification and quantification of minor compounds, especially those that interfere with the functionality of the ingredients, must also be investigated. Finally, other protein fractionation processes, such as dry fractionation, can be explored and compared with classical aqueous fractionation processes to better understand how different processes for obtaining protein fractions can impact the functionality of ingredients obtained from pigeon pea.

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Appendix

Detailed summary of the thesis in French

Résumé détaillé de la thèse en français

1.Contexte

Parmi les graines de légumineuses, plusieurs espèces sont sous-utilisées, appelées cultures orphelines ou cultures mineures, et présentent un grand potentiel pour le développement agronomique, les besoins nutritionnels et la sécurité alimentaire (SAMAL et al., 2023). Bien que ces espèces soient bien connues dans leurs pays d'origine pour leur impact socio-économique, elles sont considérées comme sous-utilisées car elles sont encore négligées par les décideurs politiques, les chercheurs agricoles traditionnels, les sélectionneurs de plantes, les services de vulgarisation, les fournisseurs de technologie et les consommateurs en général (POPOOLA et al., 2020 ; VERBEECKE et al., 2023). De plus, elles comprennent des espèces dont les réseaux d'approvisionnement en graines sont inexistantes ou pas encore développés (ALI & BHATTACHARJEE, 2023). Or, ces espèces peuvent être résistantes à différentes conditions environnementales, capables de survivre dans des environnements secs et à des températures extrêmes (SAMAL et al., 2023).

Le pois d'Angole (*Cajanus cajan* L. Millsp.) est une légumineuse très résistante à la sécheresse, originaire des pays émergents d'Asie et d'Afrique (TAPAL et al., 2019). La production mondiale de pois d'Angole est d'environ 5,5 millions de tonnes, ce qui représente environ 6% de la production mondiale de légumineuses. Selon l'Organisation des Nations unies pour l'alimentation et l'agriculture (FAO), dans son rapport sur l'économie mondiale des légumineuses, les pois d'Angole font partie des légumineuses les plus importantes en termes de quantité de consommation et de production mondiale (FAO, 2019). L'Inde est le plus grand consommateur et producteur de pois d'Angole, représentant environ 90% de la production mondiale de cette légumineuse (TAPAL et al., 2019). Sa haute valeur nutritionnelle, avec une teneur en protéines variant de 18 à 28% de sa composition en graines (SUN et al., 2020), fait du pois d'Angole une source potentielle de production d'ingrédients riches en protéines. Dans la plupart du monde, cependant, le pois d'Angole est encore peu utilisé dans la consommation humaine. Lorsqu'il a été introduit en Amérique, il était utilisé pour nourrir les pigeons sur l'île de la Barbade, dans la mer des Caraïbes, d'où vient son nom en anglais, « pigeon pea » (VAN DE MAESEN, 1990). Au Brésil, pendant de nombreuses années, l'espèce était principalement utilisée pour nourrir le bétail, de sorte que ce n'est que ces deux dernières décennies qu'elle a fait l'objet d'études pour développer des variétés plus adaptées à la consommation humaine (GODOY et al., 2005 ; MIANO et al., 2020). Sa production, cependant, reste limitée aux petits producteurs. En fait, le pois d'Angole est l'une des légumineuses dont la production est presque entièrement limitée aux agriculteurs à petite échelle. L'une des façons de stimuler sa production à grande échelle est de

surmonter les éventuelles difficultés de traitement des graines et une meilleure compréhension de leur composition protéique.

Certaines difficultés dans le traitement des légumineuses telles que le pois d'Angole impliquent le phénomène difficile à cuire et la présence de facteurs antinutritionnels. Des facteurs antinutritionnels tels que les inhibiteurs de trypsine, les phytates, les tanins, les saponines, entre autres, peuvent entraver l'action des enzymes digestives sur les protéines, compromettant ainsi leur digestibilité et leur absorption par le corps (SAHNI et al., 2020 ; SUN et al., 2020). Des prétraitements tels que le trempage et l'ébullition font partie des principales méthodes technologiques pour réduire les effets des facteurs antinutritionnels dans les légumineuses (CODA et al., 2015 ; ZHOU et al., 2023). Bien que de tels prétraitements aient déjà montré leur efficacité dans la réduction des composés antinutritionnels dans le pois d'Angole (DEVINDRA et al., 2012 ; ONWUKA, 2006), ils peuvent affecter la structure des protéines et la fonctionnalité des ingrédients à base de graines prétraitées, constituant un sujet qui nécessite encore des recherches approfondies.

