UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
INSTITUTO D BIOCIÊNCIAS – RIO CLARO

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS (BIOLOGIA CELULAR, MOLECULAR E MICROBIOLOGIA)
(Mestrado)

Produção e purificação da proteína recombinante humana SLPI a partir da microalga *Chlamydomonas reinhardtii*

Bárbara Luísa Chagas da Silva

Rio Claro – SP
2023

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Bárbara Luísa Chagas da Silva

Orientador: Prof. Dr. Augusto Ducati Luchessi

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Prof. Dr. AUGUSTO DUCATI LUCHESSI (Participaçao Virtual)
Faculdade de Ciencias Aplicadas / Universidade Estadual de Campinas

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Prof. Dr. FERNANDO MOREIRA SIMABUCO (Participaçao Virtual)
Departamento de Bioquímica / Universidade Federal de São Paulo

Prof. Dr. IGOR LUCHINI BAPTISTA (Participaçao Virtual)
Faculdade de Ciências Aplicadas / Universidade Estadual de Campinas

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Rio Claro, 28 de fevereiro de 2023

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“Conheça todas as teorias, domine todas as técnicas, mas ao tocar uma alma humana, seja apenas outra alma humana.”
(Carl Jung)

RESUMO

O inibidor de protease secretado por leucócitos (SLPI) foi primeiramente descrito em 1986. Desde então, é alvo de diversos estudos devido às suas variadas funções. A proteína SLPI desempenha diversos papeis no organismo humano, sendo que a principal é sua ação inibidora de serina – proteases. Devido à essa função, a SLPI têm um importante papel na manutenção da homeostase entre proteases e antiproteases no trato respiratório humano e na pele, agindo principalmente em respostas anti-inflamatórias contra agentes danosos ao tecido. Adicionalmente, a proteína SLPI também já foi caracterizada em mecanismos de cicatrização, metabolismo ósseo, câncer e apresenta propriedades antimicrobianas, antifúngicas e antivirais. Por ser uma proteína multifuncional, o potêncial terapeutico da SLPI é bastante explorado por pesquisadores e vem sendo testado em diferentes campos da ciência, como doenças respiratórias, neuroregeneração, cicatrização de feridas, e cardioproteção em situações de isquemia/reperfusão. Devido ao crescente interesse em pesquisas biotecnológicas visando a proteína SLPI como um bioproduto, diversas empresas passaram a produzir e comercializar a proteína em diferentes sistemas de expressão e produção. Porém, a SLPI ainda é vendida somente para fins de pesquisa científica à um custo muito alto. O mercado biotecnológico de produção e expessão de proteínas recombinantes têm avançado bastante nos últimos anos, e diversas plataformas estão sendo desenvolvidas, como plantas, animais transgênicos, células humanas e microalgas. As microalgas vem sendo muito utilizadas em pesquisas para biorremediação, desenvolvimento de ração e suplemento alimentar, e não há muito tempo, ganharam notável evidência como promissoras plataformas para produção de proteínas recombinantes. Esses organismos apresentam grande potencial como plataformas biotecnológicas para desenvolvimento de bioprodutos, pois são seres eucariotos, unicelulares, de rápido crescimento e com baixo custo de meio de cultura. À vista disso, o presente projeto teve como objetivo a produção e purificação da proteína recombinante humana SLPI a partir da microalga *Chlamydomonas reinhardtii.*

Palavras – chave: SLPI; purificação; *Chlamydomonas reinhardtii;* proteína recombinante.

ABSTRACT

The secretory leukocyte protease inhibitor (SLPI) was first described in 1986. Since then, it has been the subject of several studies due to its varied functions. The SLPI protein plays several roles in the human body, the main one being its inhibitory action on serine-proteases. Due to this function, SLPI play an important role in maintaining homeostasis between proteases and antiproteases in the human respiratory tract and in the skin, acting mainly in anti-inflammatory responses against tissue-damaging agents. Additionally, the SLPI protein has also been characterized in healing mechanisms, bone metabolism, cancer and has antimicrobial, antifungal and antiviral properties. As it is a multifunctional protein, the therapeutic potential of SLPI is widely explored by researchers and has been tested in different fields of science, such as respiratory diseases, neuroregeneration, wound healing, and cardioprotection in situations of ischemia/reperfusion. Due to the growing interest in biotechnological research, targeting the SLPI protein as a by-product, several companies started to produce and commercialize the protein in different expression and production systems. However, SLPI is still only sold for scientific research purposes at a very high cost. The biotechnological market for the production and expression of recombinant proteins has advanced a lot in recent years, and several platforms are being developed, such as plants, transgenic animals, human cells and microalgae. Microalgae have been widely used in research for bioremediation, feed development and food supplement, and not long ago, they gained the spotlight as promising platforms for the production of recombinant proteins. These organisms have great potential as biotechnological platforms for the development of bioproducts, as they are eukaryotic, unicellular, fast-growing beings with low cost of culture medium. In view of this, the present project aimed at the production and purification of the recombinant human protein SLPI from the microalgae Chlamydomonas reinhardtii.

Keywords: SLPI; purification; *Chlamydomonas reinhardtii*; recombinant protein.

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1 INTRODUÇÃO

O inibidor de protease secretado por leucócitos (SLPI), é uma proteína catiônica não glicosilada com ação contra serina proteases, servindo para controlar a liberação excessiva de proteases secretadas. Os inibidores de proteases podem ser divididos em dois grupos: inibidores "sistêmicos" e inibidores de "alarme". Os inibidores "sistêmicos" são sintetizados principalmente no fígado e incluem o inibidor da α1-proteinase e a antiquimotripsina. Já os inibidores de “alarme” são sintetizados e secretados localmente no local da lesão e são produzidos em resposta às citocinas primárias, como a interleucina (IL) -1β e o fator de necrose tumoral (TNF) (SALLENAVE, 2000). A SLPI atua em ambos os grupos de inibidores, sendo considerada uma proteína multifacetada.

A SLPI é um polipeptídeo formado por 107 aminoácidos e possui um peso molecular de 11.726 kDa. A estrutura terciária da SLPI lembra um “boomerang”, em que cada braço representa um domínio (C-terminal e N-tetminal). Os domínios homólogos são denominados domínios *whey acidic protein* (WAP) e são caracterizados por oito resíduos de cisteína que formam quatro ligações dissulfeto intramoleculares. Em 1988 Grutter et al publicaram a estrutura do domínio WAP II (C-terminal) da proteína SLPI acoplada com a α-quimiotripsina bovina (GRÜTTER et al., 1988). Posteriormente, Koizumi et al. (2008) e Fukushima et al. (2013) também elucidaram, em alta definição, a estrutura do domínio WAP II da proteína em complexo com a elastase de neutrófilo humana e tripsina, respectivamente. Estes estudos confirmaram estudos anteriores que descreveram que o domínio WAP II é o responsável pela ação inibitória da SLPI contra serina proteases e que o sítio de inibição está localizado entre os resíduos 67 e 74 (EISENBERG et al., 1990a; FUKUSHIMA; KAMIMURA; TAKIMOTO-KAMIMURA, 2013; KOIZUMI et al., 2008; KRAMPS et al., 1990).

Além de sua atividade antiprotease, a SLPI demonstrou propriedades antibacterianas, antivirais, antifúngicas e anti-inflamatórias, além de participar de respostas imunes inatas e adaptativas. A proteína SLPI é produzida por células epiteliais humanas (NUGTEREN; SAMSOM, 2021), células traqueais, alveolares brônquicas e bronquiolares, e em células mielóides, como macrófagos, granulócitos,

células dendríticas e plaquetas, em neurônios e astrócitos e também em células musculares esqueléticas (JIN et al., 1997; LILO et al., 2013; NUGTEREN; SAMSOM, 2021; ODAKA et al., 2003; SCHULZE et al., 2004; WANG et al., 2003). Além disso, foi relatado que SLPI é expressa em adipócitos e tecido adiposo, onde poderia desempenhar função moduladora relevante na resolução da inflamação (ADAPALA; BUHMAN; AJUWON, 2011). Está presente em altas concentrações em diversas secreções, incluindo secreções salivares e nasais, bronquiais, além de muco cervical e intestinal (NUGTEREN; SAMSOM, 2021).

Já foi demonstrado atividade antibacteriana, antifúngica e antiviral da proteína SLPI. A atividade antibacteriana foi primeiramente descrita em 1980 e em 1996 Hiemstra et al. (1996) demonstrou a atividade contra *Escherichia coli* e *Staphylococcus aureus.* Wiedow et al. (1998) testou a atividade antibacteriana da SLPI contra *Pseudomonas aeruginosa*, *Staphylococcus aureus*, e *Staphylococcus epidermidis*, microorganismos altamente presentes na pele humana, e atestou que a SLPI promove uma barreira antimicrobiana. A atividade antiviral da SLPI foi observada contra o vírus da imunodeficiência humana tipo 1 (HIV-1). (HIEMSTRA et al., 1996; WIEDOW et al., 1998).

Alto nível de SLPI na saliva de bebês de 1 mês de idade foi associado à reduzido nível de infecção por HIV-1, quando expostos ao vírus por meio do leite materno, assim como a presença de SLPI no fluido vaginal mostrou inibição da infecção de bebês pelo vírus HIV-1 quando nascidos através de parto normal (FARQUHAR et al., 2002; PILLAY et al., 2001b). A expressão de SLPI em glândulas salivares foi relacionada à baixa taxa de transmissão viral entre indivíduos, mais especificamente do vírus HIV-1 (WAHL et al., 1997). Ademais, a atividade anti-HIV-1 da SLPI presente na saliva já foi demonstrada *in vitro* (MCNEELY et al., [s.d.]). A atividade da proteína SLPI contra o vírus do papiloma humano (HPV) também já foi reportada. Woodham et al (2012) mostrou que a a incubação da SLPI com células epiteliais diminui a infecção dessas células com o vírus HPV através da ligação da SLPI à Anexina A2 (HOFFMANN et al., 2013; WOODHAM et al., 2012). A atividade antifúngica do inibidor de protease leucocitária secretora (SLPI) foi avaliada contra os patógenos *Aspergillus fumigatus* e *Candida albicans.* Em *Candida albicans* foi mostrado que a SLPI pode modular diferentes fatores de virulência como atividade

proteolítica, hidrofobicidade, adesão a epitélio e expressão de manoproteínas (CURVELO et al., 2014).

A produção heteróloga de SLPI recombinante em *E. Coli* é disponível comercialmente e vem sendo utilizada em estudos de função estrutural bem como em estudos de aplicações terapêuticas, como por exemplo, no tratamento de inflamação pulmonar, lesão isquêmica, reperfusão em transplante cardíaco e em tuberculose (FAKIOGLU et al., 2008; GIBBONS et al., 2009; GOMEZ et al., 2009; NERNPERMPISOOTH; PROMPUNT; KUMPHUNE, 2017; PROMPUNT et al., 2018a, 2018b; TOMEE et al., 1998). No entanto, a produção da proteína SLPI humana em bactérias implica em limitações para sua aplicação terapêutica, pois existe a possibilidade de contaminação por endotoxinas, pode occorrer variação e oxidação da proteína, além de potencialmente afetar a atividade proteolítica da SLPI (KONOPKA et al., 1999). Ademais, o processo de purificação da proteína SLPI contribui para o seu alto custo (cerca de US$ 355,00 por 100 µg – R&D Systems) (EISENBERG et al., 1990a; HAMAKER et al., 1996; HARCUM; COLLINS; SEELY, 1993).

Assim, as questões associadas com a produção de SLPI recombinante expressa em bactérias indicam a necessidade de um sistema de expressão alternativo que seja capaz de fornecer consistentemente SLPI na sua conformação nativa a um custo mais acessível. Neste contexto, é proposto realizar a expressão de SLPI humana (rhSLPI) na microalga *C. reinhardtii*.

2 OBJETIVO

Este projeto tem como objetivo a produção e purificação da proteína humana recombinante SLPI a partir da microalga *C. reinhardtii.*

2.1 Objetivos específicos:

- Revisão da literatura sobre a proteína SLPI (funções, aplicações e produção) e definição do objeto de estudo;
- Confirmação de linhagens de *C. reinhardtii* rhSLPI-positivas;
- Seleção e manutenção do clone com maior expressão de rhSLPI;

- Purificação da proteína rhSLPI produzida pelas linhagens transformadas de *C. reinhardtii*.

3 CAPÍTULO 1: ARTIGO DE REVISÃO

3.1 Metodologia

3.1.1 Artigo de revisão

A revisão bibliográfica foi realizada no período de abril de 2022 a janeiro de 2023, a partir de buscas na base de dados PubMed. Os critérios de inclusão englobaram artigos de revisão bibliográfica, ensaios clínicos e artigos experimentais publicados até o ano de 2022 que abordavam como tema central a proteína SLPI. Foram utilizados o total de 144 artigos. Os termos utilizados para pesquisa incluíram “SLPI”, “Secretory leukocyte protease inhibitor”, “SLPI antiprotease ativity”, “SLPI production”, “SLPI therapeutic”, “SLPI in wound healing”, “SLPI anti-inflamatory activity”, “SLPI antibacterial activity”, “recombinant protein production in *Chlamydomonas reinhardtii*”, “recombinant protein production”, “SLPI and cancer”, “heterologous protein production in microalgae”. O critério de inclusão utilizado consistiu em artigos que abordavam sobre as funções da proteína SLPI relacionadas à ação anti-protease, antiinflamatória, antimicrobiana e de metabolismo ósseo. Além da estrutura, aplicações e produção da proteína SLPI, e plataformas biotecnológicas para produção de proteínas recombinantes. Artigos sobre a proteína SLPI em contextos diferentes do proposto não foram incluídos. Não houve restrição quanto à nacionalidade de trabalhos. Foi utilizada a ferramenta anti-plágio, Turnitin, para validação do texto.

3.2 Resultados

3.2.1 Manuscrito de revisão intitulado “SLPI: functions, applications, and production”.

SLPI: functions, applications, and production

REVIEW

Bárbara Luísa Chagas da Silva[1, 2]; Letícia Fudimori da Silva[1], Augusto Ducati Luchessi[1, 2]

[1] Laboratory of Biotechnology, School of Applied Sciences, University of Campinas (UNICAMP), Limeira, São Paulo, Brazil.

