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EFFECTIVENESS INACTIVATION OF TRYPSIN INHIBITOR FROM BRAZILIAN CULTIVARS OF BEANS (*PHASEOLUS VULGARIS* L.)*

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■ABSTRACT: The trypsin inhibitor, an antinutritional factor, which is abundant in dycotiledoneous and monocotyledoneous, is usually inactivated by heating treatment. The influence of pressure-cooking (121°C and 141kPa) for 30 min on, trypsin inhibitors concentration and inhibitors reactivation from ten Brazilian beans varieties of *Phaseolus vulgaris* L. namely: IAPAR-14, IAC-Carioca, Rudá, Corrente, IAC-Aruã, IAPAR-16, IAPAR-57, IAC-Carioca Pyatã, Carioca, Aporé, were investigated. The inhibitors reactivation was evaluated in comparison with the activity of raw and pressure-cooking. For raw the *in vitro* protein digestibility mean values ranged from 40% (in Carioca cultivar) to 60% (in IAC-Aruã cultivar), showing an increase from 11% to 37% using the autoclaving at 121°C and 141kPa. Among ten cultivars studied the trypsin inhibitor activity varied from 36.18UTI.mg^{-1} for IAC-Aruã to 63.33UTI.mg^{-1} for IAPAR-16. Trypsin inhibitor activity was totally inactivated by pressure-cooking. The study of the trypsin inhibitors reactivation using double-digestive pepsin-pancreatin enzymes *in vitro* showed a recovering activity from 34% up to 100%. Native inhibitor is resistant to double- digestive pepsin-pancreatin proteolysis, whereas autoclaving to 121°C.30 min^{-1} results in a non-native conformation that is susceptible to proteolysis, improving the digestibility and inactivate differentially the activity of trypsin inhibitors. The results of the thermal treatment of the beans show inactivation of the inhibitors, which may be due to formation of high molecular weight aggregates with other substances of the grain. The pepsin-pancreatin digestion of the inactivated inhibitor restores the activity, probably due to its retention by the digested fragments.

■KEYWORDS: Antinutritional factor; heat treatment; *in vitro* protein digestibility; inhibitor reactivation.

INTRODUCTION

The beans seeds of *Phaseolus vulgaris* L. are important source of protein, dietary fiber, fat, starch, flavonoids and vitamin for human diet.[8,11,12,15, 21, 23, 26] In spite of these favorable features, the bean also contains antinutritional factors that are not good for human and animal nutrition, such as trypsin inhibitors, lectin, tannins, and phytate.[3,11,12,32] In addition, feed trypsin inhibitor can reduce nutrient uptake, animal growth[14,16,31] and is considered an effective pancreatic hypertrophy agent.[13,16,17,31] On the other hand, it is well established that protease inhibitors can prevent cancer.[9,20,28]

Although trypsin inhibitor activity has been partially or fully inactivated by different treatments[18] and the inactivation process might be attributed to formation of high molecular weight aggregated among trypsin inhibitor and others proteins,[7,8] beans nutritional value have been improved by thermal inactivation treatment,[8,11,18] high-pressure,[31] extrusion cooking,[21] natural fermentation,[15] pressure-cooking (121°C.30 min^{-1}) or germination.[12] In addition, it has been shown that purified inhibitor is resistant to inactivation[24, 29] and trypsin inhibitor purified from rice lost its activity after 2 hours of *in vitro* digestion with pepsin and pancreatin.[27] An important point that remains inconclusive is that if this protein is resistant to inactivation and become active into digestive systems of humans and animals.

Due to the importance of animal and human nutrition, many investigations have been carried out to develop treatments in order to reduce or even remove the content of antinutritional factors and enhance the nutritional quality. These changes differ widely depending on the technology and conditions involved. [15]

Therefore, the study of tripsyn inhibitor reactivation from *Phaseolus vulgaris* L. submeted to pressure-cooking might provide new information about efficiency of thermal treatment, or necessity of a combination of several treatments.