La composition protéique du pois d'Angole est également un sujet qui nécessite des études supplémentaires. Les globulines représentent environ 60% des protéines du pois d'Angole, la 7S globulin étant sa principale protéine (SINGHI & JAMBUNATHAN, 1982). Les graines de pois d'Angole contiennent également de la glycine 11S, de l'albumine 2S et d'autres protéines en proportion plus faible (KRISHNAN et al., 2017). Des études ont été menées sur la production de concentrés et d'isolats protéiques de pois d'Angole, avec l'évaluation de leurs propriétés fonctionnelles (ADENEKAN et al., 2018 ; FERNÁNDEZ SOSA et al., 2021, 2023 ; MWASARU et al., 1999a ; Mwasaru et al., 1999b). La méthode de solubilisation alcaline suivie d'une précipitation isoelectrique est la méthode d'extraction la plus utilisée pour obtenir des concentrés et des isolats protéiques. De telles méthodes permettent la récupération d'extraits protéiques riches en globulines, mais qui contiennent également d'autres contaminants tels que d'autres groupes de protéines, des sucres, des composés phénoliques, des phospholipides, entre autres (GRAVEL & DOYEN, 2023). Pour comprendre le rôle de la globuline 7S du pois d'Angole dans de tels mélanges complexes, la purification par des techniques chromatographiques peut être utilisée. Krishna & Bhatia (1985) ont été les premiers à proposer l'isolement et la purification à l'échelle du laboratoire de la 7S globulin du pois d'Angole. Cette étude a permis d'identifier que la 7S globulin du pois d'Angole est une glycoprotéine et a une sous-unité d'environ 70 kDa et deux sous-unités d'environ 57 kDa, avec un poids moléculaire de 180 kDa à l'état natif. Cette étude pionnière a présenté les fondements de l'étude de la composition en protéine du pois d'Angole. Cependant, pour évaluer également la fonctionnalité de ces protéines, il est nécessaire de les obtenir en quantités suffisantes en passant d'un processus de purification à l'échelle analytique vers un processus à l'échelle préparative.

2.Stratégie d'étude

2.1 Objectifs de l'étude et approche expérimentale

L'objectif de cette thèse était d'extraire, de caractériser et d'évaluer les propriétés fonctionnelles des fractions protéiques du pois d'Angole. L'approche expérimentale des différentes parties de l'étude était la suivante :

- La première partie a étudié l'impact des prétraitements des graines sur la fonctionnalité des concentrés protéiques de pois d'Angole. Le trempage des graines a été réalisé dans de l'eau à température ambiante pendant 24 heures, tandis que l'ébullition a été effectuée en cuisant les graines trempées dans de l'eau bouillante pendant 30 minutes. Après les prétraitements, les graines ont été séchées au four et broyées pour produire de la farine. Les concentrés protéiques de pois d'Angole ont été obtenus à partir de la farine en utilisant la méthode de solubilisation alcaline (pH 12,0) suivie de la précipitation isoelectrique (pH 4,5). Les farines et les concentrés protéiques ont été caractérisés en fonction de leur composition en protéines, glucides, lipides et composés mineurs. Pour évaluer la fonctionnalité des concentrés protéiques, la capacité de rétention d'eau et d'huile, l'indice d'aptitude à l'émulsification, l'indice de stabilité d'émulsion, la capacité moussante, la stabilité de la mousse et la concentration minimale de gélification ont été déterminées;
- La deuxième partie a consisté en la purification de la globuline 7S du pois d'Angole à une échelle préparative, en utilisant un ensemble de techniques de chromatographie liquide avec désalage de l'extrait protéique suivie de chromatographie échangeuse d'anions et de chromatographie d'exclusion de taille. La pureté des fractions obtenues a été déterminée par la teneur en azote, l'analyse par électrophorèse et les mesures de fluorescence intrinsèque. Les propriétés physicochimiques des fractions purifiées telles que le facteur de conversion azote-protéine, le coefficient d'extinction, l'accroissement de l'indice de réfraction, la solubilité, la mobilité électrophorétique et la distribution de taille ont été déterminées en laboratoire et les données expérimentales obtenues ont été comparées avec les valeurs calculées à partir d'études prédictives utilisant la séquence et la composition en acides aminés des fractions protéiques;
- Dans la troisième partie, les propriétés interfaciales air/eau et moussantes des fractions protéiques du pois d'Angole ont été évaluées. Des extraits de protéines brutes ont été obtenus à partir d'une méthode d'extraction douce suivie d'une désalinisation de l'extrait. La globuline 7S purifiée a été utilisée comme référence. Pour les études de propriétés interfaciales, un tensiomètre à goutte a été utilisé. Des gouttes ont été formées à l'extrémité d'une aiguille exposée à l'air et des balayages d'amplitude de déformation de la surface de 10, 20 et 30% ont été effectués. La propriété moussante a été évaluée à l'aide d'un système de deux seringues couplées, l'une contenant la solution protéique et l'autre contenant de l'air. Après le mouvement de va-et-vient des seringues, les mousses ont été produites et la capacité moussante ainsi que sa stabilité au fil du temps ont été

mesurées. Des agents moussants d'origine animale tels que l'isolat de protéine de lactosérum, l'albumine de sérum bovin et la lysozyme ont été utilisés comme témoins.