[2] Institute of Biosciences, São Paulo State University (UNESP), Rio Claro, São Paulo, Brazil

Abstract

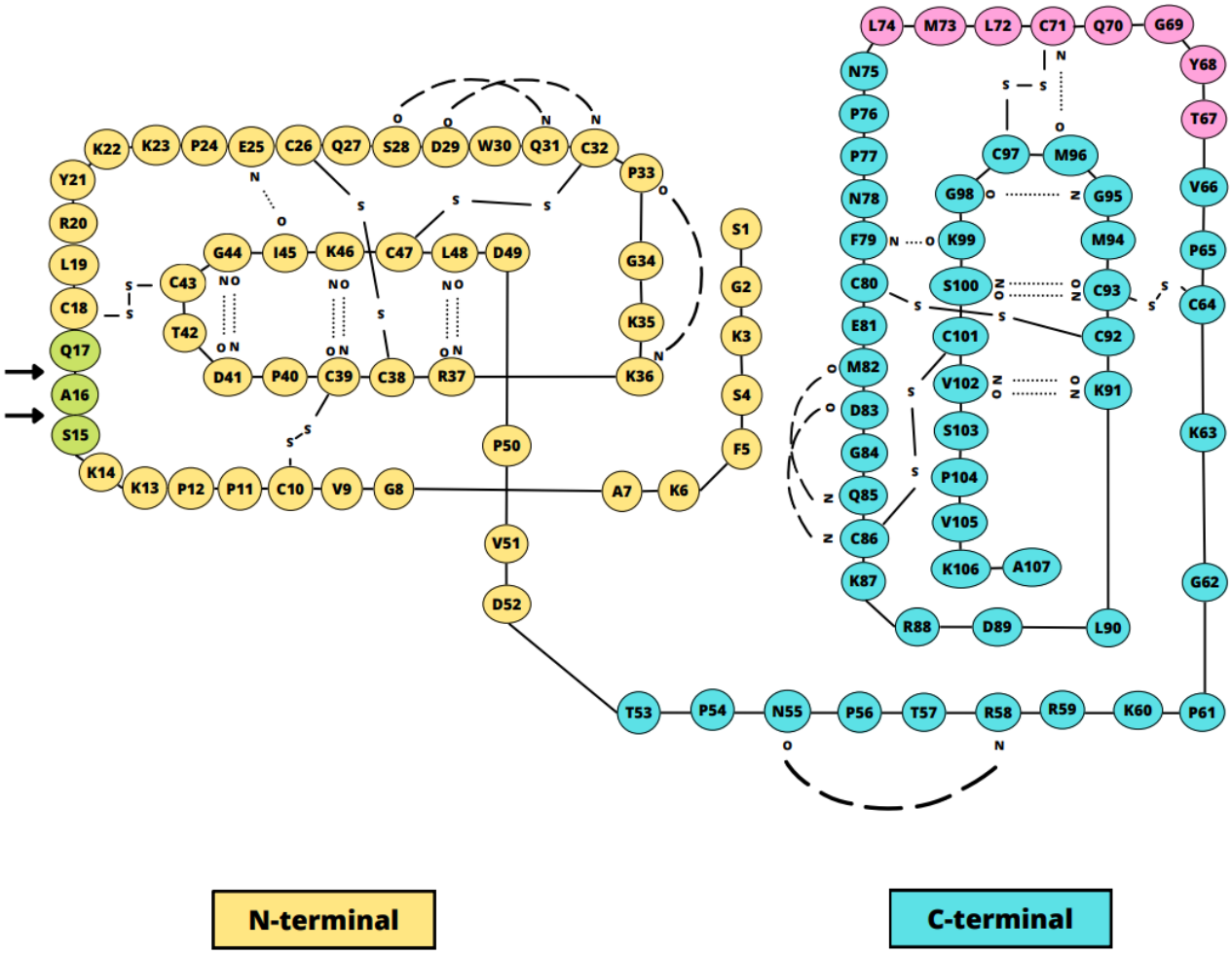
Since it was isolated and characterized, the different applications of the Secretory leukocyte protease inhibitor (SLPI) have been investigated, mainly its performance as a potential biopharmaceutical. Its antiprotease function is an essential ally for tissues during the inflammatory response, as it controls the action of proteases such as elastase, cathepsin G, chymase, and tryptase. In addition to acting on mechanisms of inflammation, SLPI also showed a role in wound healing, bone metabolism, cancer, and properties such as antimicrobial, antifungal, and antiviral activity. *In vitro* and *in vivo* studies show a therapeutic role of SLPI in lung diseases, neuron regeneration, and cardioprotective effect on ischemia/reperfusion. Given its panorama as a bioproduct, several groups and companies aim to develop systems for its heterologous production. In this sense, we analyzed studies of recombinant SLPI in *E. coli, P. pastoris, S. cerevisiae,* and Baculovirus, to finally unravel the potential of this production in microalgae.

Introduction

Secretory leukocyte protease inhibitor (SLPI) is a human protein associated with the immune system. It is a cationic protein with a molecular weight of 11.726 kDa. It has a polypeptide chain formed with 107 amino acids with an N-terminal transglutaminase substrate domain and a core of four C-terminal disulfides. The

terminal aspects of the protein inspired the name of the trappin gene family, and each of the terminals has distinct functions. The C-terminal nucleus is responsible for inhibiting serine proteases, while the N-terminal seems to stabilize or mediate the activation of the protease inhibitory function (YING; SIMON; KEMME, 1994), (SCHALKWIJK; WIEDOW; HIROSE, 1999), (STETLER; BREWER; THOMPSON, 1986).

The tertiary structure of SLPI has a boomerang form, with each arm carrying one domain. The homologous domains are denominated whey acidic protein (WAP) domains. The WAP domain is characterized by eight cysteine residues that form four intramolecular disulfide bonds. Koizumi et al. (2008) and Fukushima et al. (2013) have elucidated, in high resolution, the structure of the SLPI WAP II (C-terminal) domain complexed with human neutrophil elastase (NE) and porcine pancreas trypsin, respectively. The structures confirm early studies that described that the SLPI WAP II domain is responsible for the inhibitory function of SLPI, and the site of inhibition is localized between residues 67-74 (T67-L74) (Fig. 1) (KRAMPS et al., 1990), (EISENBERG et al., 1990b). Eisenberg et al. (1990) reveal that SLPI's inhibitory activity against serine proteases is mediated by Leu^{72} residue, located within the inhibitory loop. Other studies show that mutation at $Leu^{72,}$ Met^{73}, and Leu^{74} within the inhibitory loop, could cause SLPI to lose its protease-inhibitory function(ZABIEGLO et al., 2015).



The evolutionary history of SLPI/ (WAP) four-disulfide core family (WFDC) has already been tracked (HURLE; SWANSON; GREEN, 2007),(CLAUSS; LILJA; LUNDWALL, 2005). Those studies shows that most genes of the WFDC family are conserved in all species analyzed, but there are also species-specific gains and losses of genes (CLAUSS; LILJA; LUNDWALL, 2005). The conservation of different WFDC domains varies considerably, which lead to an uneven alignment of the SLPI protein even in species evolutionarily closed as it shows in figure 2.

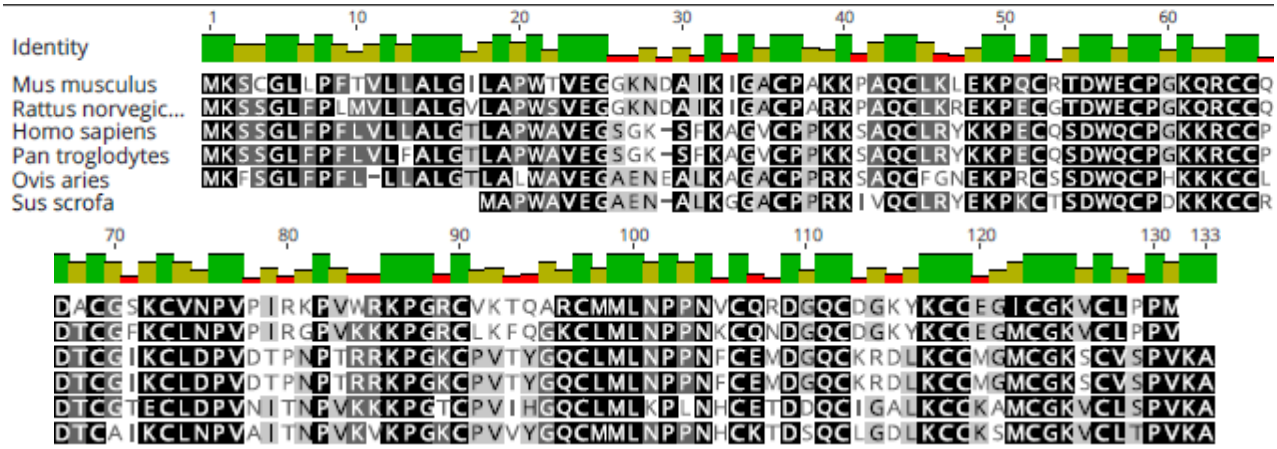
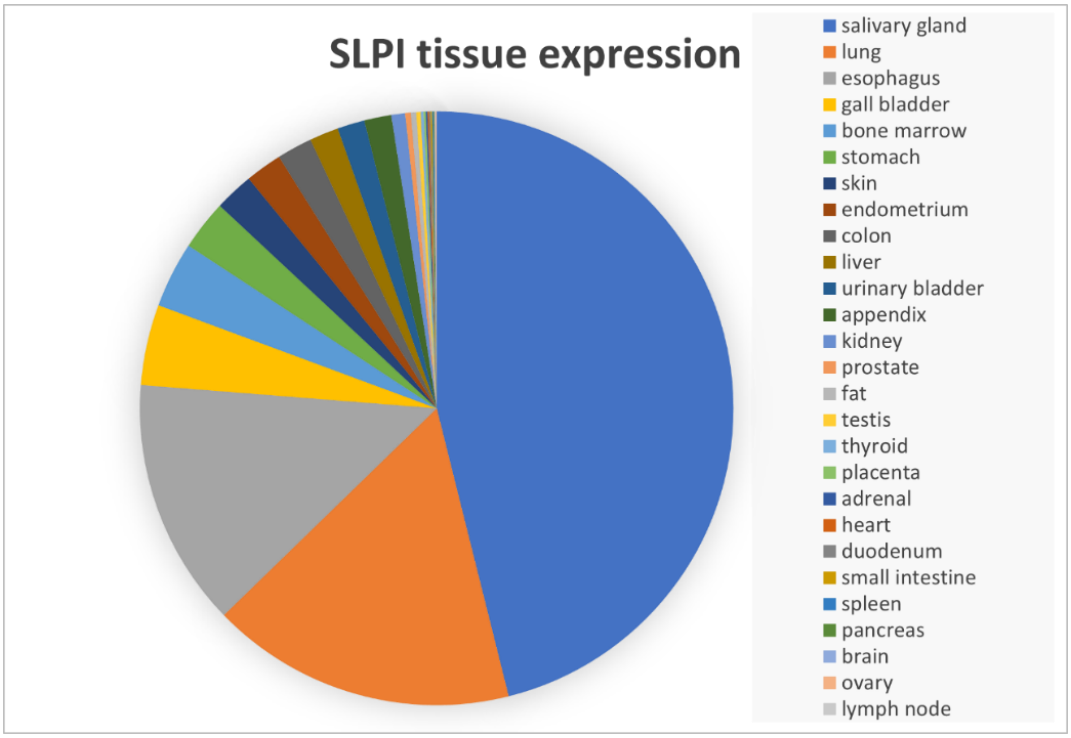


Figure 2. SLPI protein sequence alignment of 5 species: Mus musculus (mouse), Rattus norvegicus (rat), Homo sapiens (human), Pan troglodytes (chimpanzee), Ovis aries (sheep) and Sus scrofa (pig). The alignment was made using the Geneious Prime program, with the Clustal Omega alignment option. The species are ordered according to the sequence's similarities inferred by the program.

SLPI is present in several tissues (Fig.3); it is a widely expressed protein in the human epithelium, including mucous membranes, as well in the salivary glands, respiratory tract, and cells of the immune system, like neutrophils and macrophages (MARUYAMA et al., 1994), (SALLENAVE et al., 1997). SLPI was also found at high concentrations in bronchial secretions and cervical and intestinal mucus (THOMPSON; OHLSSON, 1986), (KRAMPS; FRANKEN; DIJKMAN, 1984), (WALLNER; FRITZ, 1974), (BERGENFELDT, 1996).



1. SLPI inhibition of serine proteases: mechanism of action

Proteases cleave protein substrates from the N- or C- terminus, or in the middle of the substrate, through the binding of the protease active site to the substrate residues flanking the cleavage site. The active site residues in the protease are composed of contiguous pockets termed subsites (SCHECHTER; BERGER, 1967). Each subsite pocket binds to a corresponding residue in the substrate sequence. Thus, amino acid residues in the substrate sequence are consecutively numbered outward from the cleavage sites as -P4-P3-P2-P1-P1'-P2'-P3'-P4'-..., where the scissile bond is located between the P1 and P1' positions. The subsites in the active site are correspondingly labelled as ...-S4-S3-S2-S1-S1'-S2'-S3'-S4' (“PROSPER: Protease substrate specificity webserver”, [s.d.])

Fukushima, K et al (2013) solved the crystal structure of 1/2SLPI with porcine pancreas trypsin (PPT) and compared the binding modes of two other complexes, already published (HNE: human neutrophil elastase, and chymotrypsin). They showed that 1/2SLPI strongly inhibits not only HNE and chymotrypsin, but also PPT and BPT (bovine pancreas trypsin). In all complex structures analyzed (1/2SLPI–PPT, 1/2SLPI–

HNE and SLPI–chymotrypsin), the Leu72 residue of 1/2SLPI was located in the S1 pocket as a P1 residue. A main part of the affinity and inhibitory activity of 1/2SLPI depends on the P1 residue which penetrates deeply into the S1 pocket of the proteases.

Fukushima, K et al (2013) showed that the intermolecular hydrogen-bonding patterns between main-chains of 1/2SLPI and PPT are very similar to the 1/2SLPI–HNE and SLPI–chymotrypsin complexes. The differences between the three complexes with 1/2SLPI are found in the interaction in the P5 Tyr68 residue, because de side-chain of this residue takes different conformations. 1/2SLPI changed the P5 Tyr68i conformation to accommodate each S5 pocket. Since the enzymes have not changed the structure of the S5 site by 1/2SLPI binding. This flexibility causes the inhibitory mechanism as 1/2SLPI can inhibit various serine proteases broadly. Possibly, 1/2SLPI controls the inhibitory activity by using moieties from P2′ to P5 and can be adapted to various proteases (FUKUSHIMA; KAMIMURA; TAKIMOTO-KAMIMURA, 2013)

1.1 Anti-inflammatory activity

The antiprotease function of SLPI was the first to be described and the most relevant in humans because it is intrinsically related to its role in the immune response. During an inflammatory landscape, leukocyte cells release serine proteases for containment, which end up destroying the inflamed organ tissue as well. SLPI acts against these serine proteases protecting the tissue from further injuries. The SLPI-inhibited proteases include elastase and cathepsin G from human neutrophils, chymase and tryptase. It has been documented that SLPI is the main inhibitor of elastase in the upper respiratory tract. In that regard SLPI acts as a protective agent and proves to be relevant in the control of inflammatory responses (STETLER; BREWER; THOMPSON, 1986)(WALTER; PLOTNICK; SCHECHTER, 1996), (SONG et al., 1999), (SALLENAVE et al., 1994), (SALLENAVE et al., 1997), (WRIGHT et al., 1999).

In addition to directly inhibiting serine proteases, SLPI can also act as a suppressor of signal transduction for synthesizing enzymes such as interstitial collagenase, also known as matrix metalloproteinases 1 (MMP1) and gelatinase B, also known as matrix metalloproteinases 9 (MMP9), a family protein responsible for

cellular matrix degradation (20). Furthermore, it has been described that SLPI can stop the activation of proenzymes, for example, by attenuating the conversion of plasminogen to plasmin at the cell surface. Plasmin generation at the cell surface is generally associated with the invasive ability of tumor cells, so its attenuation by SLPI may have a therapeutic potential (WEN et al., 2011)

Another important mechanism by which SLPI controls the inflammatory response is through NF-κβ inhibition. The inflammatory signaling pathway leads to a cascade of reactions that culminate in the release of transcription factors that regulates the production of pro-inflammatory cytokines. SLPI can inhibit the NF- κβ activation through different ways. It was shown that SLPI prevents LPS-induced degradation of IkBα in U937 monocytes cells, preventing the NF- κβ to enter the cell nucleus (TAGGART et al., 2002). The localization of NF- κβ to the nucleus is regulated by two main proteins: IkBα and IkBβ. In unstimulated cells, the NF- κβ dimers are sequestered in the cytoplasm by these two proteins. However, during the inflammatory response, the IkBα and IkBβ become phosphorylated, which leads to its degradation by proteasome trough ubiquitination (NELSON et al., 2004), (TAGGART et al., 2005). With the degradation of the regulatory proteins, the NF- κβ are free to translocate to the nucleus and regulate gene transcription. SLPI can also bind and block NF- κβ DNA binding sites (24). SLPI can enter the nucleus and bind to the promoter region of proinflammatory immune response genes (IL-8 and TNF-α) in the NF- κβ region. Furthermore, through its anti-inflammatory functions, SLPI downregulates TNF-α, one of the activating factors of NF- κβ signaling pathway (TANG et al., 2020).