In this paper we report the trypsin inhibitors concentration in ten cultivars of *Phaseolus vulgaris* and the study of the trypsin inhibitors reactivation using double-digestive pepsin-pancreatin *in vitro* of bean grains samples

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submitted to pressure-cooking. The degree of hydrolysis and amino acid composition were performed in the IAC-Aruã cultivar due to higher recovery of inhibitor activity after digestion.

MATERIAL AND METHODS

Bean Cultivars

Ten Brazilian varieties of *Phaseolus vulgaris* L. namely: IAPAR-14; IAC-Carioca; Rudá; Corrente, IAC-Aruã, IAPAR-16, IAPAR-57, IAC-Carioca Pyatã, Carioca, Aporé were chosen for this study.

Sample Preparation

Bean grains were soaked in distilled water (1:5 p:v) for 12 hours under laboratory conditions at 26±5ºC and submitted to pressure-cooking at 121°C and 141 kPa for 30 min, according established previously by Carvalho et al.[8] After cooling, the erlenmeyer was immersed in crushed ice, dried in an oven with air flow forced circulation at 60ºC for 18 hours and grounded into a fine powder using a grinder. The powder was sieved through a screen and stored at -20ºC. Flour bean without pressured-cooked was included in each experiment to quantify the inactivation degree of trypsin inhibitor in each varieties cultivars.

Crude Extract

The trypsin inhibitors extraction was carried out at 26±5ºC Heat treated and untreated samples were homogenized (1:50 $w.v^{-1}$) with volumes of 0.025M HCl, with starring for 1h filtered in Whatman nº 2 paper and then the filtrate was used in the enzyme inhibition assay.

Assay of Inhibitory Activity

Inhibitory trypsin activity was assayed discontinuously, at 37ºC, in a Hitachi U-1000 spectrophotometer by following the liberation of p-anilide at 410nm. The reaction was initiated by addition of the substrate and stopped with 0.1mL of 30% acetic acid. Standard conditions were 0.1mL the trypsin ($0.11mg.mL^{-1}$ in 0.001N HCl), after 10 min of incubation 0.7mL 1mM BAPNA (N-benzoyl-DL-arginine *p*-nitroanilide) previously prepared in 50mM Tris.HCl buffer, pH 8.2, containing 20mM $CaCl_2$ in a final volume of 1.0mL.[19] The inhibitory activity was measured by the difference in enzyme activity with and without the inhibitor presence. One trypsin unit (TU) was defined as an increase of 0.01 unit of absorbance at 410nm by 100mL of reaction medium. The results were expressed as Units Trypsin Inhibited (UTI) per milligram of sample.

In vitro protein digestibility and inhibitor reactivation

In vitro hydrolysis of raw and pressure-cooking cultivar extracts was carried out as described by Akerson & Stahman[2] using a double-digestive pepsin-pancreatin. After hydrolysis, proteins that were not hydrolyzed were precipitate with TCA, centrifuged at 12.000xg for 20 min, at 4ºC, filtrated at Watman nº 2 paper and used for determination of digestibility and assay of inhibitor reactivation.

The protein was measured using the semimicro-Kjeldahl procedure and expressed as percent of the total N, by using the following formula:

Were: dN – nitrogen; aN – auto digestible nitrogen; – tN - sample total nitrogen.

Limited proteolysis with pepsin-pancreatin

Raw and pressure-cooking protein samples of IAC-Aruã were incubated using double-digestive pepsin-pancreatin.[2] The hydrolyzed protein was analyzed measuring the amount of α-amine groups liberated with TNBS[1] using L-leucine as standard.

Gel filtration chromatography

Raw and pressure-cooking extract of IAC-Aruã cultivar were fractionated on a 1.50 × 120cm Sephadex G-75 chromatography column, equilibrated and eluted with 50mM Tris.HCl buffer, pH 7.5. Fraction of 4.2mL was collected and protein 280nm and trypsin inhibitor activity assayed. The fraction with activity were pooled, frozen in liquid nitrogen and stored at -20ºC for a period of one month without appreciable loss of activity.