3. Résultats

3.1 Impact du prétraitement des graines sur la composition, la qualité nutritionnelle et la fonctionnalité des ingrédients à base de pois d'Angole

Deux prétraitements des graines ont été utilisés dans ce projet, le trempage suivi ou non d'un traitement thermique, en raison des avantages du point de vue de l'élimination des composés antinutritionnels présents dans le pois d'Angole et de l'amélioration conséquente de leur digestibilité et de leur qualité nutritionnelle (DEVINDRA et al., 2012 ; ONIMAWO & AKPOJOVWO, 2006).

Le trempage des graines pendant 24 heures à température ambiante a réduit les niveaux d'oligosaccharides de la famille du raffinose (RFOs) d'environ 25 %, l'activité des inhibiteurs de la trypsine (TIA) de 31 % et les niveaux de composés phénoliques solubles totaux (TSPC) d'environ 16 %. Le phénomène principal responsable de ces réductions est le lessivage de ces composés dans l'eau de trempage (CARTER & MANTHEY, 2019).

La combinaison de trempage et traitement thermique pendant 30 minutes a intensifié davantage la réduction des RFOs, du TIA et du TSPC dans les graines de pois d'Angole, réduisant respectivement de 37 %, 86 % et 57 % par rapport à la farine témoin (sans prétraitement). En plus du lessivage des composés qui se produit pendant le trempage, d'autres réactions se produisent pendant traitement thermique, telles que : l'hydrolyse des oligosaccharides en disaccharides et monosaccharides, l'inactivation des inhibiteurs de protéase par la chaleur, l'oxydation des acides phénoliques, la libération d'acides libres à partir de leurs formes conjuguées, et la formation de complexes entre les polyphénols et d'autres composés, tels que les protéines (FLEURIET, 2003 ; ONIGBINDE & AKINYELE, 1983).

L'élimination des facteurs antinutritionnels du pois d'Angole par la combinaison de trempage et traitement thermique est bénéfique pour la qualité nutritionnelle et la digestibilité de ses graines et des ingrédients qui en découlent, car les oligosaccharides peuvent causer des problèmes de flatulence (DEVINDRA et al., 2012 ; RAKSHIT et al., 2015), tandis que les inhibiteurs de la trypsine entravent l'action des enzymes digestives sur les protéines et certains composés phénoliques peuvent également agir pour inhiber l'action enzymatique et réduire l'absorption des nutriments (CHIGWEDERE et al., 2019).

D'autre part, les prétraitements de trempage et traitement thermique ont également entraîné la perte de composés bénéfiques pour la santé, tels que le tocophérol et les minéraux. Le trempage a réduit les niveaux de tocophérol dans la farine de pois d'Angole de 65 %, tandis que sa combinaison avec traitement thermique a réduit sa teneur de 75 % par rapport à la farine témoin. Le tocophérol est un antioxydant naturel qui peut se dégrader lors des procédés alimentaires. Dans des graines telles que les haricots et les pois, leur dégradation est associée à l'action de l'enzyme α -tocophérol, qui catalyse les différentes formes de tocophérol, nécessitant de l'oxygène moléculaire et des phospholipides pour son action (BARLOW & GAUNT, 1972 ; MURILLO et al., 1976). Bien

que la majeure partie de la dégradation des tocophérols ait pu se produire pendant le trempage, il est possible que cette dégradation ait également eu lieu au cours des premiers moments du prétraitement par traitement thermique, jusqu'à ce que les enzymes perdent leur activité. De plus, les niveaux de lipides totaux, de lipides neutres et de phospholipides ont également diminué avec les prétraitements. Une perte considérable de minéraux a également été observée, en raison du lessivage de ces composés pendant le traitement. La teneur en cendres a diminué de 5 et 33 % pour les traitements de trempage et de trempage+ traitement thermique, respectivement.

Pour produire des concentrés protéiques à partir de farines prétraitées, la méthode d'extraction de solubilisation alcaline suivie de la précipitation isoelectrique a été utilisée. La teneur en protéines variait de 62 à 68 % et tous les extraits protéiques obtenus étaient des fractions riches en globuline, comme observé dans l'analyse SDS-PAGE, où la prédominance des principales sous-unités α et β de la globuline 7S du pois d'Angole a été observée, avec des poids moléculaires d'environ 64 et 49 kDa, respectivement.