The ability of SLPI to enter the cell nucleus is not fully elucidated. Miller et al (1989) first describe it in bacterial DNA. They pointed out that the toxicity caused by SLPI in *Escherichia coli* was due to SLPI binding to bacterial nucleic acids via charge interactions inhibiting protein translation. Taggart et al (2005) showed the SLPI ability to enter the U937 cell nucleus *in vitro* and bind to DNA in NF- κβ sites.(TAGGART et al., 2005). The exact mechanism of protein transduction into cell isn't clear, but it was hypothesized that it might be the arginine-rich nature of SLPI that permits it to interact with the negatively charged cell membrane and be internalized (MITCHELL et al., 2000). Ghasemlou et al (2010) showed *in vivo* that systemically delivered rSLPI translocate to the nucleus of leukocytes of mice treated with rSLPI after spinal cord injury. The treatment showed a significant improvement in locomotor recovery and reduction in secondary tissue damage (GHASEMLOU et al., 2010).

McElvaney et al. (1992) pointed out not only the effect of SLPI on the recruitment of neutrophils during inflammation but also the specific interaction of this protein and the decrease in the expression of pro-inflammatory cytokines, such as IL-8, interrupting the inflammatory cycle in human bronchial epithelial cells in cystic fibrosis (MCELVANEY et al., 1992). On the other hand, SLPI seems to positively regulate the expression of the anti-inflammatory cytokine IL-10 (SANO et al., 2000).

In addition to its role in the inflammatory response, SLPI also plays a vital role in wound healing. Ashcroft et al. (2000) demonstrated in *in vivo* studies the relevance of this protein in skin healing since its absence resulted in the delay of this process, together with the maintenance of the inflammatory response and elastase activity, through an increase in TGF-β and less matrix accumulation (ASHCROFT et al., 2000).

1.2 Respiratory tract

The role of SLPI in lung disease treatment has been extensively studied in the last decade, mainly in cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD), since SLPI is an anti-protease naturally produced in the lung and acts against exacerbating agents of these diseases.

SLPI is produced in the lungs by bronchial epithelial cells, alveolar macrophages, and neutrophils and is primarily responsible for anti-elastase activity in the respiratory tract. In airway epithelial cells, SLPI production is increased in the presence of neutrophil elastase and defensins, while the presence of TGF-β inhibits its production (NUGTEREN; SAMSOM, 2021).

In cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD), one of the causes of lung destruction is the imbalance in the proportion of proteases and protease inhibitors. When proteases lyse collagen and elastin, chemotactic peptides are released, resulting in the accumulation of neutrophils and increased production of proteases. SLPI limits neutrophil-induced lung destruction by inhibiting neutrophil elastase (NE) and attenuating IL-8 production in bronchial epithelial cells. In the CF bronchial surface, the NE released by neutrophils induces the bronchial epithelium to secrete IL-8, which in turn recruits additional neutrophils to the bronchial surface in a self-perpetuating process (NAKAMURA et al., 1992). However, as neutrophil elastase also inactivates SLPI, the protective role of SLPI is lost when high levels of neutrophil elastase are present, as in patients with COPD and CF. Furthermore, patients with CF

are commonly chronically infected with *Pseudomonas aeruginosa*, an elastase-producing bacterium. Although SLPI exerts antimicrobial activity against *Pseudomonas aeruginosa*, both the bacterium and neutrophil elastase can cleave and inactivate SLPI, further contributing to the natural imbalance of the disease (NUGTEREN; SAMSOM, 2021).

Aiming to develop drugs against lung diseases such as COPD and CF, the production of recombinant SLPI (rSLPI) is a promising target for the clinical-scientific sector. In *vitro,* SLPI can ameliorate the mucus hypersecretion characteristic of COPD and CF disease (GRIFFIN et al., 2007)**.** Furthermore, the use of rSLPI reduces disease severity in COPD model animals (MULLIGAN et al., 1993), (RUDOLPHUS et al., 1993), (FORTEZA et al., 2001).

Delivery of rSLPI directly into the lungs in an aerosolized form has already been tested *in vivo* in different studies (MCELVANEY et al., 1993), (WRIGHT et al., 1999), (MCELVANEY et al., 1992), (GIBBONS; MCELVANEY; CRYAN, 2010). Despite showing efficiency in reducing neutrophil elastase activity in airway epithelial lining fluid and reducing IL-8 mRNA expression in bronchial epithelial cells from patients with CF, aerosol deposition in the lungs requires not only appropriate size particles, but that sufficient amounts of the drug reach the lung area to be treated, including poorly ventilated areas. Treatment of patients with CF or emphysema with aerosolized recombinant SLPI was shown to be effective only in well-ventilated lung areas (ZANI et al., 2011), (STOLK et al., 1995).

Furthermore, studies have shown that aerosolized rSLPI does not accumulate on the respiratory surface after inhalation and instead migrates rapidly to the interstitium of the lung, making it necessary repeated dose every 12h (VOGELMEIER; GILLISSEN; BUHL, 1996), (MCELVANEY et al., 1993).

To circumvent the rapid inactivation of rSLPI by cathepsins and immediate release from the lungs, researchers developed liposome-encapsulated rSLPI. Encapsulated rSLPI within liposomes composed of 1,2-dioleoyl-sn-glycero-3-[phospho-L-serine]/cholesterol (DOPS/Chol), named DOPS–rSLPI, was shown to be protective against cathepsin L-induced SLPI inactivation and stable in storage for up to 5 months (GIBBONS; MCELVANEY; CRYAN, 2010). The dry powder DOPS–rSLPI was tested *in vitro* and *in vivo* in a guinea pig asthma model and showed a reduced clearance rate of rSLPI of the respiratory tract and increased lung residence time (GIBBONS et al., 2011). There are no recent publications of clinical trials using

encapsulated or aerosolized rSLPI formulations. The development of drugs to treat diseases via inhalation dates to 1990 and is still studied today. As it is a quick, easy, and painless method for patients, this route of administration is highly sought after by researchers. Still, the development of highly effective and durable inhaled drugs in the respiratory tract is challenging.

Furthermore, more stable variants of SLPI that are less susceptible to neutrophil elastase degradation and oxidation but still bind to bacterial lipopolysaccharide (LPS) and inhibit NF-κB signaling, a transcription factor that induces the expression of various pro-inflammatory genes, including those encoding cytokines and chemokines (LIU et al., 2017a) have been developed. Camper et al. (2016) showed that engineered variants of SLPI are more resistant to protease degradation while retaining antiprotease and anti-inflammatory properties similar to the SLPI-Wide-type protein. These SLPI variants may hold enhanced therapeutic potential. In particular, one of these variants may be of therapeutic benefit in reducing the possibly damaging inflammatory response to infection in the lung, as demonstrated by its greater efficiency in a murine model of pulmonary disease compared with the proteolytically sensitive SLPI-WideType protein (CAMPER et al., 2016).

1.3 Wound healing

Concerning wound healing, it has already been observed that SLPI participates in the various stages of this process: blood clotting, cleaning and sterilization of the wound by inflammatory cells, and growth and remodeling of new tissues.

Its participation in blood clotting was seen in SLPI-deficient mice, as they had longer blood clotting time. In cleaning and sterilizing wounds, rats deficient in SLPI subject to skin injury had delayed wound healing in addition to high elastase activity, inflammation, high TGF-β activity, and high TNF-α expression. However, this state was reversed with the application of exogenous SLPI (MAJCHRZAK-GORECKA et al., 2016), (ASHCROFT et al., 2000). Munadziroh et al. (2022) recently showed the rSLPI potential for skin injury healing. In the study, the administration of rSLPI in the injured area increased CD163 and FGF-2 expression dose-dependent while decreasing the IL-1 and IL-6 expression, accelerating the wound healing process. The cluster of differentiation 163 (CD163) is expressed on macrophages, precisely the M2 type, which produces anti-inflammatories cytokines. The expression of CD163 on

macrophages is a determinant for macrophage polarization to become dominant to the M1 type. The role of rSLPI in this process is that the rSLPI mechanism functions as an anti-inflammatory and inhibits the production of INF-γ induced by NF-κβ. rSLPI interacts with the receptor on the macrophage surface and inhibits the downstream activation of NF-κβ, preventing the secretion of pro-inflammatory cytokines like IL-1 and IL-6.

Fibroblast growth factor 2 (FGF-2) is also involved in wound healing. The macrophages M2 can produce precursors for fibroblast activation and collagen synthesis necessary for the wound healing process, such as FGF-2. Administration of rSLPI increased FGF-2 expression, leading to increased fibroblast proliferation and accelerating wound healing (MUNADZIROH et al., 2022).

Another function of SLPI in wounded tissues was observed in thymic stromal lymphopoietin (TLSP)-deficient mice with dextran sulfate sodium (DSS)-induced colitis. SLPI was profoundly reduced in the colon of TLSP-deficient mice, leading to excessive colonic neutrophil elastase activity, decreased mucosal healing, and death. However, the administration of recombinant SLPI in TLSP-deficient mice with DSS-induced colitis reduced the mortality rates, suggesting that SLPI reduces protease-induced injury in the damaged intestine (REARDON et al., 2011)**.**

Following its role in tissue regeneration, SLPI showed to act in neurological regeneration. Researchers showed that wild-type mice treated daily with recombinant SLPI in the first week after spinal cord injury exhibited long-lasting notable neuroprotective effects like improved locomotor control and reduced secondary tissue damage. In addition, improved sparing of spinal cord tissue, increased number of ventral horn neurons, decreased myelin loss, and extended serotonergic innervation was also observed. The rSLPI administered intraperitoneally was shown to localize in the nucleus of circulating leukocytes and was detected in the injured spinal cord. Moreover, rSLPI reduces activation of NF-κβ and expression of TNF-α, suggesting that treatment with recombinant SLPI could be an appropriate therapy after spinal cord injury (GHASEMLOU et al., 2010).

Another study shows that SLPI administered exogenously can overcome inhibition by Central Nervous System (CNS) myelin proteins like myelin-associated glycoprotein (MAG) and enhance regeneration of transected retinal ganglion cell axons in rats. Adult rats subjected to unilateral optic nerve crush were treated with human SLPI injections right after the injury had robust regeneration of retinal ganglion cell

axons in contrast to saline-treated rats that exhibited negligible axonal growth beyond the lesion site. This study also shows that exogenous SLPI localizes to the nuclei of neurons *in vitro* and *in vivo* and that internalization of SLPI is required for its ability to overcome myelin-associated glycoprotein (MAG) inhibition (HANNILA et al., 2013).

1.4 Ischemia/reperfusion

The role of SLPI in ischemia/reperfusion is widely investigated. In cardiac ischemia/reperfusion (I/R), which has an overproduction of protease enzymes secreted from infiltrated leukocytes, cardiomyocytes, and cardiac fibroblasts, cellular necrosis events are observed in and around the ischemic area to which SLPI was shown to be promising for the treatment or prevention of I/R injuries and post-ischemic inflammation. In cardiac transplantation, Schneeberger et al. (2008) showed that when recombinant SLPI was added to the preservation solution, the absence of myocardial contraction early after transplantation was reversed. In addition, the levels of rSLPI were inversely correlated with protease and TGF-β levels, showing a dual inhibitory effect (SCHNEEBERGER et al., 2008).

Several other researchers have shown the cardioprotective effect of rSLPI in ischemia/reperfusion (PAIYABHROMA; NERNPERMPISOOTH; KUMPHUNE, 2018), (KONGPOL et al., 2019), (PROMPUNT et al., 2018b), (PROMPUNT et al., 2018c). Mongkolpathumrat et al. (2021) showed the post-ischemic treatment with SLPI in an in vivo I/R model, where recombinant human (rh) SLPI (rhSLPI) was systemically injected during coronary artery occlusion or at the onset of reperfusion. The results show that post-ischemic treatment with rhSLPI could significantly reduce infarct size, Lactate Dehydrogenase (LDH) and Creatine kinase-MB (CK-MB) activity, inflammatory cytokines, and protein carbonyl levels and improve cardiac function. The cardioprotective effect of rhSLPI is associated with the attenuation of p38 MAPK phosphorylation, Bax, caspase-3 and -8 protein levels, and enhancement of pro-survival kinase Akt and ERK1/2 phosphorylation (MONGKOLPATHUMRAT et al., 2021). In the most recent study of this group, the researchers showed that the role of SLPI in ischemia/reperfusion is not just associated with its role as an anti-protease. The study shows that a mutated form of SLPI, unable to exert its anti-protease function, could reduce cardiac cell injury in an *in vitro* hypoxia/reoxygenation. The mutated protein could also reduce infarct size and improve the cardiac function of rats subjected

to *in vivo* myocardial I/R injuries, showing that the anti-protease activity of SLPI may not be essential for cardioprotection since the mutated form had a similar effect. The anti-protease deficient recombinant human SLPI could, then, be safer in clinical practice for not interfering with basal serine protease activity since it has been reported that proteases inhibitors may cause several side effects such as hyperglycemia, hemolytic anemia, spontaneous bleeding in hemophiliac patients and metabolic side effects on cholesterol and triglycerides (MONGKOLPATHUMRAT et al., 2022), (HUFF, 1997), (L, 1997).

2. Bone metabolism

Recently, the functional role of SLPI in bone metabolism was described. SLPI is highly expressed by parathyroid hormone (PTH) induction in osteoblasts. It is vital for the osteoanabolic action of PTH since it can promote a PTH-mediated switch from bone resorption to bone formation. SLPI acts within osteoblasts and enhances bone formation by controlling gene expression. Also, it increases the cell-cell communication between osteoblasts and osteoclasts, enhancing the adhesive force to neighboring osteoblasts and suppressing osteoclastic function (MORIMOTO et al., 2021).

Choi et al. (2016) first reported the function of SLPI in osteoblast differentiation and mineralization on a titanium (Ti) surface *in vitro*. The study shows that SLPI increases preosteoblasts differentiation during osteoblastogenesis and stimulates mineralization on a Ti surface. One of the biggest challenges in the fixation of implants is the osseointegration between osteoblasts and a Ti surface. Hence, the activity of SLPI during differentiation and mineralization of pre-osteoblasts can reduce osseointegration failure on a Ti surface and therefore increase the success of implantation. It is now necessary to carry out an *in vivo* study to show how this finding would work in a living tissue model and then take it to a clinical trial (CHOI et al., 2016).

3 Antibacterial, antifungal, and antiviral activity

In addition, studies show more activities of this protein, such as antibacterial, antiviral, and antifungal action. The activity against bacteria was recognized in the 1980s, and a few years later, Hiemstra et al. (1996) demonstrated this activity in *Escherichia coli* and *Staphylococcus aureus* (HIEMSTRA et al., 1996). Wiedow et al. (1998) tested antibacterial activity on *P. aeruginosa*, *S. aureus*, and *S. epidermidis*,

microorganisms widely present on human skin, and it was reported that SLPI produced a constant antimicrobial barrier. This activity appears to be dose-dependent and mainly related to the N-terminus of the protein (MILLER et al., 1989), (WIEDOW et al., 1998).