Amino acid composition

Proteins from IAC-Aruã treated and untreated were hydrolyzed with 6N HCl at 110°C for 24 hours.[22] The amino acid composition was determined in a Dionex DX 300 Ion Chromatography System by using a cation exchange column and a ninhydrin derivation post-column. The amino acid standard was used to determine the samples amino acid concentrations. Yearly average and standard deviations were determined for each amino acid. Alls determination was carried out at Protein Chemistry Center of USP-Ribeirão Preto

RESULTS AND DISCUSSION

Protein digestibility was investigated in ten bean cultivars of treated and untreated *Phaseolus vulgaris* L. As shown at Table 1, there was a significant difference ($p<0.01$) among digestible protein that ranged from 40% (Carioca) to 60% (IAC-Aruã). These results were accordingly to Carvalho et al.[8] who reported values from 47% for cultivar OC and 59% for Parana. Similar value for protein digestibility (36.3-56%) has also been reported by Salunke & Kadam.[25] On the other hand, these values were lower than those reported (65.6-80.7%) by different cultivars of *Phaseolus vulgaris* growth in difference countries.[15,26,30]

These variations in beans digestibility can be influenced by specie, cultivar and environmental conditions.[10,11,18,26]

When compared to other food proteins, the beans have low digestibility due to their resistance to enzymatic hydrolysis.

The pressure-cooking at 121ºC for 30 min increased digestible protein *in vitro* (P<0.01) from 11 to 37%. These results are quite similar to that reported by Carvalho et al., [8] in which digestibility at some conditions showed an increase of 32.6% using the heating process.

There was a significant difference of trypsin inhibitor (P<0.001) among cultivars studied. The IAC-Aruã (Table 2) shows 36.18UTI.mg^{-1} while IAPAR-16 for 63.33UTI. mg^{-1}. The medium value for trypsin inhibitor widespread in almost varieties is about 46UTI.mg^{-1}. Was found in other cultivars of bean growth in different countries.[5,8,11,15,18,26] The values of trypsin inhibitor harmful to animals are not yet fully known. So, cultivars with trypsin inhibitor with a lower activity and/or more susceptible to inactivation is expected to increase the nutritional quality of grains. Although trypsin inhibitor activity was totally inactivated by pressure-cooking at 121ºC for 30 min, there was a significant difference (P<0.001) in trypsin inhibitors reactivation after pressure-cooking and incubating *in vitro* using a double digestive pepsin-pancreatin (Table 2). The recovering ranged from 19.24UTI/mg for IAC-Carioca Pyatã to 36.12UTI/mg for IAC-Aruã. According to Roychaudhuri et al.[24] the reversibility or not of thermal

Table 1 – Digestible protein *in vitro* (%) of raw and autoclaved beans of ten cultivars of *Phaseolus vulgaris.*

Cultivars	Raw beans	Autoclaved beans	% of digestibility autoclavated
IAPAR	55,0 ± 1,4^{ab}	61,0 ± 2,7^{bcd}	11,0
IAC-Carioca	49,0 ± 4,2^{bcd}	62,0 ± 1,4^{bcd}	27,0
Rudá	53,0 ± 2,8^{abc}	58,0 ± 4,0^{cd}	13,0
Corrente	53,0 ± 2,8^{abc}	60,0 ± 1,6^{cd}	13,0
IAC-Aruã	60,0 ± 4,2^{a}	73,0 ± 2,8^{a}	22,0
IAPAR-16	53,0 ± 2,8^{abc}	60,0 ± 1,6^{cd}	13,0
IAPAR-57	48,0 ± 4,2^{bcd}	63,0 ± 2,5^{bc}	31,0
IAC-Carioca Pyatã	56,0 ± 1,4^{ab}	68,0 ± 2,4^{ab}	21,0
Carioca	40,0 ± 3,0^{d}	55,0 ± 3,0^{d}	37,0
Aporé	45,0 ± 4,0^{cd}	56,0 ± 1,4^{cd}	24,0
F	10,17**	14,51**	-
CV	6,16	4,02	-
DMS	9,12	7,15	-

All values are means of triplicate ± standard deviation.
**- means with different superscript within a column indicate statistically significant differences (P<0.01).