Les lipides constituaient une fraction significative des concentrés protéiques de pois d'Angole. Le concentré protéique obtenu à partir de graines crues, sans prétraitement, avait une teneur en lipides d'environ 12 g pour 100 g, cette valeur étant trois fois plus élevée que celle présentée par la farine de pois d'Angole. L'utilisation de graines trempées et bouillies a montré un effet significatif sur la réduction de la teneur en lipides des concentrés protéiques, présentant une teneur en lipides de 4 g pour 100 g. Les lipides neutres et les phospholipides ont également suivi la réduction observée pour la teneur totale en lipides, de sorte que les phospholipides n'ont pas été observés dans le concentré protéique produit avec des graines trempées et bouillies. Ces résultats jouent un rôle important dans la fonctionnalité de ces concentrés protéiques, car les phospholipides, par exemple, peuvent contribuer aux propriétés émulsifiantes (XIA et al., 2022).

La fraction glucidique variait de 9 à 15 g pour 100 g pour les concentrés protéiques. Alors que les valeurs des polysaccharides non-amylacés (NSP) n'étaient pratiquement pas affectées par les prétraitements des graines utilisées dans la production de ces extraits, variant de 2,8 à 3,6 g pour 100 g, les teneurs en saccharose et en oligosaccharides de la famille du raffinose (RFOs) étaient réduites, tandis que la teneur en amidon augmentait. La réduction des RFOs contribue à la qualité nutritionnelle des concentrés protéiques, réduisant davantage les niveaux observés pour la farine de pois d'Angole. Comparativement, alors que la farine témoin (sans prétraitement) présentait une teneur en RFOs d'environ 11 g pour 100 g, le concentré protéique produit avec des graines trempées et bouillies a montré une réduction de 72 % de ces valeurs, avec une teneur en RFOs d'environ 3 g pour 100 g. En plus de cet impact d'un point de vue nutritionnel, l'augmentation de l'amidon observée dans ce concentré protéique a également eu un impact sur sa fonctionnalité, principalement en ce qui concerne sa capacité de rétention d'eau, sa concentration minimale de gélification et sa stabilité d'émulsion, qui étaient supérieures à celles du concentré protéique obtenu à partir de graines sans prétraitement.

Enfin, la combinaison de trempage+ traitement thermique avec le processus de fractionnement aqueux a abouti à un TIA de 0,6 $\mu\text{g TId mg}^{-1}$ d'échantillon, équivalant à une réduction d'environ 93 % par rapport à la farine témoin (sans traitements). Parmi les mécanismes impliqués dans cette réduction plus importante figurent l'effet du traitement thermique sur l'inactivation des inhibiteurs de la trypsine combiné à l'élimination possible des inhibiteurs de la trypsine lors de l'étape de précipitation des protéines.

3.2 Purification de la globuline 7S du pois d'Angole par chromatographie préparative

3.2.1 Processus de purification

La purification de la 7S globuline du pois d'Angole a d'abord été optimisée à l'échelle analytique, dans le but d'isoler au mieux la protéine cible. Différentes colonnes de chromatographie échangeuse d'ions ont été testées, ainsi que les valeurs de pH d'extraction et les paramètres chromatographiques. L'extrait protéique à un pH de 8,0 a été utilisé, de même que la colonne d'échange anionique Q, en raison des meilleurs résultats de rétention des protéines. L'étape de désalage de l'extrait protéique a été ajoutée avant la séparation dans la colonne d'échange anionique pour éliminer les composés mineurs présents dans l'extrait. Après avoir optimisé le processus à l'échelle analytique, la purification de la 7S globuline a été transférée à l'échelle préparative. À l'échelle préparative, la purification a suivi deux routes différentes : (i) la dialyse des fractions protéiques contre un tampon de carbonate d'ammonium ; (ii) le remplacement de la dialyse par une injection dans une colonne de filtration sur gel équilibrée avec un tampon de carbonate d'ammonium. Le processus comprenait trois étapes de chromatographie : (i) la désalinisation de l'extrait protéique ; (ii) la chromatographie échangeuse d'ions ; (iii) la chromatographie d'exclusion de taille. Alors que le processus contenant la dialyse a entraîné une hydrolyse partielle inattendue de la 7S globuline, le processus avec élimination du sel lors de la filtration sur gel était efficace pour maintenir la 7S globuline dans son état natif. L'analyse SDS-PAGE a révélé que la 7S globuline dans son état natif a des sous-unités d'environ 64 et 49 kDa, tandis que la 7S globuline dans un état partiellement hydrolysé a des sous-unités d'environ 49 et 20-25 kDa. L'analyse protéomique a montré que les bandes autour de 20-25 kDa sont composées d'un mélange entre les chaînes α et β de la 7S globuline, confirmant la coupure des bandes identifiées dans l'état natif.