The antimicrobial activity of SLPI has been attributed mainly to the N – terminal domain of the protein. When isolated, the C – terminal domain presents very low antibacterial activity in comparison to the N – terminal domain, which conferred to the N-terminus the antibacterial activity of the protein. However, the intact SLPI exhibited better antibacterial properties than its domains alone, implying that only the whole molecule confers the structural machinery necessary for the full activity of SLPI. (HIEMSTRA et al., 1996), (TOMEE et al., 1997). The mechanism of antimicrobial activity of SLPI has not been properly elucidated yet, but these properties have been proposed to be related to the cationic nature of its WAP domains. The N – terminal domain has a higher cationic charge (+7) than the C – terminal domain (+5), which could drive initial interactions between SLPI and anionic bacterial lipids, leading to destabilization of microbial membranes (SCOTT; WELDON; TAGGART, 2011). The possible involvement of the cationic properties in the SLPI activity is supported by observations that high salt concentrations significantly reduce SLPI's antimicrobial capacity (TOMEE et al., 1997).

Other possible mechanism of antimicrobial actions has been reported. Studies indicated that SLPI can directly bind to bacteria. Cooper et al (2012) demonstrated that SLPI is bactericidal to the gonococci through direct binding to the gonococcal – Opa (outer membrane opacity) protein. The direct interaction between SLPI and Opa proteins is required for bacterial attenuation since Opa – negative gonococci were resistant to the bactericidal effect of SLPI (67). SLPI was also reported to bind to bacterial nucleic acids via charge interactions and inhibit translation, when over-expressed in SGE61 *E. coli* strain, causing bacterial toxicity (63).

Antiviral activity has been observed and investigated in studies with human saliva and human immunodeficiency virus type 1 (HIV-1). SLPI inhibited the infection of monocytes by HIV-1; the protein interaction occurs during transmission in the host cell and not with viral proteins. Thus, SLPI could interrupt the beginning of the virus-cell cycle through a mechanism independent of its antiprotease activity (68). SLPI binds to human macrophages via annexin II, a membrane phospholipid-binding protein and cellular fusogenic factor for HIV-1. Therefore, SLPI disrupts the interaction of the human-derived phosphatidylserine in the HIV-1 envelope with annexin II in

macrophages (69). In addition, SLPI interacts with scramblase 1 and 4 (PLSCR1 and PLSCR4), inhibiting its interaction with CD4, the main T – lymphocyte receptor for HIV – 1 entry. SLPI compete with CD4 for the same binding region on PLSCR1/4. However, the mechanism in which the PLSCR1/4/CD4/SLPI axis regulates HIV – 1 infection still to be fully elucidated (70).

The HIV – 1 inhibition by SLPI was also evaluated in the vaginal mucosa to assess the perinatal transmission of HIV-1; in this way, women who had their babies infected had vaginal fluid samples that contained a lower concentration of SLPI than women who had their babies uninfected (71). SLPI activity against the Human papillomaviruses (HPV) was also reported. Woodham et al (2012) showed that SLPI incubation with epithelial cells reduces HPV entry into these cells through binding to the Annexin A2, which is associated with HPV infection of epithelial cells (72), (73).

Antifungal activity was also assessed against the pathogens *Aspergillus fumigatus* and *Candida albicans*; the action against fungi was compared to the human defense system and lysozyme in a dose-dependent manner, and *in vitro* studies suggest the relevance of this activity *in vivo*, mainly in the lung. In *Candida albicans*, it was shown that SLPI can modulate different virulence factors of Candia albicans like proteolytic activity, hydrophobicity, adhesion to the epithelium, and mannoprotein expression (74). The molecular mechanism was predominantly associated with the N-terminus of the protein, and the possible explanation would be its characteristics, such as greater cationicity and the presence of positively charged amino acids (65) (Table 1).

Table 1 *Antimicrobial activity of SLPI*

Activity	Microorganism	Reference
Antibacterial	*P. aeruginosa*	(Wiedow, et al 1998)
	S. aureus	(Hiemstra et al 1996); (Wiedow, et al 1998)
	S. epidermidis	(Wiedow et al 1998)
	E. coli	(Hiemstra et al 1996); (Wiedow, et al 1998)
Antiviral	Human Immunodeficiency Virus Type 1 (HIV-1)	(Pillay et al. 2001); (McNeely et al. 1997).
	Human papillomaviruses (HPV)	(Woodham et al, 2012), (Hoffmann et al, 2013)
Antifungal	*Aspergillus fumigatus*	(Tomee et al 1997)
	Candida albicans	(Tomee et al. 1997)

4 SLPI role in cancer

SLPI is also related to cancer and has been extensively studied because of its contradictory roles in different types of cancer. Although proteases are necessary for tumor expansion and invasion, and SLPI acts to combat these proteins, studies demonstrate overexpression of serine protease inhibitors in several types of cancer, contributing to tumor growth and metastasis. Still, SLPI also shows some functions that may be protective against cancer (75). The controversial role of SLPI in cancer promotion and protection is evident in breast, ovarian, lung, and head and neck squamous cell carcinoma (HNSCC) cancers, where SLPI is shown to act in both ways. In all those cancers mentioned above, SLPI was shown to either induce apoptosis of cancer cells, inhibit tumor growth and invasion, or inhibit cell migration, but also promote metastasis, cell proliferation and migration, and tumor growth (76), (77), (78), (79), (80), (81), (82), (83), (84), (85). In gastric, colorectal, pancreatic, prostate, and endometrial cancers, SLPI has been demonstrated to act only in the promotion of tumor growth and metastasis until now; its role in the prevention of those cancers is unknown (54), (86), (87), (88), (89), (84). Recently, Nugteren et al. (2022) suggested that the role of SLPI in colorectal cancer (CRC) is different depending on the disease stage. In stage III CRC, SLPI is unfavorable for tumors after administering adjuvant

chemotherapy, while in the distant metastatic stage, SLPI expression may be beneficial. In both scenarios, SLPI may be helpful due to the formation of vessel-like structures which provide a blood supply to hypoxic regions of the tumor and through the anticoagulant action. In the first scenario, it can make it more accessible to chemotherapy. In the second, it can help the cancer cells to proliferate (90).

5 Production

Since it was first isolated and characterized in 1986, researchers have produced SLPI in different expression systems aiming for a system that delivers high protein yields for clinical applications.

5.1 *Escherichia coli*

E. coli is the most popular expression system for heterologous protein production. Since it was characterized, SLPI has been widely produced. Meckelein et al. (1990) directly cloned the C-terminal domain of SLPI in *E. coli* using the signal sequence of alkaline phosphatase. Still, the signal peptide processing in their system was incorrect. They achieved 0.5 – 1.0 mg/l bacterial culture (91). Masuda et al. (1996) also expressed SLPI in *E. coli* using a fusion protein expression system. They reported that the system provides highly purified protein, achieving a 50 mg/l culture (92). In 2017 Munadziroh et al. produced a soluble SLPI in the presence of its native signal peptide. The expression was achieved upon induction at ITPG concentrations of 50 µM or less. The presence of its native signal peptide may have slightly altered the protein's secondary structure. Still, it did not affect its biological activity (93).

Despite being an easy system expression, the production in *E. coli* has some problems. Since it started to be produced, researchers noticed that the SLPI produced in *E. coli* was not always functional. The protein is accumulated intracellularly in an inactive form because the disulfide bonds are not formed correctly, and after cell disruption, the protein is only partially soluble. Because of that, it is necessary a renaturation step for the protein to be perfectly folded and fully functional (94), (95). It was also reported that SLPI binds to mRNA and DNA in *E. coli* via charge interactions, which may cause cell toxicity (63).

Nowadays, human SLPI produced in *E. coli* is sold by several companies, and despite being an easy and high-yield production system, the complications related to it contribute to its high costs (355,50 dollars per 100 µg, by R&D Systems).

5.2 *Pichia pastoris*

Pichia pastoris is a methylotrophic yeast widely used for protein expression using recombinant DNA techniques. To overcome the problems in *E. coli* production, Zhiguo et al. (2009) constructed a *Pichia* expression system to produce recombinant SLPI. The construction consisted of a mature SLPI, lacking its native signal peptide in frame with the N-terminal *Saccharomyces cerevisiae* Matalpha prepro peptide leader and a C-terminal region encoding the c-myc epitope and polyhistidine tag. The coding sequence was controlled by the AOX1 promoter, induced under methanol growth conditions. The recombinant protein was directed to the extracellular medium for easy purification, which was carried out by affinity techniques because of the polyhistidine tag added in the C-terminal domain. The highest level of SLPI expression obtained was 3.8 mg/l when cultivated at 30°C in a growth medium containing 1% methanol. After a scale-up of 10-liter fed-batch fermentation, the group got a yield of approximately 26 mg/l of the SLPI fusion protein (SLPI-myc-his6). The biological activity was tested against trypsin and compared with the bacterial-derived SLPI. The results showed that *P. pastoris*-produced protein could perform its function (96).

The same group tried to increase the expression of SLPI in *Pichia pastoris* by overexpression of PDI protein. Protein disulfide isomerase (PDI) is a multifunctional protein involved in the folding, assembly, and post-translational modification of proteins in eukaryotes; it also increases the rate of oxidation and disulfide bond formation (97). The group created a *Pichia* strain containing vectors with both SLPI and PDI genes under the control of the AOX1 promoter. The overexpression of PDI increased secretion levels over eightfold compared with strains harboring normal levels of PDI (endogenous PDI) and secreted approximately eight times SLPI (~1,70 mg/l). The overexpressed-PDI strain also increased the inhibitory SLPI power compared to normal-levels-PDI strains, probably because of the complete disulfide formation and better folding in SLPI peptides from PDI overexpression (98).

5.3 *Saccharomyces cerevisiae*

Based on what has been exposed about the importance and the various applications of SLPI, some studies have proposed to produce this protein in organisms capable of synthesizing this byproduct. The production of heterologous SLPI in Saccharomyces cerevisiae yeast was already documented in 1989; Stetler et al. (1989) transformed with SLPI using two systems from yeast proteins. The invertase system resulted in the production of both processed and active and inactive SLPI; however, both forms were trapped in the cell, and treatment with other reagents was necessary to remove the active form of the protein. The a-factor system did not allow the expression of complete and processed SLPI. Still, it was possible to express and secrete a truncated form of SLPI (CLPI) capable of acting as an elastase inhibitor, although it may have other altered properties. More recently, Ulfa et al. (2018) cloned and transformed SLPI into S. cerevisiae, identified by western blotting, using another signal peptide, HM1, but failed to make the yeast able to secrete the protein (99), (100).

5.4 Baculovirus

Another production system tested was recombinant baculovirus infection in Sf21 insect cells. Human SLPI was fused with an N-terminal polyhistidine tag and expressed in the cells but not secreted. Thus, it was necessary to lyse the cells and perform a digestion process to remove the 6×His-tag. Regarding the activity of the protein, its anti-protease role was observed with chymotrypsin and cathepsin G. Finally, the SLPI yield by this system reached 27.3 µg/10 mL of cell lysate, 7.6 µg/1.7 mL from the first round of purification and 1.9 µg/1.7mL from the second round of purification (101).

Nowadays, recombinant SLPI is sold by several companies using different expression systems (Table 2). The most used system is the protein produced in *E. coli*, sold by four companies (R&D Systems, Abcam, Bosterbio, and AMSBIO). Two companies sell the protein produced in insect cells (SinoBiological and Elabscience), and one company uses de cell-free expression system wheat germ (Abnova).

Table 2 *SLPI production by several companies and different expression systems*

Expression system	Companies	Price per 50 µg
E. coli	R&D Systems	$177,75
	Abcam	$385,00
	Bosterbio	$549,00
	AMSBIO	$120,00
Wheat germ	Abnova	$1.675,00
Insect Cells (baculovirus)	SinoBiological	-
	Elabscience	$402,50

6. Others expression systems

Besides the expression system platforms mentioned above, there are others widely used for therapeutic recombinant protein production like mammalian cells, transgenic animals, plants, and microalgae.

6.1 Mammalian cells

Among all the expression systems, mammalian cells are the most used to produce recombinant protein-based biopharmaceuticals (102). Mammalian cells are the choice to produce complex products because of the ability to synthesize proteins that are similar to those naturally occurring in human. The most used mammalian cells are Chinese hamster ovary cells (CHO), mouse myeloma cells (NS0), and Baby Hamster Kidney cells (BHK). The mammalian production system has numerous advantages like posttranslational modifications (PTMs), high yields, low infectibility by human pathogenic virus, and existing regulatory approval. But there are some disadvantages too, like complex growth requirements, long production timescale, difficult to scale-up and high product cost (103). Despite the safety and efficacy of recombinant protein expression in murine cells, their glycosylation pattern is different from human-type glycosylation, because they do not have the machinery required. These differences in glycosylation pattern may be immunogenic in humans. Because

of that, scientists are using human cells lines (HEK293, HKB11, PER.C6, HeLa, and CAP cells) to produce biopharmaceuticals. A human cell line enhances the expression of proteins with human-like PTMs. These cells have shown efficient production in laboratory scale, but the cultivation on a commercial scale is still in development (104).

6.2 Transgenic animals

The use of whole animal as a host for recombinant protein production is also promising, attractive and is already being used. The advantages combine the low cost of production, high productivity, and quality of the proteins. They can produce extremely complex proteins in high quantity and quality. Moreover, it is possible to produce multiple biopharmaceuticals or vaccines in a single animal. Nowadays, two main ways of sourcing proteins are in use: milk from transgenic mammals (such as sheep, goats, cows, rabbits, or pigs) and eggs from transgenic chickens. But there are some alternative systems such blood, urine, and seminal plasma (105). Atryn® (rEVO Biologics Inc.), a recombinant antithrombin produced in the milk of goats, was the first recombinant protein biopharmaceutical produced in transgenic animal. It was approved in 2006 by European Medicines Evaluation Agency (EMA) and in 2009 by Food and Drug Administration (FDA) (106). However, the production of transgenic animals faces some ethical issues.

6.3 Plants

Besides the traditional expression systems based on microorganisms, several groups have tested land plants for recombinant proteins productions (107).

Using plants to produce recombinant pharmaceutical proteins, named as Plant molecular Farming (PMF), is not a new concept. In 1986 Human growth hormone, initially produced in tobacco and sunflower, was the first plant-derived recombinant therapeutic protein developed (108). In 2012 the FDA approved the human use of ELELYSO™ (taliglucerase alfa) (Protalix BioTherapeutics, Karmiel, Israel), a PMF-derived enzyme used in an enzyme replacement therapy to treat adult patients with Gaucher disease (First plant-made biologic approved). Since 1994, more than 100 pharmaceutical proteins have been expressed and characterized in plants (109). A recent survey has listed fewer than 40 plant-derived recombinant products and their clinical status (110).

The most used plant species for recombinant protein production are Tobacco, mainly for its rapid scale up potencial; Cereal seeds, for its dry intercellular environment and protein storage vesicles. Maize (111), rice (112) and wheat are the most used. Wheat is also used for recombinant protein production as a cell-free system, named wheat germ cell-free system (WGCFS). WGCFS can express large and complex proteins in their near native forms and is suitable for the expression of both prokaryotic and eukaryotic proteins. Furthermore, it commonly doesn't require codon optimization. This system has been widely used nowadays to overcome the obstacles of cell-living systems that leads to low yields of proteins (113). It also is an expression system used to produce recombinant SLPI by Abnova™. The main disadvantage of WGCFS is it costs, it is a very expensive system, and difficult to scale up.