Table 2 – Trypsin inhibitor activity (UTI.mg^{-1}) contente of raw and autoclaved and subject to proteolysis double-digestive pepsin-pancreatin of ten cultivars of *Phaseolus vulgaris.*

Cultivars	Raw	ASP	Recovery (%)
IAPAR	38,18^{de}	32.42^{b}	85
IAC-Carioca	44,00^{d}	33,08^{b}	76
Rudá	44,08^{cd}	26,00^{c}	59
Corrente	41,31^{de}	34,00^{ab}	82
IAC-Aruã	36,18^{e}	36,12^{a}	100
IAPAR-16	63,33^{b}	32,49^{b}	51
IAPAR-57	55,12^{b}	32,03^{b}	58
IAC-Carioca Pyatã	38,32^{b}	28,74^{d}	75
Carioca	56,47^{de}	19,24^{bc}	34
Aporé	50,30^{bc}	23,91^{cd}	47
F	54,12**	25,43**	-
CV	2,16	1,95	-
DMS	6,24	5,63	-

**- means with different superscript within a column indicate statistically significant differences (P< 0.01).
ASP = autoclaved and subject to proteolysis.

denaturation of trypsin inhibitor depends on the rate of the renaturation.

The reactivation studies showed an average recovering percentage of 68.22%. The highest value observed was for IAC-Aruã (100%) following by the IAPAR-14, with efficiency of 85% as well as by Jourdan et al.[18] for inactivation of trypsin inhibitor activity from Brazilian varieties of beans. This observation is in agreement with Carvalho et al.[7] and has been interpreted as non specific interactions among inhibitor with other grain components, but not due to inhibitor thermal inactivation. Taken together, these results show that inhibitors of digestive enzymes are highly resistant to denaturation by thermal treatment and its activity is restored during the digestion process. Since it remains to be clarified if the reactivation of trypsin inhibitor alter the nutritional value and shows an adverse toxic effect on human, treatment to reduce or even remove the antinutritional factors of bean have been looking forward.[15,21,31]

The amino acids composition of raw and pressure-cooking IAC-Aruã (Table 3) was similar to those reported for different cultivars of *Phaseolus vulgaris.* [8, 23] This cultivar showed high levels of aspartic acid, glutamic acid, lysine, proline, leucine but is limiting in sulfur amino acids. The values obtained for methionine (0.62), cystein (0.37) and for lysine (7.43) of raw and autoclaved samples were not significantly modified by treatments. Despite its low content in sulphur amino acids, the presence of high level of lysine suggest that bean can be used as a good complement of those cereals deficient in lysine.[4]

The amino acid content, expressed as mile moles of amino acids.gram^{-1} of proteins (h_{tot}) was not significantly modified by treatment which is 8.31 in the raw and 8.27mM.g^{-1} protein to the treated samples, respectively (Table 3), and are in accordance to those reported by Candido[6] who found h_{tot} of 8.0mM.g^{-1} protein.

In spite of both raw and pressure-cooking IAC-Aruã beans have showed similar hydrolysis degree by pepsin at least for 180 minutes (Figure 1), the pressure-cooking at 121ºC for 30 min increased the susceptible to proteolysis by pancreatin and the digestibility increases from 59% to 63%. It should be stressed that trypsin inhibitor activity was not modified by autoclaving at 121ºC for 30 min, and was quite similar to those reported for crude extracts. However, these results do not exclude unequivocally the possibility that the inhibitor might be structurally modified without loss of its activity. Since it activity depend of native configuration, only after structural studies is possible determine whether there are changes in the native configuration of the inhibitor.