L'hydrolyse sélective de la chaîne α de la 7S globuline du soja par des protéases endogènes a également été observée par Morita et al. (1994). De plus, selon les auteurs, la chaîne β était résistante à l'hydrolyse, un résultat également observé dans notre étude. La cause de l'hydrolyse de la 7S globuline du pois d'Angole n'est pas claire, mais la présence de protéases endogènes dans la farine peut aider à comprendre ce résultat. Une hypothèse pourrait être que tout au long du processus de purification, la protéase responsable du clivage des protéines est également concentrée et atteint une concentration optimale pour son action. Le fait que le processus avec injection de l'extrait directement dans la colonne de filtration sur gel empêche l'hydrolyse de se produire peut être dû au temps plus court de ce processus par rapport à la dialyse. Nous avons également constaté que cette hydrolyse inattendue observée tout au long du processus

de purification affecte la fonctionnalité de la 7S globuline, du moins en ce qui concerne ses propriétés moussantes. La capacité de moussage de la 7S globuline native était d'environ 95 %, tandis que la 7S globuline partiellement hydrolysée présentait une valeur d'environ 82 %. La stabilité de la mousse était également réduite par l'hydrolyse. Alors que la 7S globuline native présentait une stabilité de la mousse après 120 minutes de 69 %, la 7S globuline partiellement hydrolysée réduisait la stabilité de la mousse à 55 %. L'aspect des mousses formées par les deux fractions différait également, la 7S globuline partiellement hydrolysée présentant des bulles de tailles plus hétérogènes que la 7S globuline dans son état natif. Nos résultats mettent en évidence la difficulté de maintenir les protéines dans leur état natif tout au long des processus de purification, en particulier à plus grande échelle. Nous avons proposé un protocole de purification plus rapide, sans l'étape traditionnelle de dialyse, qui évitait l'hydrolyse de la 7S globuline. Par conséquent, le processus d'élimination du sel lors de la filtration sur gel contribue à préserver les protéines dans leur état le plus natif et à préserver leur fonctionnalité.

3.2.2 Composition des fractions purifiées

La 7S globuline purifiée a présenté une pureté d'environ 90 %. L'analyse SDS-PAGE a montré les deux bandes principales correspondant aux chaînes α (64 kDa) et β (49 kDa) de la 7S globuline. Le processus de purification a abouti à une teneur totale en glucides d'environ 2,3 à 2,9 g pour 100 g. Le mannose était le monosaccharide prédominant dans la fraction purifiée, avec des niveaux de 1,4 à 1,7 g pour 100 g. Ce résultat suggère des interactions mannose-protéine, car la 7S globuline du pois d'Angole est décrite comme une glycoprotéine (KRISHAN et al., 2017 ; KRISHAN et al., 1985). Le fait que le mannose soit présent dans les fractions purifiées, même avec les étapes de séparation chromatographique et de dialyse, suggère qu'il n'est pas à l'état libre, mais plutôt lié aux protéines.

Dans cette thèse, nous avons utilisé des mesures de fluorescence pour surveiller l'apparition, les spécificités et l'intensité de fluorescence de fluorophores endogènes présents dans les fractions protéiques du pois d'Angole. Les matrices de fluorescence d'excitation-émission tridimensionnelles (3D-EEM) ont montré deux régions de fluorescence pour les échantillons de pois d'Angole : l'une correspondant à l'émission de protéines (λ_{Ex} = 270-290 nm, λ_{Em} = 300-360 nm correspondant principalement à l'émission de tryptophane/tyrosine), et une autre région se référant à l'émission de fluorescence de composés non-protéiques (λ_{Ex} $\approx$ 400-440 nm, λ_{Em} = 450-500 nm pour l'état solide et λ_{Ex} $\approx$ 300-340 nm λ_{Em} = 440-480 nm pour l'état liquide).

Étant donné que la deuxième région de fluorescence observée se réfère à des composés non protéiques, son élimination a été l'un des axes d'étude de cette thèse dans le but d'améliorer l'extraction et l'isolement des protéines cibles. Nous n'avons pas identifié de tels composés dans ce travail, mais en fonction de leurs motifs de fluorescence, on s'attend à ce qu'il s'agisse de composés phénoliques libres et/ou complexés avec d'autres composés (LOCQUET et al., 2018). La désalinisation des extraits protéiques s'est avérée efficace pour éliminer ces composés, comme cela a été observé par l'élimination totale de cette deuxième région dans les fractions purifiées de la globuline 7S.

Le processus de purification a également contribué positivement à l'élimination des inhibiteurs de trypsine, qui présentaient des valeurs de 3,6 et 1,4 $\mu\text{g TId mg}^{-1}$ d'échantillon pour la 7S globuline native et partiellement hydrolysée, respectivement. Nos résultats ont montré que la fraction non retenue dans la colonne d'échange anionique est constituée de l'inhibiteur de trypsine 1A, indiquant qu'une partie considérable des inhibiteurs de trypsine est éliminée à cette étape de la séparation chromatographique. Une différence dans le TIA a également été observée en ce qui concerne les processus de purification utilisés, la dialyse étant plus efficace pour réduire le TIA que son remplacement par l'injection directe de la fraction purifiée dans la colonne de filtration sur gel.