Recently, Buntru et al (2014) developed a new cell-free expression system platform based on tobacco BY-2 cells (114). The tobacco BY-2 cells are prepared in batches, which provide cell material for lysate preparation. The lysate preparation consists in the removal of the cell wall and other cell components unnecessary for protein production, leaving only the protein production machinery and mitochondria. The BY-2 lysates (BYL) are then used to express proteins. The researchers reported a yield of 3 mg/ml after lysate preparation and reaction optimization (115). The BYL system is now commercialized by the germany company LenioBio and is marketed under the brand name ALiCE® (116), (117).

Legumes has also been a target for recombinant protein production, because of its advantage of removing the nitrogen requirement in their fertilizer, reducing the cultivation cost. Production in soybean (118), and alfalfa (119) has already been documented. Another category is fruits and vegetables crops. This system advantage includes orally delivery of the protein with minimal processing. Production in lettuce (120), tomato (121) and potato (122) has already been reported (103). Recently, Angiotensin Converting Enzyme 2 (ACE2) expressed in lettuce chloroplasts for delivery via chewing gum to prevent infection and transmission of severe acute respiratory syndrome coronavirus (SARSCoV-2) was approved by the FDA for a Phase I/II clinical trial (IND154897, NCT05433181, IRB 851459) (123), and the researchers are studying optimization of biomass and target protein yield for Phase III clinical trial (124).

Plants and plant cells have some advantages over bacterial and mammalian platforms for production of recombinant proteins including therapeutic proteins. These advantages include the low cost, middle speed of recombinant protein production at a large scale, well-understood manufacturing practices, ability to synthesize complex proteins, low risk of human pathogen contamination, and others.

The main advantage of land plants is the size of the plantation, you can seed hectares of corn, for example, but the system also face some challenges: difficulties in controlling the transgene expression level (it may vary in plant organs, plant tissues, or subsequent generations), differences in protein glycosylation in term of N-glycan composition between plants and animals that can affect the distribution, stability, activity and immunogenicity of therapeutic proteins (125). Furthermore, the purification stage is more complex since secondary metabolites or pesticides must be removed (105).

Despite being very promising, the plant-based expression system still has some issues to overcome, like de yield, purification, regulatory approval, and biosafety concerns.

6.4 Microalgae

Microalgae have been used as a model organism to comprehend genic expression mechanisms regulated by light and nutrients, structural and functional characterization of photosynthetic complexes, and other cellular mechanisms (126). But only in the past years microalgae have been in the spotlight as a recombinant protein expression system. The production of recombinant protein in microalgae has innumerous advantages, they can be grown in contained bioreactors, they commonly double their biomass within 24 hours, and they can progress from initial transformation to large-scale protein production in only a few weeks. They are also single-cell types, leading to less protein accumulation variation and easier downstream processing (127). In addition, as eukaryotes, algae possess the chaperones and cellular machinery required to fold complex proteins that bacteria and yeasts cannot process properly (128).

The most used model algae are *Chlamydomonas reinhardtii*. All its three genomes have already been sequenced (chloroplast, nucleus, and mitochondria), and the tools for genetic manipulation and protein super expression in the *C. reinhardtii*

chloroplast are well established (129), (130). Several compounds were successfully produced in *Chlamydomonas reinhardtii,* including complex mammalian proteins, vaccines, antibodies, and others (131), (132), (133), (134), (135).

Recombinant protein production in *C. reinhardtii* is primarily done in the chloroplast due to its more straightforward genetics and the size of this organelle (up to 70% of the cell's volume). Several recombinant proteins have been expressed in the chloroplast. Usually, this method reaches yields ranging from 0.5 to 5% of total soluble protein (134), (136). The disadvantage of recombinant protein production in chloroplasts is that some post-translational modifications are not added to the proteins, like glycosylation, and proteins expressed in chloroplasts cannot be addressed to different compartments in the cell (137). Unlike the chloroplast, recombinant protein in the nucleus can be addressed to subcellular localizations and thus receive post-translational modifications (138). But the yield achieved in nuclear transformation is much lower than in chloroplast transformation, corresponding to 0.5% of total soluble protein. Another difficulty in successful nucleus transformation and expression is that the nuclear genome codon usage of *C. reinhardtii* is very biased, and the frequency of some codons is so low that they are not used, making the expression of transformants difficult and unstable. Thus, codon usage optimization for nucleus transformation is indispensable (139).

The obstacles to microalgae expression systems are the limited availability of molecular tools for genetic engineering, generally low expression levels of recombinant proteins, and the absence of standard procedures for molecular transformation. But several researchers are already developing technologies and strategies to circumvent these problems. For example, some molecular toolkits have been developed recently (pChlamy4, pJR, and pOptimized) (140), (141), (138), (142). Among them, a cooperation of several laboratories developed a molecular cloning toolkit based on golden gate cloning (143). It contains promoters, UTRs, terminators, tags, reporters, antibiotic resistance genes, and introns cloned in various positions (144). This work will significantly accelerate the biotechnology research of *C. reinhardtii*.

As for the improvement of recombinant protein production in microalgae, YasinTorres-Tiji et al. (2022) increased the production of the recombinant protein ICAM-1 in *C. reinhardtii* by manipulation of the culture medium. They examined the nutrient imbalance in the media over time to identify limiting factors in biomass accumulation under fed-batch growth. By supplying extra sulfur, calcium, iron, and

yeast extract to the medium, the biomass increased in wild-type microalgae, and the production of recombinant protein increased in mutant algae. The group achieved a titer of 47 mg/l of active protein (137). Another group, Neupert et al., reported the creation of UV-mutated *C. reinhardtii* strains, where the expression of GFP was significantly enhanced (142).

Conclusions

Secretory leukocyte protease inhibitor (SLPI) has innumerous applications in the medical field, as explained here. Since it was first characterized in 1986, researchers have been trying to understand how this protein works and its hole in the human body. SLPI's multifaceted holes in the immune system, respiratory diseases, viral and bacterial infections have aroused the interest of scientists for its potential as a pharmaceutical product. Thus, innumerous works have been and are still being done to produce this protein in a productive, profitable, and low-cost way. Nowadays, SLPI production is done by several companies, mainly using *E. coli* as a production and expression system, but for research purposes only. New production platforms are emerging, like transgenic animals, plants, and microalgae. They are a promising biotechnological platform for recombinant protein production. It has innumerous advantages over the systems used today, and technologies are being developed to overcome the obstacles that make heterologous production difficult.

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4 CAPÍTULO 2: PRODUÇÃO E PURIFICAÇÃO DA PROTEÍNA RECOMBINANTE HUMANA SLPI A PARTIR DA MICROALGA *CHLAMYDOMONAS REINHARDTII*

4.1 Metodologia

4.1.1 Construção dos plasmídeos e transformação da microalga *C. reinhardtii*

As etapas de construção dos plasmídeos, transformação e obtenção dos clones positivos foram realizadas pelo professor Dr. Augusto Ducati Luchessi no laboratório do Dr. Steve Mayfield na Universidade da California, San Diego, EUA.

Para transformação da microalga *Chlamydomonas reihardtii*, foram realizadas duas construções plasmidiais – pAJB3 – SLPI e pAJB3 – SLPI – FLAG (Figura. 1). O plasmídeo pAJB3 foi obtido do laboratório do Dr. Steve Mayfield da Universidade da Califórnia, San Diego, USA. O plasmídeo pAJB3 contém os genes para resistência à ampicilina AmpR e origem de replicação bacteriana. Para a expressão do gene codificador da proteína SLPI e seleção das microalgas tranformadas, foram sintetizados dois cassetes para introdoção no plasmídeo.

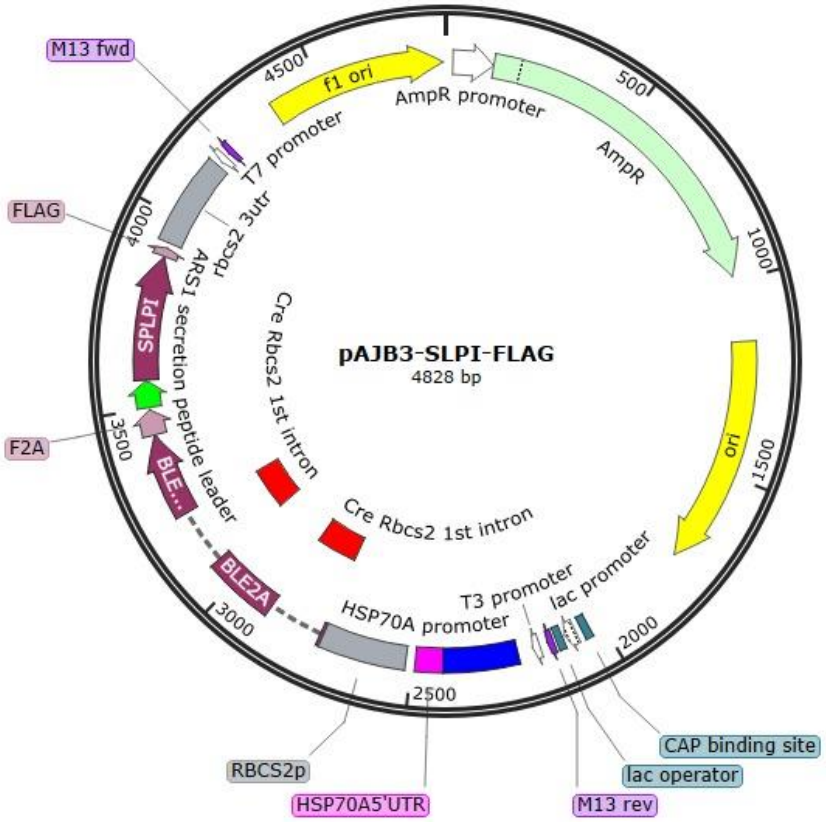
O cassete do plasmídeo pAJB3 – SLPI - FLAG foi sintetizado pela Integrated DNA Technologies – IDT e inserido no plasmídeo de forma que no sentido 5’-3’ têm-se: um gene de resistência antizeocina para microalga BLE2A intercalado pelo primeiro íntron do gene RBCS2 para melhor expressão do cassete; um peptídeo sinal de secreção da própria *C. Reinhardtii* ARS1; um sítio de auto-clivagem F2A; a região codificadora da proteína SLPI humana otimizada para *C. reinhardtii* (“codon usage”), sem o peptídeo sinal de origem, fusionada ao peptídeo FLAG (DYKDDDDK). O cassete também apresenta a sequência não tranduzida 3’ UTR e promotor do gene RBCS2, além do promotor HSP70A. Segundo estudos, tais sequências melhoram a expressão de gene heterólogos no nucleo da *Chlamydomonas reinhardtii* (BAIER et al., 2018; LÓPEZ-PAZ et al., 2017; LUMBRERAS; STEVENS; PURTON, 1998).

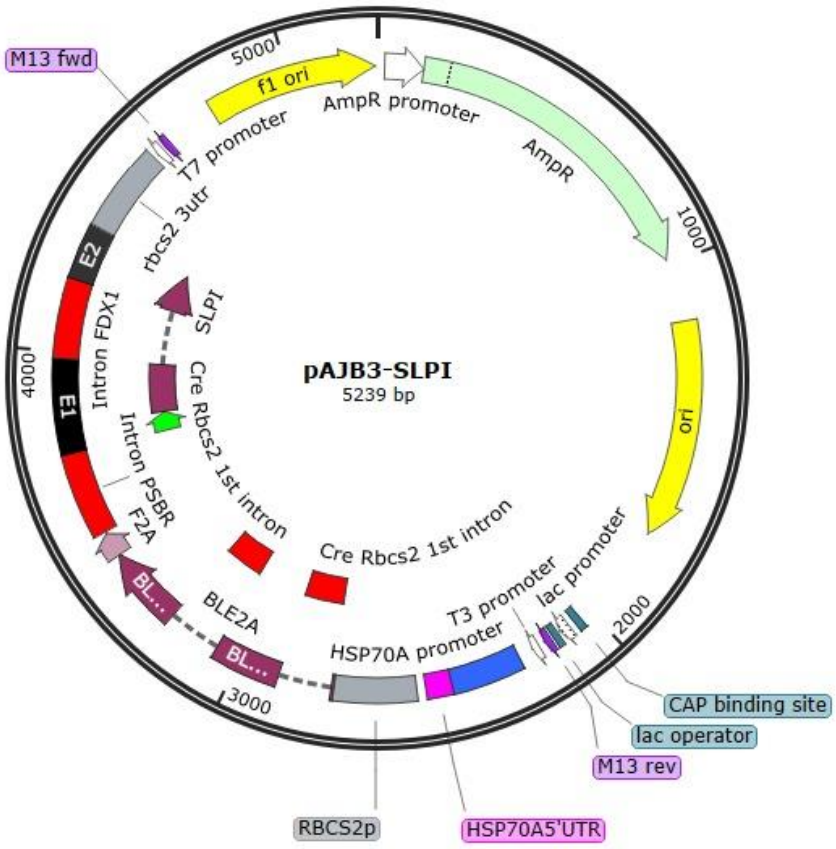
O segundo cassete, para o plasmídeo pAJB3 – SLPI, também apresenta a sequência não tranduzida 3’ UTR e promotor do gene RBCS2, juntamente do promotor HSP70A. Porém, diferentemente do primeiro cassete, o segundo não apresenta o peptídeo FLAG, e contém sequências intrônicas adicionais dos genes FDX1 e PSBR que intercalam os exons do gene SLPI (LÓPEZ-PAZ et al., 2017).

A proteína SLPI possui o próprio peptídeo sinal. Após a sintese, a proteína se encontra na sua forma imatura, apenas após a clivagem do peptídeo sinal que direciona a proteína para o complexo de golgi, é que a proteína se torna madura. Porém, para a expressção em microalga, além da otimização do códon para expressão em C. reinhardtii, foi utilizado um peptídeo sinal de secreção da própria *C. reinhardtii*, ARS1, para garantia da secreção da proteína para o meio extracelular. O sítio de auto-clivagem F2A é um peptídeo de origem viral - *foot-and-mouth disease virus (FMDV)* – que causa a clivagem de longas sequências. A clivagem ocorre através de um mecanismo conhecido como “salto ribossomico” em que o ribossomo salta a formação de uma ligação peptídica levando à clivagem da sequência (LIU et al., 2017). Dessa forma, o peptídeo F2A, libera a proteína rhSLPI das outras sequências fusionadas. O peptídeo FLAG foi adicionado a fim de gerar clones que produzissem a proteína fusionada ao peptídeo, como uma maneira opcional de obtenção e purificação da rhSLPI do meio de cultura da microalga. O gene de reistência à zeocina, BLE2A, tem a finalidade de selecionar a células transformadas das não transformadas, no meio de cultura seletivo contendo zeocina.

A transformação da microalga *C. reinhardtii* foi feita por eletroporação baseado no kit Invitrogen™ MAX Efficiency™ Transformation Reagent for Algae (Invitrogen™ A24229). Os parâmetros utilizados para transformação foram 500 V, 50 µF e 800 Ω. Após a transformação, as células foram plaqueadas em placas TAP contendo 15 µg/ml de zeocina, para seleção das células transformadas. Entre os clones gerados, dois clones apresentavam maior produção e secreção de rhSLPI, o clone βH3 (rhSLPI) e o clone IVB1 (rhSLPI-FLAG). Dessa forma, esses foram os clones escolhidos para o desenvolvimento desse projeto.

Figura 1. Representação dos plasmídeo pAJB3 - SLPI -FLAG e PAJB3-SLPI utilizado para transformação da microalga Chlamydomonas reinhardtii. A construção foi feita pelo programa SNAPGENE.