Chromatography on Sephadex G-75 column (Figure 2) of raw extracts and of the samples pressure-cooking (121°C and 141kPa) for 30 min and treated with a double-digestive pepsin-pancreatin showed that inhibitor of crude extract was eluted at fraction of 127mL. On the other hand, when the treated sample was chromatographed under the same conditions, the inhibitor was eluted at 176mL volume. Our results shows that the native inhibitor is resistant to proteolysis with double-digestive pepsin-pancreatin, whereas pressure-cooking at 121ºC for 30 min results in a non-native conformation that is significantly susceptible to proteolysis by both enzymes, improving the digestibility and inactivate differentially the activity of trypsin inhibitors. The results of the thermal treatment of the beans show inactivation of the inhibitors, which may be due to formation of high molecular weight aggregates with other substances of the grain. The pepsin-pancreatin digestion of the inactivated inhibitor restores the activity, probably due to its retention by the digested fragments.

Table 3 – Amino acids composition of raw and autoclaved beans of IAC-Aruã.

Amino acids	Raw beans		Autoclaved beans	
	g.100g^{-1}prot.	m mM.g^{-1} prot.	g.100g^{-1} prot.	mM.g^{-1} prot.
ASP	14,74	1,11	13,52	1,02
TRE	4,62	0,39	4,39	0,37
SER	7,18	0,68	6,55	0,62
GLU	23,36	1,59	22,66	1,54
PRO	7,83	0,68	7,51	0,65
GLY	4,62	0,62	6,91	0,92
ALA	5,00	0,56	4,45	0,50
CYS	0,37	0,03	0,84	0,07
VAL	3,62	0,31	3,61	0,31
MET	0,62	0,04	0,60	0,04
ILE	2,44	0,19	2,41	0,18
LEU	7,25	0,55	6,97	0,53
TYR	3,37	0,19	3,79	0,21
PHE	5,56	0,34	5,29	0,32
LYS	7,43	0,51	7,09	0,49
HIS	2,69	0,17	2,58	0,17
ARG	5,68	0,33	5,41	0,31
TRP	0,37	0,02	0,36	0,02
H_{tot} [1]	-	8,31	-	8,27

[1] mile moles of amino acids.gram^{-1} of proteins.

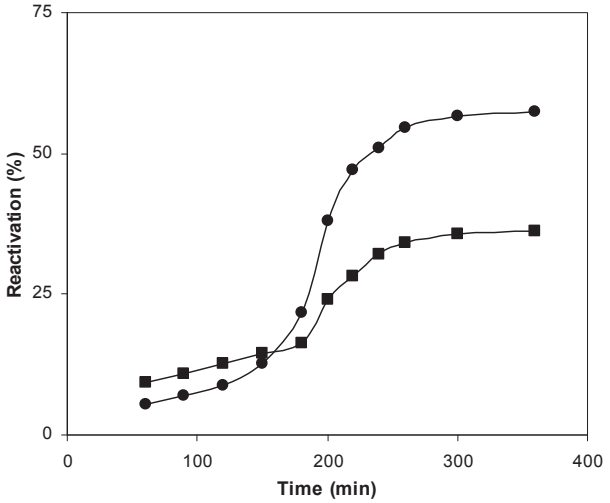

FIGURE 1 – Time-course of reactivation (%) in (●) raw and (■) pressure-cooking IAC-Aruã beans subject to proteolysis with pepsin for 180 minutes followed of pancreatin for 180 minutes. Data is plotted as % reactivation versus time of incubation. Results represent the means of three determinations as described under experimental procedures.