3.2.3 Propriétés physico-chimiques des fractions purifiées

La 7S globuline native a été caractérisée pour certaines propriétés physico-chimiques, telles que le facteur de conversion N/P, les spectres UV-Vis, le coefficient d'extinction, l'incrément d'indice de réfraction, la solubilité, la mobilité électrophorétique et la distribution de taille. Les données expérimentales obtenues ont été comparées avec les valeurs obtenues par des études prédictives, en utilisant les séquences disponibles de la 7S globuline du pois d'Angole et la composition en acides aminés des échantillons.

Le facteur de conversion N/P calculé était égal à 5,85, légèrement au-dessus de la valeur prédite de 5,7. Le spectre d'absorption UV-visible tracé à partir des séquences de la 7S globuline du pois d'Angole a montré une absorption plus élevée de la chaîne α par rapport à la chaîne β . L'absorption maximale pour les deux chaînes était de 274 nm. Le spectre UV-visible tracé à partir des données expérimentales de la 7S globuline purifiée du pois d'Angole présentait le même profil que les données prédictives. Cependant, un décalage de 3 nm a été observé pour la fraction purifiée en laboratoire, qui présentait des absorptions maximales égales à 278 nm. L'absorption maximale observée pour la 7S globuline suggère la contribution de la tyrosine et du tryptophane. Cependant, le profil de courbe avant le pic d'absorption maximale suggère également la contribution de la phénylalanine (HENDRICKS & JULIAN, 2015 ; PACE et al., 1995). La composition en acides aminés de la 7S globuline du pois d'Angole montre une teneur significative en phénylalanine de 98 mg g⁻¹, respectivement. Les teneurs en tyrosine et en tryptophane sont inférieures à celles de la phénylalanine, étant d'environ 30 et 0,9 mg g⁻¹, respectivement. Ces valeurs sont également en accord avec celles obtenues par des études prédictives. Les coefficients d'extinction déterminés à 280 nm variaient de 0,34 à 0,48 cm⁻¹ g⁻¹ L pour la 7S globuline. Ces valeurs concordent avec le coefficient d'extinction à 280 nm calculé à partir des séquences de la 7S globuline, la chaîne α présentant un coefficient égal à 0,37 cm⁻¹ g⁻¹ L et la chaîne β un coefficient de 0,31 cm⁻¹ g⁻¹ L. Les coefficients d'extinction déterminés étaient plus bas pour les pH 4,0 et 5,0 et plus élevés pour le pH 6,0.

En ce qui concerne l'incrément d'indice de réfraction (dn/dc), les valeurs variaient de 0,185 à 0,239 pour la 7S globuline du pois d'Angole. Selon les données théoriques de la séquence de la globuline, les valeurs de dn/dc pour la chaîne α et la chaîne β étaient respectivement de 0,192 et 0,191. La valeur d'incrément d'indice de réfraction généralement utilisée pour les protéines est de 0,185 (ZHAO et al., 2011). Les valeurs

expérimentales sont, dans la plupart des conditions, légèrement supérieures aux valeurs théoriques. Une valeur de dn/dc plus élevée peut être liée à la présence de composés non protéiques dans les fractions purifiées.

À faible force ionique, la solubilité de la 7S globuline était d'environ 41-50% à pH 4.0, diminuant à 7-17% à pH 5.0 pour ensuite augmenter progressivement avec le pH jusqu'à atteindre une solubilité maximale à pH 8.0, qui variait de 67-81%. À haute force ionique, la solubilité des protéines était plus élevée que les solubilités déterminées à faible force ionique. De plus, à des concentrations élevées en sel, la 7S globuline présentait une solubilité > 90% à presque tous les pH évalués, sauf à pH 6.0 où la solubilité était d'environ 70%. La plus faible solubilité observée à pH égal à 5.0 et 6.0 pour des conditions de faible et haute force ionique, respectivement, peut s'expliquer par la proximité du point isoélectrique de la 7S globuline, qui est égal à 6.2 pour la chaîne α et 6.1 pour la chaîne β , selon des études prédictives.

La mobilité électrophorétique de la 7S globuline variait de 1 260 à -1 351 $\mu\text{m cm s}^{-1} \text{ V}^{-1}$. Il n'y avait aucun effet du pH sur la mobilité électrophorétique des échantillons à des pH de 4.0 à 6.0 ($p > 0,05$). À pH 5.0, la mobilité électrophorétique était proche de zéro ce qui suggère un point isoélectrique proche de 5.0. Cette valeur est inférieure à la valeur prédite (6.2). La distribution de taille de la 7S globuline présentait une distribution bimodale, avec une population autour de 10-20 nm et une autre entre 100-300 nm. La 7S globuline montrait une plus grande population autour de 300 nm lorsqu'elle était évaluée à pH 6.0. En revanche, des pH plus alcalins (7.0-8.0) déplaçaient cette population autour de 100 nm, indiquant que le pH influence l'état d'agrégation de la 7S globuline.