4.1.2 Linhagens de *Chlamydomonas reinhardtii* e condições de cultivo

As linhagens de *C. reinhardtii* selvagens (CC124) e produtoras de rhSLPI (IVB11 e βH3) foram cultivadas em meio líquido tris-acetato fosfato (TAP) sob agitação 125 rpm ou contendo ágar, conforme descrito anteriormente (YAGI et al., 2016). O meio líquido é utilizado para amplificação das microalgas e o meio ágar para seleção dos transformantes e manutenção da cepa. As culturas foram incubadas a 25° C sob luz constante. As microalgas foram cultivadas em escala laboratorial (tubos de ensaio contendo 10 mL até biorreatores de 15 L) antes da purificação da rhSLPI.

4.1.3 Purificação da proteína rhSLPI por cromatografia de troca iônica

Sobrenadante de 4 L de culturas de microalgas de 1 dia após a fase estacionária de crescimento, avaliada por densidade óptica a 750 nm, foram obtidas após centrifugação da cultura a 1800 xg por 10 minutos a temperatura ambiente. A seguir, 2 L do sobrenadante foi filtrado com Whatman® glass microfiber filters, binder free, Grade GF/C, Circles 90 mm (Merck, Darmstadt, Germany) seguido de filtração em ExpressTM Plus (Millipore, Burlington, MA, USA). O clarificado foi acidificado com HCl até atingir o pH próximo a 6,4 (para purificação de rhSLPI de ponto isoelétrico estimado em 8,66). A seguir, o material obtido foi submetido a cromatografia de troca iônica utilizando o equipamento fast protein liquid chromatography (FPLC) ÄKTAprime plus (Cytiva) e coluna HiTrap SP HP cation exchange chromatography column, 5 mL (GE healthcare Life Sciences, Chicago, IL, USA). Foram testados dois tampões diferentes para análise da eficácia de eluição da proteína de interesse - Tampão TRIS e Tampão Fosfato.

O tampão de ligação utilizado consistiu em 20 mM TRIS e o tampão de eluição consistiu em 20 mM TRIS contendo 1 M NaCl, sendo que o pH equivale ao mesmo utilizado para acidificação das amostras de sobrenadantes. Para o tampão Fosfato, o tampão de ligação consistiu em 50 mM de fosfato monossódico e o tampão de eluição em 50 mM de fosfato monossódico contendo 1 M NaCl. Para a eluição foi utilizado o método isocrático e coletado 15 mL em frações de 1 mL.

4.1.4 Immunoblotting

Sobrenadantes de cultura de microalgas, e amostras da purificação por cromatografia de troca iônica foram fracionadas por eletroforese em gel de

poliacrilamida (12%) contendo SDS (SDS-PAGE), utilizando tampão de corrida (50 mM Tris; 0,38 M Glicina; 1,8 mM EDTA; 0,1%SDS) a 120V. Em seguida, as proteínas foram transferidas para membrana de PVDF a 100 V por 55 minutos, utilizando tampão de transferência (24,8 mM Tris; 192 mM Glicina; 10% Metanol). A membrana foi bloqueada a temperatura ambiente por 40 minutos em tampão TBS-T (10 mM Tris-HCl; 150 mM NaCl e 0,02% Tween-20), contendo 5% leite desnatado. Em seguida, a membrana foi incubada a temperatura ambiente por 4 horas com diluições adequadas de anticorpos primários de interesse diluídos em TBS-T contendo 5% leite desnatado. Para detecção de SLPI foram utilizados polyclonal anti-SLPI produced in rabbit (HPA027774, Sigma Life Science, St. Louis, MO, USA). Após esse período, a membrana foi lavada quatro vezes por 5 minutos com TBS-T e incubada a temperatura ambiente por uma hora com anticorpos secundários policlonais anti-rabbbit IgG conjugados à peroxidase diluídos em TBS-T contendo 5% leite desnatado. Após quatro novas lavagens de 5 minutos com TBS-T, a membrana foi tratada com substrato quimioluminescente *Pierce ECL Western Blotting Substrate* (Thermo Scientific) para detecção da HRP (Horse Red Peroxidase) e permitir a visualização da proteína de interesse.

4.1.5 SDS-PAGE e coloração com prata

Para a coloração dos géis com prata, a eletroforese foi realizada como descrita no item anterior. Após a corrida, os géis foram submetidos à coloração com prata que constitui em cinco etapas com cinco soluções diferentes. Primeiramente, os géis foram incubados na solução 1 (metanol 50%; ácido acético 12%; formaldeído 37%) em temperatura ambiente sob agitação constante por 30 minutos, seguido de lavagem com água destilada três vezes. Logo após, os géis foram incubados na solução 2 (etanol 35%) por 5 minutos sob agitação constante, seguido de lavagem com água destilada três vezes. Após a solução 2, os géis foram incubados na solução 3 (10 mg Tiossulfato de sódio; 50 mL H2O M.Q) por 3 minutos sob agitação constante, seguido de lavagem com água destilada três vezes. Em seguida, os géis foram incubados na solução 4 (100 mg Nitrato de Prata; 38 µL formaldeído 37%; 50 mL H_2O M.Q) por 10 minutos sob agitação constante, seguido de lavagem com água destilada três vezes. E por último, foi feita a incubação na solução 5 (3g Carbonato de sódio; 1 mL solução 3; 25 µL formaldeído 37%; 50 mL H_2O M.Q) até o aparecimento das bandas de proteínas.

4.1.6 Precipitação de proteínas do sobrenadante com Sulfato de Amônio

Sobrenadante de culturas de microalgas de 1 dia após a fase estacionária de crescimento, avaliada por densidade óptica a 750 nm, foram obtidas após centrifugação da cultura a 1800 xg por 10 minutos a temperatura ambiente. A seguir, o sobrenadante foi filtrado 3x com Whatman® glass microfiber filters, binder free, Grade GF/C, Circles 90 mm (Merck, Darmstadt, Germany) seguido de filtração em ExpressTM Plus (Millipore, Burlington, MA, USA). O sobrenadante clarificado foi precipitado com sulfato de amônio (SA). Foram avaliadas diferentes concentrações do sal, variando entre 10% e 90% de duas maneiras distintas: (1) diversas concentrações (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% e 90%) de sulfato de amônio foram testadas para precipitação da proteína SLPI. A preciptação foi realizada durante a noite (aproximadamente 16 horas de incubação) a 4ºC sob agitação constante. Cada concentração de SA foi testada separadamente. No dia seguinte, os tubos foram centrifugados a 4500 xg, por 1h a 4°C. (2) a precipitação foi realizada em gradiente de sulfato de amônio (10% - 90%). O período de incubação em cada concentração utilizada (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% e 90%) foi de 40 min a temperatura ambiente sob agitação constante. Após cada incubação, a amostra foi centrifugada a 4500 xg, a 4°C por 30 min, o precipitado foi solubilizado em PBS e o sobrenadante foi submetido a uma nova preciptação com a concentração seguinte de SA. Todos os precipitados de proteínas obtidos em cada etapa das precipitações 1 e 2 foram solubilizados em 100 µL de PBS para análise por immunoblotting.

4.1.7 Precipitação de proteínas do sobrenadante com acetona

Acetona foi adicionada às amostras de sobrenadantes em quantidade suficiente para concentração final de 80% e a mistura mantida a -20ºC durante uma noite. As amostras foram centrifugadas a 7197xg por 40 minutos a 4ºC e as proteínas precipitadas foram solubilizadas em ureia 4 M para posterior análise por immunoblotting.

4.1.8 Diálise

O volume obtido após a purificação por cromatografia de troca iônica foi submetido à diálise utilizando tubo de diálise de celulose com tamanho de poro de 3,5K MWCO (88244, Thermo Scientific™). A diálise foi realizada em PBS. A troca do

tampão foi realizada duas vezes em um intervalo de 3h, e após a segunda troca, o béquer foi mantido na geladeira a 4°C durante a noite sob agitação constante.

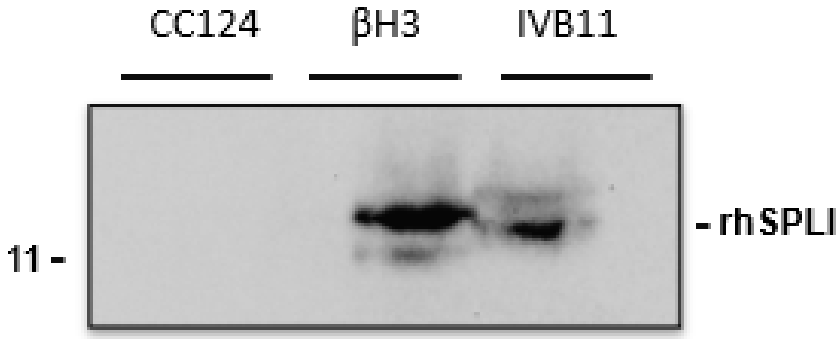
4.2 Resultados

4.2.1 Clones produtores de rhSLPI

Para as diferentes linhagens de *Chlamydomonas reinhardtii* cultivadas no laboratório, foi realizado western blotting (Figura 2) visando análise dos clones que mais expressam a proteína recombinante, rhSLPI. O *western blotting* foi realizado após precipitação das proteínas do sobrenadante de cada linhagem em cultura com acetona. Foram escolhidos dois clones produtores de rhSLPI – IVB11 e βH3 – para a realização dos experimentos de purificação da proteína recombinante. O clone IVB11 contém o peptídeo FLAG (DYKDDDDK) fusionada à SLPI, enquanto o clone βH3 produz a proteína sem nenhum marcador. É possível observar, na figura 2, que o clone βH3 apresenta maior conteúdo da proteína rhSLPI, em comparação ao clone IVB11. O motivo para essa diferença pode estar na inserção do gene SLPI no genoma da microalga. O gene pode ter se inserido em diferentes locais do genoma nos diferentes clones, influenciando a quantidade de proteína produzida e secretada por cada clone (ZHANG; WANG; WANG, 2021).

A curva de crescimento das linhagens CC124 – WT, IVB11 – produtor de rhSLPI, e βH3 – produtor de rhSLPI, foi realizada para analisar o comportamento de cada clone e se há alterações no crescimento e tamanho de células entre os diferentes clones.

Figura 2. Western blotting do precipitação de 10 mL do sobrenadante dos clones CC124, βH3 e IVB11.



A curva de crescimento foi realizada ao longo de 10 dias e foi analisado o tamanho das células em µm, o número de células, e a densidade óptica em 750 nm. É observado que o clone IVB11aumenta significativamente em número de células (Figura 3), em relação ao clone βH3 e ao controle, CC124, como mostrou o teste estatístico One-Way ANOVA seguido do teste Bonferroni, em que a comparação do clone IVB11 com os outros dois apresentou valor de $p < 0.05$. Porém todas as linhagens seguem o mesmo padrão de curva, em que no sexto dia as células atingem o platô. O comportamento do gráfico de número de células, é observado também no gráfico de densidade óptica (Figura 4), onde após uma fase de crescimento exponencial, as células atingem o platô. Porém, diferentemente do número de células, não há variação significativa no comportamento dos clones na análise de densidade óptica pois os testes estatísticos não apresentaram valor de $p < 0.05$, contradizendo o comportamento do clone IVB11 na Figura 3.

Tal contradição pode ser explicada, talvez, pelo tamanho das células (Figura 5). Em que, apesar de não haver diferença estatística no comportamento dos clones no gráfico de tamanho, as células do clone IVB11 apresentam moderada redução no tamanho das células em relação aos outros dois clone (βH3 e CC124). Dessa forma, o aumento no número de células, compensa pela redução no tamanho, resultando no comportamento observado no gráfico de densiade óptica.

Outra possibilidade para a discrepância no comportamento dos gráficos das figuras 3 e 4, é a secreção de substâncias no meio de cultura. Assim como a proteína rhSLPI é secretada no meio de cultura pela microalga, a *Clamydomonas reinhardtii* pode secretar outros componentes no meio juntamente com a proteína. Tais compostos, podem estar influenciando na leitura da densidade óptica, interferindo na leitura.

Figura 3. Gráfico representando o número de células das três linhagens, CC124 (vermelho) - WT, IVB11 (verde) e βH3 (preto) – produtoras de rhSLPI, analisado ao longo de 10 dias.

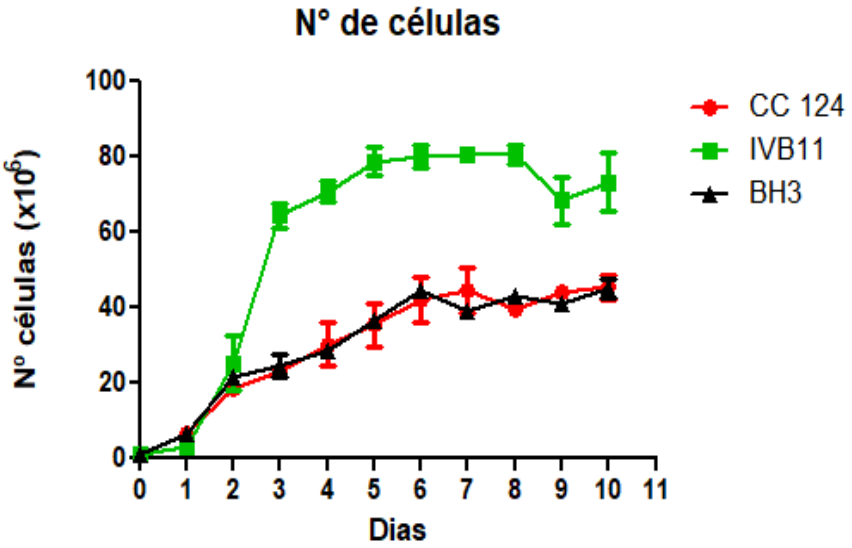


Figura 3. Gráfico representando a densidade óptica das três linhagens, CC124 (vermelho) - WT, IVB11 (verde) e βH3 (preto) -produtoras de rhSLPI, analisado ao longo de 10 dias

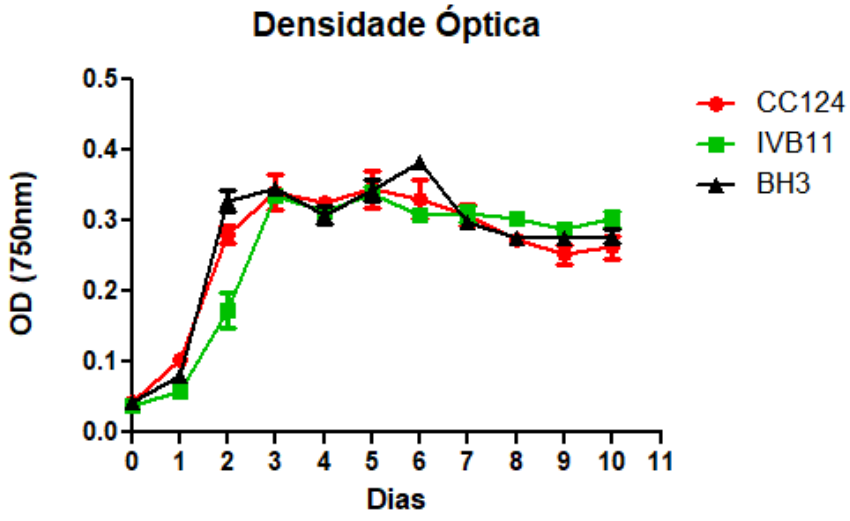
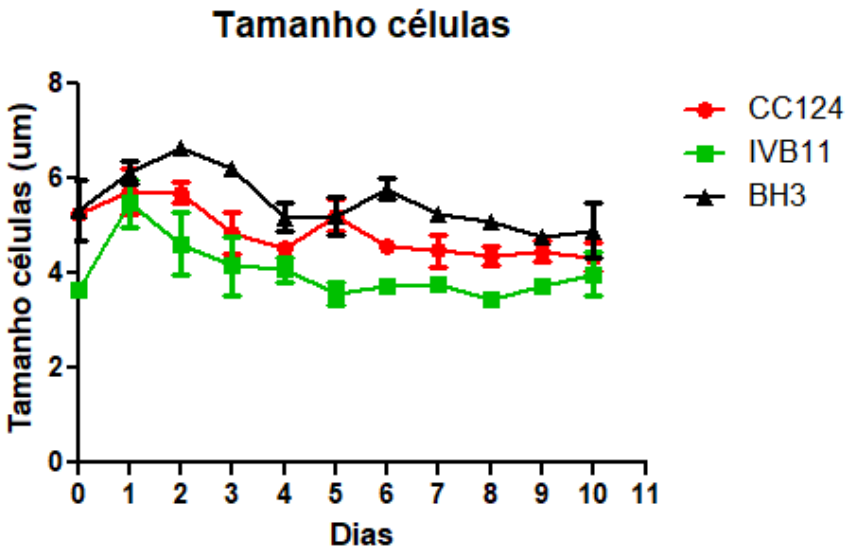


Figura 4. Gráfico representando o tamanho das células das três linhagens, CC124 (vermelho) - WT, IVB11 (verde) e βH3 (preto) – produtoras de rh SLPI, analisado ao longo de 10 dias.