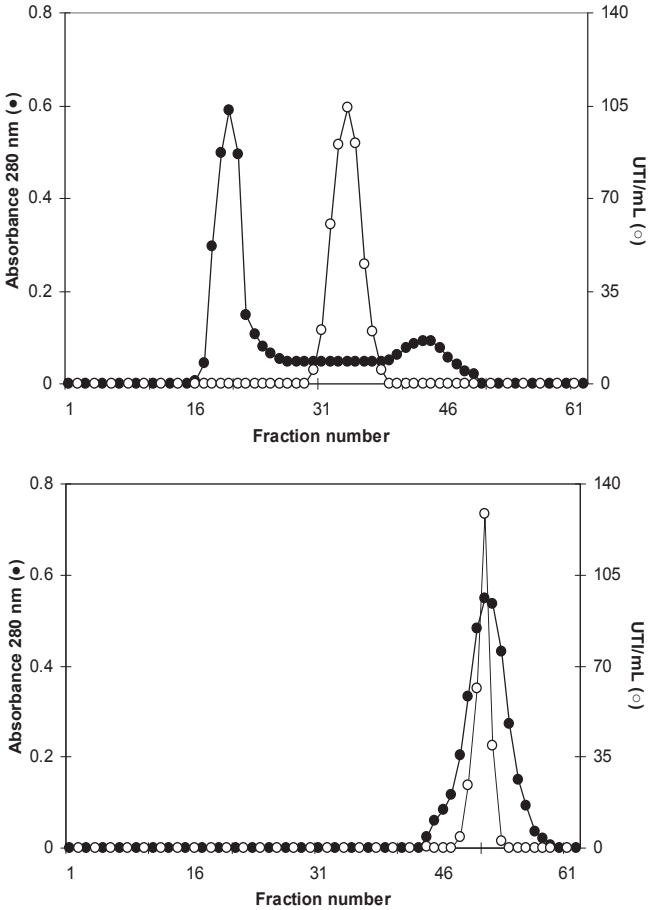

FIGURE 2 – Chromatography in Sephadex G75 column (1.50 x 120cm) from extracts of: (a) untreated; (b), autoclavedand incubated separately using a double-digestive pepsin-pancreatin. The elutionwas carried out with with Tris-HCl 50mM, pH 7,5, buffer. Samples of 4.2mL were collected.

PAIVA, K. C.; CARVALHO, M. R. B.; PIZAURO JUNIOR, J. M. Eficácia da inativação do inibidor de tripsina em cultivares brasileiras de feijão (*Phaseolus vulgaris L.*). **Alim. Nutr.**, Araraquara, v. 22, n. 3, p. 331-337, jul./set. 2011.

■RESUMO**:** O inibidor de tripsina, um fator antinutricional abundante em dicotiledoneas e monocotiledoneas, geralmente é inativado pelo tratamento térmico. O efeito do cozimento a 121°C e 141kPa durante 30 minutos sobre a concentração do inibidor de tripsina e a reativação de dez cultivares brasileiras de feijão (*Phaseolus vulgaris*L.) denominadas IAPAR-14, IAC-Carioca, Rudá, Corrente, IAC-Aruã, IAPAR-16, IAPAR-57, IAC-Carioca Pyatã, Carioca, Aporé foram investigados. A reativação dos inibidores foi avaliada em comparação com a atividade das amostras cruas e autocolavadas. A digestibilidade *in vitro* das proteínas variou de 40% (cultivar Carioca) a 60% (cultivar IAC-Aruã), apresentando aumento de 11% a 37% com o aquecimento a 121°C e 141kPa. Dentre as dez cultivares estudadas a atividade do inibidor de tripsina variou de 36,18UTI. mg^{-1} para IAC-Aruã a 63,33UTI.mg^{-1} para o IAPAR-16. O inibidor de tripsina foi totalmente inativado pelo aquecimento a 121°C e 141kPa. O estudo de reativação da atividade dos inibidores de tripsina após digestão enzimática *in vitro* com as enzimas pepsina e pancreatina mostrou uma recuperação da atividade entre 34% a 100%. O inibidor na sua forma nativa é resistente a digestão simultânea das enzimas proteolíticas pepsina e pancreatina, enquanto o tratamento térmico a 121°C e 141kPa resulta em conformação susceptível a proteólise, melhorando a digestibilidade e inativando diferencialmente a atividade do inibidor de tripsina. Os resultados do tratamento térmico do feijão mostram a inativação dos inibidores, o que pode ser devido à formação de agregados de alto peso molecular com outras substâncias do grão. A digestão por pepsina-pancreatina do inibidor inativado recupera a atividade, decorrente de sua provável retenção pelos fragmentos digeridos.

■PALAVRAS–CHAVE**:** Fator antinutricional; tratamento térmico; digestibilidade *in vitro* da proteína; reativação do inibidor.

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