L'hydrolyse partielle de la 7S globuline a augmenté les valeurs de toutes les propriétés physico-chimiques observées, par rapport à la 7S globuline native et aux valeurs prédites à partir de la séquence et de la composition en acides aminés. De plus, la solubilité a diminué avec l'hydrolyse partielle. Comme la solubilité est un facteur important dans la fonctionnalité des protéines, ce résultat suggère une fonctionnalité réduite de cette fraction par rapport à la 7S globuline native. Cela concorde avec ce que nous avons observé pour les propriétés moussantes et, probablement, ce qui serait observé pour d'autres propriétés telles que la capacité d'émulsion et la formation de gel.

3.3. Propriétés interfaciales air/eau et propriétés moussantes de la globuline purifiée et de l'extrait protéique brut de pois d'Angole.

Dans la dernière étape de cette thèse, les propriétés interfaciales air/eau et les propriétés moussantes des fractions protéiques du pois d'Angole ont été évaluées. Les extraits protéiques bruts ont été obtenus par un processus d'extraction doux suivi de la désalification de l'extrait. Deux extraits ont été produits : l'un à partir de graines commerciales (cv. inconnue) (EPE-C) et l'autre à partir de graines avec une lignée pure (cv. g18-95) (EPE-E). La 7S globuline purifiée a été utilisée comme référence.

Des gouttes de suspensions protéiques ont été formées à l'extrémité d'une aiguille exposée à l'air. Les échantillons ont été équilibrés pendant 10800 s pour permettre aux protéines de migrer de la solution vers l'interface (GRAHAM & PHILLIPS, 1979). Les extraits protéiques bruts ont montré une réduction plus rapide de la pression de surface

par rapport à la 7S globuline purifiée. Cela est dû à la présence d'autres petites molécules, telles que des albumines, qui migrent plus rapidement vers l'interface.

Après la formation du film interfacial, des balayages d'amplitude ont été effectués, étirant et comprimant le film interfacial avec des déformations de 10, 20 et 30% de sa surface. On a noté qu'à des concentrations de 0,1 g L⁻¹, le comportement des extraits protéiques bruts à l'interface air-eau était similaire à celui de la 7S globuline purifiée, suggérant une prédominance de la 7S globuline dans l'interface stabilisée par les deux types de fractions protéiques. Les modules élastiques E' étaient supérieurs aux modules visqueux E'' , indiquant la prédominance du comportement élastique des suspensions. Leur ratio (E''/E') avait des valeurs autour de 0,2 pour tous les échantillons, confirmant cette conclusion.

À des concentrations de 1 g L⁻¹, cependant, le comportement interfacial des extraits protéiques bruts et de la 7S globuline purifiée différait, les extraits protéiques bruts présentant des valeurs du ratio (E''/E') plus élevées, entre 0,5 et 0,8, suggérant une structure interfaciale moins élastique formée par le film interfacial. Ces résultats indiquent qu'à des concentrations plus élevées, d'autres composants des extraits protéiques bruts (autres protéines, phénols, phospholipides, sucres, etc.) peuvent rivaliser avec la 7S globuline pour l'adsorption à l'interface, ce qui se traduit par un film interfacial moins rigide (YANG et al., 2023; YANG et al., 2022).

En ce qui concerne les propriétés moussantes, la 7S globuline a montré une plus grande capacité moussante et une stabilité de mousse supérieure aux extraits protéiques bruts. Comme la concentration utilisée pour les études de capacité moussante était de 4 g L⁻¹, une plus grande contribution d'autres composants présents dans les extraits protéiques bruts est attendue, entraînant la perte du domaine de la 7S globuline à l'interface air-eau et une diminution de la rigidité du film interfacial formé. De plus, EPE-E a montré de meilleures performances que EPE-C, ce qui est probablement lié à une composition différente de composés mineurs qui s'adsorbent à l'interface air-eau. L'isolat de protéines de lactosérum (WPI) et l'albumine sérique bovine (BSA) ont été utilisés comme témoins de bons agents moussants, tandis que le lysozyme a été utilisé comme contrôle ayant des propriétés moussantes médiocres. Par rapport à la BSA, les fractions protéiques du pois d'Angole ont montré une meilleure stabilité de la mousse après 120 minutes. La 7S globuline purifiée a même surpassé le WPI en termes de stabilité de la mousse, mettant en évidence son potentiel en tant qu'alternative aux agents moussants d'origine animale.