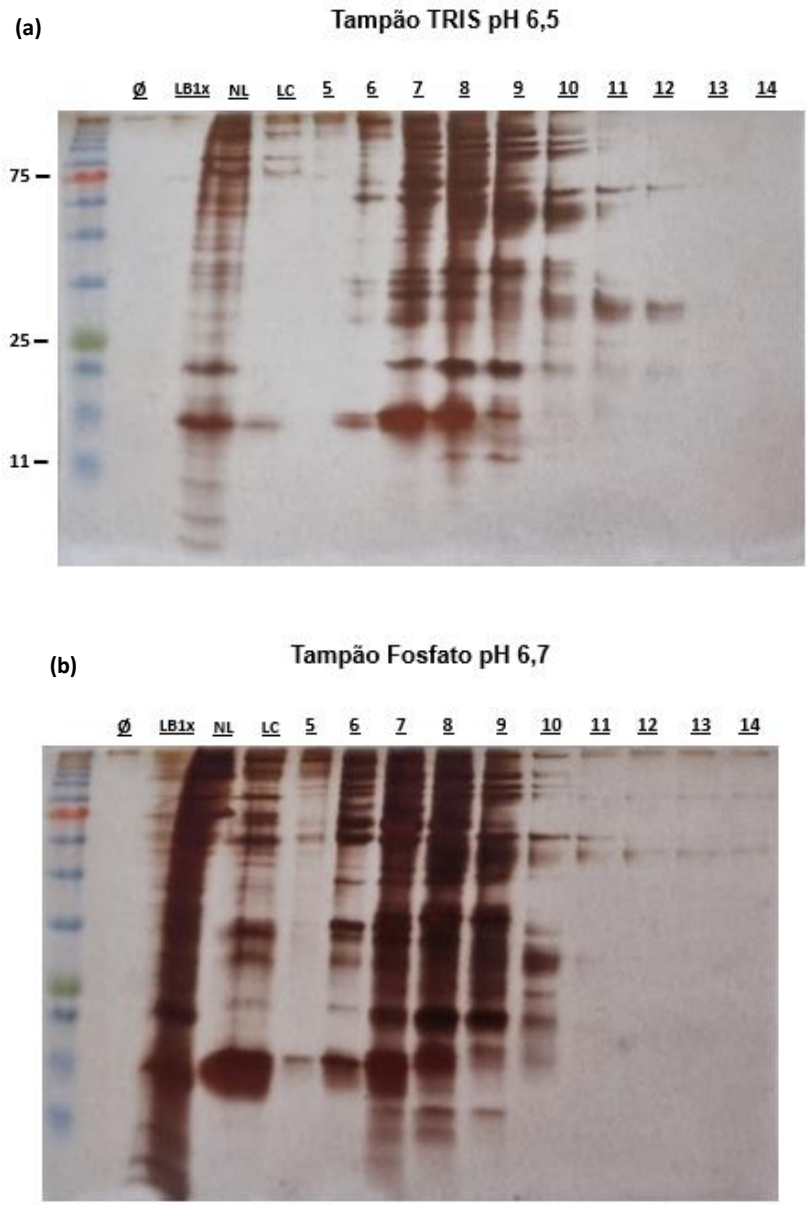
4.2.2 Primeira cromatografia

4.2.2.1 Cromatografia de troca iônica

Para a primeira cromatografia, foi utilizado o sobrenadante do clone βH3. Foram realizados SDS-PAGE seguido de coloração com prata e western blotting para constatação da eficácia de purificação da rhSLPI.

A eluição da rhSLPI na cromatografia foi realizada em um total de 15 mL divididos em frações de 1 mL. Os cinco primeiros mL não foram utilizados para detecção da proteína, pois corresponde ao volume da coluna. Dessa maneira, foram utilizadas no SDS-PAGE e western blotting, amostras a partir do 6° mL eluído até o 15° mL. No SDS-PAGE seguido de coloração com prata (Figura 6), foram analisadas as seguintes amostras: amostras oriundas da eluição da cromatografia a partir do 6° eluato, até o 15° (colunas 5 – 14), amostras oriundas do carregamento da coluna de cromatografia – “não ligado” (NL), e amostra da lavagem da coluna – “lavagem coluna” (LC).

Figura 5. SDS-PAGE seguido de coloração com prata. (a) Gél referente à cromatografia feita com tampão TRIS pH 6,5. (b) Gél referente à cromatografia feita com tampão Fosfato pH 6,7. As colunas correspondem respectivamente à: ***Ø:*** *poço vazio, LB1x, Não Ligado e Lavagem da Coluna de cromatografia. As colunas de 5 a 14 correspondem às frações da etapa de eluição*

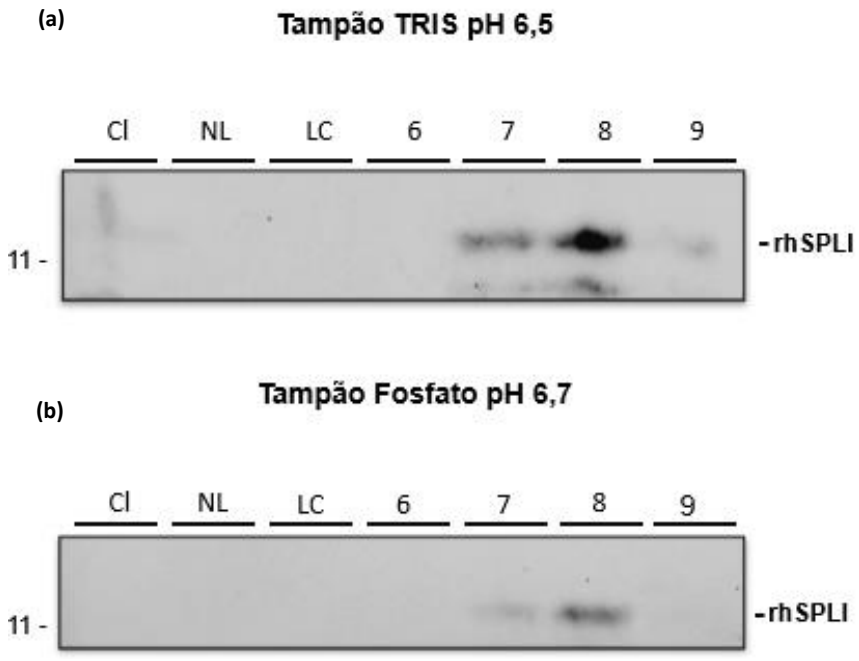


Para o *western blotting* (Figura 7) foram utilizadas as mesmas amostras oriundas da eluição utilizadas no SDS-PAGE. Porém, foi adicionada uma amostra do sobrenadante clarificado antes de passar na cromatografia – “clarificado” (Cl). No western blotting, é possível observar a proteína de interesse, rhSLPI, eluída da coluna de cromatografia no 8° e 9° mL. No SDS-PAGE é observado que foram eluídas muitas proteínas, principalmente proteínas de alto peso molecular, não permitindo a visualização da banda referente à proteína rhSLPI. Observa-se ainda, que o tampão

TRIS proporcionou um gel mais “limpo” de proteínas contaminantes, em relação ao tampão Fosfato. Como mostrado na Figura 6. Além de que, no western blotting, foi obtido um sinal mais forte na amostra oriunda da cromatografia realizada com tampão TRIS, em relação ao tampão Fosfato. Mostrando que em (a) há mais proteína. Dessa forma, os protocolos realizados a partir desse experimento, foram executados com tampão TRIS pH 6,5.

Conclui-se, dessa maneira, que a cromatografia de troca iônica foi eficiente na obtenção da proteína recombinante SLPI, porém o protocolo de purificação precisa ser otimizado, para que seja obtida uma proteína pura, livre de proteínas contaminantes.

Figura 6._Western blotting após cromatografia de troca iônica. (a) Amostras oriundas da cromatografia realizada com tampão TRIS pH 6,5. (b) Amostras oriundas da cromatografia realizada com tampão Fosfato. As colunas de respectivamente à: Clarificado, Não Ligado e, Lavagem da Coluna. As colunas de 5 a 8 correspondem à eluição da proteína.



4.2.3 Segunda cromatografia

4.2.3.1 Precipitação de proteínas do sobrenadante

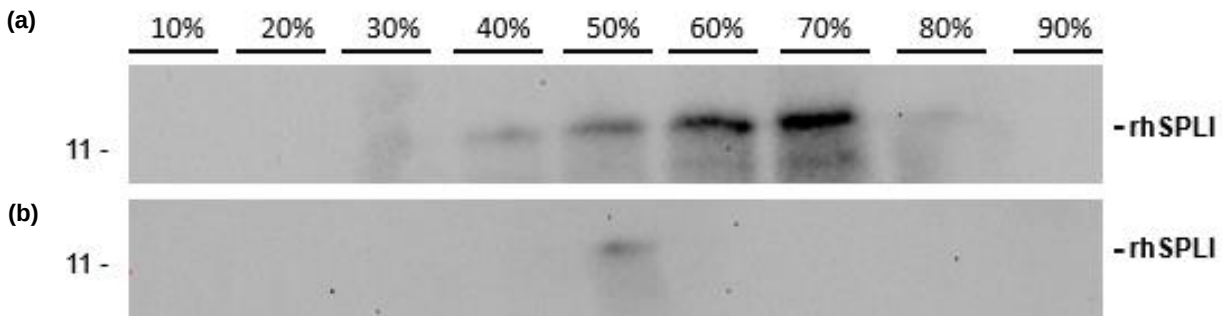
O segundo procedimento de purificação foi realizado com o clone IVB11 (rhSLPI-FLAG), pois no decorrer dos experimentos, o anticorpo utilizado para

detecção da proteína SLPI acabou. Dessa forma, foi utilizado desse experimento em diante, o anticorpo anti-FLAG para detecção da proteína. Além do mais, há interesse na purificação das duas formas da proteína produzida pela microalgas, rhSLPI e rhSLPI-FLAG. Primeiramente, foi realizada a precipitação das proteínas do sobrenadante da cultura de microalga, afim de tentar concentrar a proteína de interesse e se livrar das proteínas contaminantes, já que na primeira tentativa de purificação foi obtida um gél bastante poluído de proteínas e um concentração muito baixa da proteína rhSLPI.

A precipitação foi feita utilizando sulfato de amônio. Foram testadas diferentes concentrações do sal, entre 10 e 90% de duas maneiras distintas: (1) precipitação pontual: partindo da concentração 0%, 9 tubos correspondentes às diferentes concentrações foram utilizados para precipitação durante a noite, sob agitação constante à 4°C. (2) foi realizado a precipitação em gradiente de sulfato de amônio, de forma que a partir de 0%, o mesmo sobrenadante foi precipitado em diferentes concentrações de SA até a concentração de 90%. Cada precipitação teve duração de 45 min seguido de centrifugação à 4500 xg, à 4°C por 30min. Todos os precipitados de proteínas correspondentes à cada precipitação foi ressuspendido em PBS para análise por western blotting.

O *western blotting* (a) mostra que as amostras correspondentes às frações de 60% e 70% da provenientes da precipitação pontual de sulfato de amônio proporcionaram melhor precipitação da proteína recombinante de interesse, SLPI. A precipitação em gradiente (b) não foi eficiente e gerou apenas uma banda fraca na fração de 50% (Figura 8).

Figura 7. Western blotting da precipitação de proteínas com Sulfato de Amônio (SA). (a) precipitação pontual. (b) precipitação em gradiente.

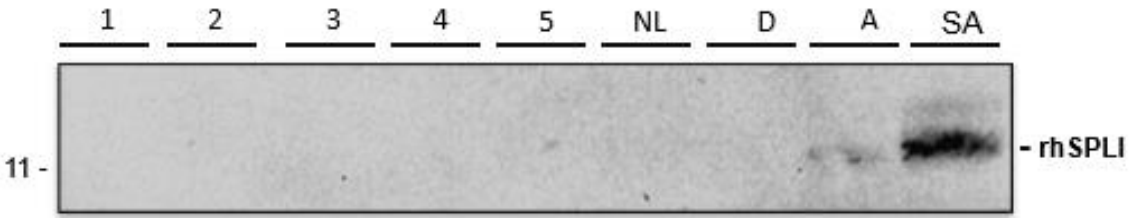


4.2.3.2 Diálise e Cromatografia de troca iônica

Após a precipitação de proteínas com sulfato de amônio (SA) 70%, devido à alta concentração salina, os precipitados de proteínas foram solubilizados em PBS e o volume final de 12 mL foi dialisado em PBS. Após a diálise, o volume da amostra passou a ser 20 mL, e foi acidificado até atingir o pH de 6,4 para ser passado na coluna de cromatografia. A segunda cromatografia foi realizada conforme a primeira, porém utilizando apenas o tampão TRIS pH 6,5. A eluição da rhSLPI foi realizada em um total de 5 mL divididos em frações de 1mL.

No *western blotting* da segunda cromatografia (Figura 9), foram utilizadas as amostras dos eluídos (colunas 1-5), amostra oriunda do carregamento da coluna com o clarificado dialisado e acidificado – "não ligado" – (NL), amostras do clarificado dialisado antes da cromatografia – "dialisado" (D) e, amostras precipitadas com SA 70% (SA) e acetona (A) usadas como controle. Não foram obtidas bandas da proteína de interesse rhSLPI referentes à cromatografia e à diálise, apenas bandas das amostras controles. Confirmando que a diálise e a cromatografia não foram bem-sucedidas.

Figura 8. Western blotting da segunda cromatografia. As colunas de 1 a 5 representam as amostras eluídas após a cromatografia. As demais colunas representam respectivamente: amostra "Não Ligada" (NL), amostra após a diálise (D), controle precipitadas com acetona (A) e sulfato de amônia 70% (SA), respectivamente.