4. Conclusions générales et perspectives

Cette thèse de doctorat avait comme objectif principal l'extraction, la caractérisation et l'évaluation des propriétés fonctionnelles des fractions protéiques du pois d'Angole. L'étude réalisée a été divisée en trois parties :

- Évaluation de l'effet du prétraitement des graines sur la composition et les propriétés fonctionnelles des ingrédients à base de pois d'Angole ;
- Purification à l'échelle préparative de la 7S globuline du pois d'Angole en utilisant une méthode douce d'extraction des protéines ;
- Évaluation des propriétés interfaciales air/eau et des propriétés moussantes des fractions protéiques du pois d'Angole.

Dans la première partie de l'étude, les prétraitements par trempage et ébullition ont modifié la composition de la farine de pois d'Angole et des concentrés protéiques. D'un point de vue nutritionnel, les prétraitements ont réduit les niveaux d'inhibiteurs de trypsine, d'oligosaccharides de la famille du raffinose et de composés phénoliques solubles dans les farines et les concentrés protéiques, contribuant ainsi positivement à une meilleure digestibilité de ces ingrédients. En termes de fonctionnalité, les concentrés protéiques obtenus à partir de graines trempées et bouillies présentaient une meilleure capacité de rétention d'eau, une concentration minimale de gélification inférieure et une stabilité d'émulsion supérieure par rapport au concentré protéique obtenu à partir de graines non traitées. Nous avons également démontré que les lipides et les glucides, ensemble, représentent environ un cinquième de la composition des concentrés protéiques, jouant un rôle significatif dans la fonctionnalité de ces ingrédients.

La deuxième partie de cette thèse est axée sur la purification à l'échelle préparative du 7S globuline du pois d'Angole. Jusqu'à présent, seules des études de purification à l'échelle analytique avaient été menées pour la purification du 7S globuline du pois d'Angole, et dans ce travail, une purification à plus grande échelle a été proposée, optimisée et réalisée, permettant la récupération de cette protéine en quantités suffisantes pour également sonder ses propriétés fonctionnelles. Tout au long du processus à grande échelle, cependant, nous avons observé une hydrolyse partielle du 7S globuline, probablement causée par l'action de certaines protéases endogènes. Remplacer l'étape de dialyse par une injection dans une colonne de filtration sur gel pour éliminer les sels et les composés mineurs s'est avéré être une alternative plus efficace pour préserver l'état natif du 7S globuline. Nous avons démontré que le 7S globuline partiellement hydrolysé présentait une capacité moussante inférieure et une stabilité de mousse plus faible par rapport au 7S globuline natif. En termes de paramètres physico-chimiques, le 7S globuline natif présentait des valeurs similaires à celles obtenues dans des études prédictives utilisant des séquences de pois d'Angole et leur composition en acides aminés. En revanche, le 7S globuline partiellement hydrolysé présentait des valeurs non conventionnelles, réitérant l'importance de l'état natif des protéines pour déterminer précisément leurs propriétés physico-chimiques.

Dans la troisième partie de cette thèse, nous démontrons le comportement différent qu'un extrait brut de protéine et une 7S globulines purifiés présentent à

l'interface air/eau et ses implications pour les propriétés moussantes. Le 7S globuline purifié présente un film interfacial plus rigide et de meilleures propriétés moussantes que les extraits bruts de protéines. Une diminution de la rigidité des films interfaciaux stabilisés par les extraits bruts de protéines peut s'expliquer par la perte du domaine du 7S globuline à l'interface et la plus grande contribution d'autres composés adsorbants, tels que les sucres, les phospholipides, les phénols, entre autres. Le type de graines utilisé dans la production des extraits protéiques a également eu un impact sur les propriétés évaluées, probablement en raison de différents niveaux de composés mineurs présents. Enfin, nous avons également démontré que les fractions riches en 7S globuline du pois d'Angole se comportent de manière similaire, voire mieux, que les agents moussants d'origine animale, tels que l'isolat de protéines de lactosérum et l'albumine sérique bovine.

Les perspectives du travail réalisé dans cette thèse impliquent d'explorer d'autres propriétés fonctionnelles des fractions protéiques du pois d'Angole, telles que les propriétés émulsionnantes et gélifiantes. L'identification et la quantification des composés mineurs, en particulier ceux qui interfèrent avec la fonctionnalité des ingrédients, doivent également être étudiées. Enfin, d'autres processus de fractionnement des protéines, tels que le fractionnement à sec, peuvent être explorés et comparés aux processus de fractionnement aqueux classiques pour mieux comprendre comment différents procédés d'obtention de fractions protéiques peuvent influencer la fonctionnalité des ingrédients obtenus à partir du pois d'Angole.

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