4.2.4 Terceira cromatografia

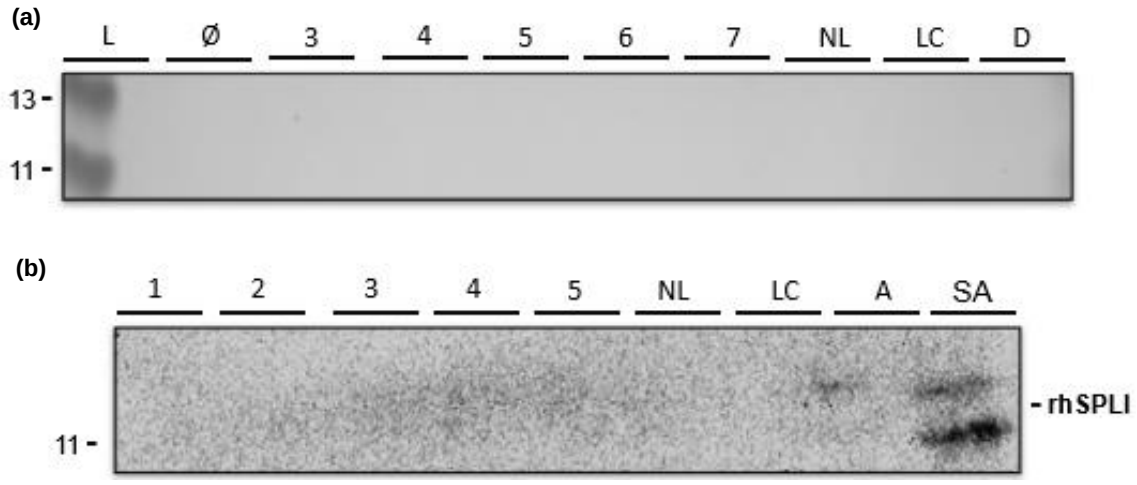
4.2.4.1 Diálise e Cromatografia de troca iônica

Para a terceira cromatografia, o clarificado pertencente ao clone IVB11, foi precipitado com SA 70%, e o precipitado foi solubilizado em H_2O MQ e ureia 4 M, diferindo do protocolo utilizado na segunda cromatografia. O volume de 12 mL foi dialisado em tampão TRIS 20 mM, ao invés de PBS, e o volume final após a diálise foi de 16 mL. A cromatografia de troca iônica foi realizada conforme as anteriores. A

eluição foi feita em um total 5 mL coletados em frações de 1 mL. No western blotting da terceira cromatografia, foram utilizadas amostras dos eluídos (colunas 3 – 7), amostra oriunda do carregamento da coluna com o clarificado dialisado e acidificado – “não ligado” (NL) –, amostra oriunda da lavagem da coluna – “lavagem coluna” (LC) – com tampão de ligação TRIS 20 mM após o carregamento e amostra do clarificado dialisado antes da cromatografia – “dialisado” (D).

Conforme a segunda cromatografia, também não apareceram bandas referentes à proteína de interesse em nenhuma das amostras (Figura 10 a). O western blotting foi repetido (Figura 10 b), utilizando precipitações com SA 70% e acetona como controles. Novamente, apenas as bandas referentes às amostras das precipitações apareceram, mostrando que a diálise e a cromatografia não foram bem-sucedidas.

Figura 9. Western blotting referente à terceira cromtografia. (a) primeiro western blotting. As colunas correspondem à: ladder (L), poço vazio (Ø), amostras eluídas (3-7), "não ligado" (NL), "lavagem da coluna” (LC) e “dialisado” (D), respectivamente. (b) segundo western blotting. As colunas correspondem à amostras eluídas (1-5), “não ligado” (NL), “lavagem da coluna” (LC), precipitação com acetona (A) e sulfato de amônio 70% (SA), respectivamente.



4.2.5 Quarta cromatografia

4.2.5.1 Cromatografia de troca iônica

Para a quarta cromatografia, foram feitas algumas mudanças no roteiro experimental. Para a cromatografia, foi utilizado o sobrenadante clarificado do clone IVB11, a precipitação de proteínas e a diálise não foram realizadas. 1 L do clarificado foi passado na coluna de cromatografia e o eluato coletado (3 mL). O mesmo

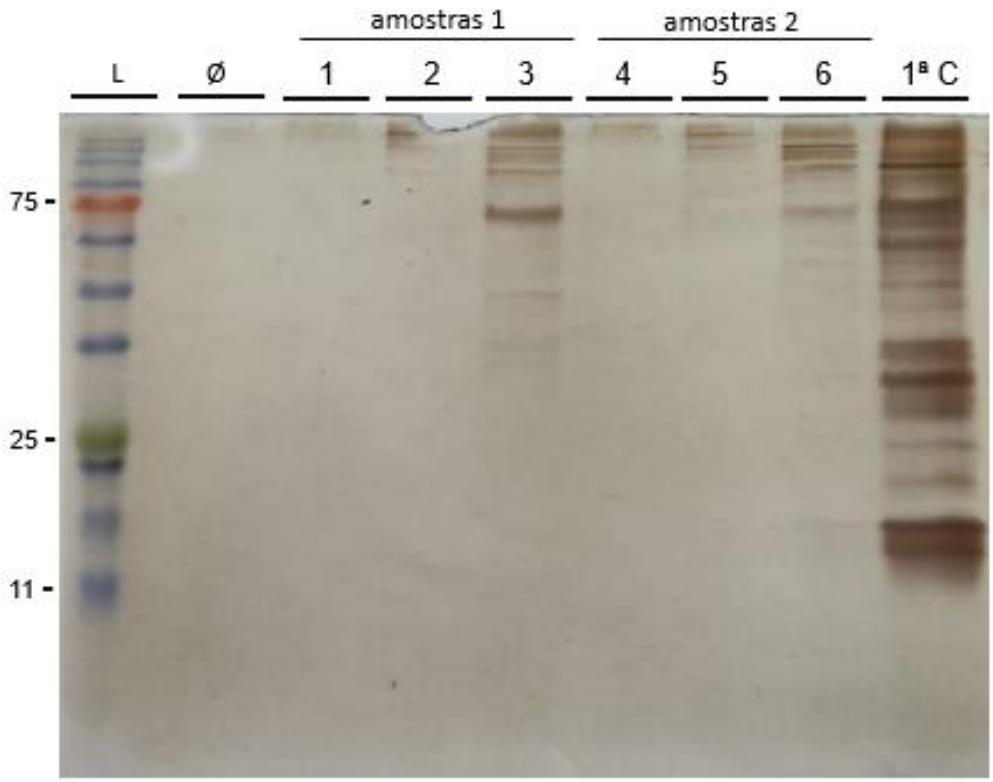
sobrenadante foi passado pela segunda vez na coluna de cromatografia, e o segundo eluído foi coletado em frações de 1 mL, em um volume total de 3 mL.

Foi realizado SDS-PAGE seguido de coloração com prata e *western blotting* com as amostras da primeira (amostras 1) e segunda passagem (amostras 2). Foi utilizada a amostra da primeira cromatografia (1ª C) como controle positivo.

O *western blotting* não deu certo, pois não apareceram bandas referente à proteína de interesse, rhSLPI. Tal problema pode ser devido à ausência da proteína na amostra ou no anticorpo utilizado que, posteriormente, se mostrou ineficiente em se ligar ao peptídeo FLAG, lingando – se fracamente. No SDS-PAGE seguido de coloração com prata (Figura 11), é possível observar que após a segunda passagem na coluna de cromatografia, há menos proteínas de alto peso molecular e mais proteínas menores, o que pode favorecer a ligação e eluição da proteína rhSLPI. Em experimentos futuros, será coletado mais frações correspondentes ao eluído, pois é possível que, com esse protocolo, a proteína de interesse seja obtida em frações mais tardias.

Observa-se também que o clone βH3 (1ª C) possivelmente expressa mais proteínas que o clone IVB11 (amostras 1 e 2). Pois, ao comparar a coluna 1ª C com a 3, ambas oriundas de apenas uma passagem da amostra na coluna de cromatografia, a coluna 1ª C contém mais proteínas. Tal observação precisa ser cuidadosamente analisada e verificada em experimentos futuros, para a certificação de que o resultado obtido não é devido à etapas anteriores à cromatografia que pode ter interferido na obtenção da proteína.

Figura 10._SDS-PAGE seguido de coloração com prata, após a quarta cromatografia. As colunas correspondem a: ladder (L), poço vazio (Ø), eluídos da primeira passagem da amostra na coluna de cromatografia (amostras 1), eluídos da segunda passagem da amostra na coluna de cromatografia (amostras 2) e amotra da primeira cromatografia (1ªC).



5 DISCUSSÃO

Desde sua descoberta, a proteína SLPI é bastante estudada devido à suas diversas funções no organismo humano (antimicrobiana, anti-inflamatória, cicatrizantes etc.). Esses estudos incluem a tentativa de produção em larga escala do inibidor de protease secretado por leucócitos, para aplicações clínicas e biotecnológicas.

Como mencionado nesse trabalho, diversos pesquisadores já produziram a proteína SLPI em diferentes organismos, na tentativa de encontrar uma plataforma de alto rendimento e baixo custo. Com essa mesma perspectiva, o presente trabalho, teve como objetivo o desenvolvimento de um artigo de revisão acerca da proteína SLPI, além da produção e purificação da proteína recombinante humana SLPI a partir da microalga *Chlamydomonas reinhardtii.*

As microalgas foram primeiramente utilizadas como organismos modelo para compreensão e caracterização do mecanismo de fotossíntese (SASSO et al., 2018).

A espécie *C. reinhardtii* foi primeiramente descrita em 1988 por Dangeard e foi tida como organismo modelo em 1950, quando os primeiros mutantes foram gerados. Hoje, é a microalga mais utilizada para pesquisas e produção de proteína recombinante (SASSO et al., 2018). Diversos fatores corroboram para a preferência em se utilizar a *C. reinhardtii* como uma plataforma para produção de proteínas recombinantes. É um microrganismo unicelular com um período de duplicação bastante curto, reduzindo o tempo desde a transformação até purificação da proteína de interesse, em comparação com outras plataformas. A *C. reinhardtii* têm a capacidade de crescer em um ambiente ausente de luz, se houver uma fonte de carbono em seu meio de cultura. Todos os seus três genomas (nuclear, cloroplasto e mitocôndria) já foram sequenciados e todos são passíveis de transformação. Além do mais, como eucariotos, essas microalgas contêm chaperonas e a maquinaria celular necessária para o correto dobramento e adição de modificações pós-traducionais necessário para proteínas complexas (FRANKLIN; MAYFIELD, 2004). Diversos compostos já foram produzidos em *C. reinhardtii,* incluindo proteínas, vacinas, anticorpos etc. (MAYFIELD; FRANKLIN; LERNER, 2003), (SUN et al., 2003), (MANUELL et al., 2007), (RASALA et al., 2010), (TRAN et al., 2013).

No presente projeto, foi apresentado a produção e purificação da proteína humana SLPI em *Chlamydomonas reinhardtii.* Com a transformação da microalga, foram gerados clones que expressam a proteína SLPI fusionada ao peptídeo FLAG, assim como clones que expressam a proteína SLPI sozinhas. Os clones escolhidos para a realização desse trabalho foram os clones IVB11 (rhSLPI-FLAG) e βH3 (rhSLPI), pois foram os clones que apresentaram maior produção de rhSLPI em estudos anteriores realizados pelo professor Dr. Augusto Ducati Luchessi na Universidade da California, San Diego, EUA. Entres os dois clones, o clone βH3 é o que aparenta expressar mais proteína recombinante no meio de cultura. Dessa forma, foi com esse clone que o estudo foi iniciado.

A primeira tentativa de purificação da proteína recombinante gerou baixas quantidades de proteína. A fim de aumentar o rendimento, foram realizadas diversas modificações no roteiro experimental ao longo das tentativas posteriores. Porém, não foi obtida a proteína de interesse nessas outras tentativas. Não se sabe qual o motivo da falha, mas algumas hipóteses foram levantadas. Após a primeira tentativa, o anticorpo usado para detecção da proteína SLPI acabou, dessa forma, alteramos o clone para o IVB11, pois assim era possível utilizar o anticorpo anti-FLAG para

detecção. A não detecção da proteína recombinante humana SLPI produzida pelo clone IVB11, pode ser devida à baixa taxa de produção da proteína por esse clone. Ao transformar a *C. reinhardtii*, o gene de interesse se insere em locais aleatórios do genoma da microalga (ZHANG; WANG; WANG, 2021). Dessa forma, os diferentes clones podem ter o gene inserido em diferentes locais do genoma, influenciando a produção, expressão e secreção da rhSLPI. Além do mais, é necessário ainda, uma avaliação da quantidade de proteína recombinante humana SLPI que pode estar sendo retida no interior da microalga. O mecanismo de secreção utilizado (peptídeo sinal) pode não ser suficiente para a microalga secretar quantidade significativas da proteína. Por esse motivo, essa linha de estudo já está sendo desenvolvida em nosso laboratório, para que seja produzido um clone capaz de secretar altas quantidades de proteína recombinante.

Outra possibilidade é a execução da técnica de purificação, é possível que com as alterações realizadas, quantidades significativas de proteína podem estar sendo perdidas. É necessário uma revisão detalhada e estudos mais aprofundados sobre os pormenores da técnica escolhida, para que seja possível a detecção do problema, caso esse esteja na execução da estratégia. A cromatografia de troca iônica (catiônica), foi a metodologia escolhida devido ao custo e a disponibilidade dos materiais necessários. Além do mais, diversos trabalhos na literatura utilizaram esse mesmo método cromatográfico para a purificação da proteína SLPI (BARANGER et al., 2011; MECKELEIN et al., 1990; VOGELMEIER et al., 1990). O que nos leva a acreditar que a não detecção da proteína após a purificação é devido, mais provavelmente, à baixa produção e secreção pelo clone.

Outros métodos de cromatografia já foram também reportados, como cromatografia por afinidade (GRAY; ALEXANDER; SHUGARS, 2002; LI et al., 2010; MUNADZIROH et al., 2017), e fase reversa (MASUDA et al., 1996; SEELY; YOUNG, 1991; STETLER et al., 1989).

Em suma, a produção e purificação da rhSLPI a partir da microalga *Chlamydomonas reinhardtii* apresenta grande potencial. São necessário estudos e análises dos experimentos já realizados para que o problema seja identificado e solucionado.

6 CONCLUSÃO

O inibidor de protease leucocitária secretora (SLPI), é uma proteína bastante estudada nos últimos anos. Sua atuação em diversos campos da fisiologia humana chama a atenção de pesquisadores das mais diversas áreas, o que levou ao desenvolvimento de estratégias para a obtenção de SLPI recombinante e sua aplicação.

A biotecnologia de microalgas é um campo de pesquisa promissor para o desenvolvimento de diversos produtos: medicamentos, biocombustíveis, alimentos, fármacos, entre outros. A biotecnologia mudará com o aprimoramento das tecnologias que permitem que as microalgas se tornem um sistema de produção como *E. coli, P. pastoris*, células de insetos e de mamíferos. As microalgas como biorreatores têm vantagens consideráveis sobre bactérias, leveduras, plantas e outros sistemas de produção de proteínas recombinantes, como baixo custo, segurança, métodos alternativos de cultura e rápida escalabilidade. Com o atual desenvolvimento de pesquisas, as microalgas emergirão como o sistema de produção de proteína recombinante mais eficiente e de alta recuperação em breve. Pensando nisso, a produção de SLPI em microalgas é um campo promissor para entidades médicas e farmacêuticas.

O presente projeto, tem como objetivo a produção e purificação da SLPI humana recombinante a partir da microalga *Chlamydomonas reinhardtii.* Foi apresentado que a técnica de purificação por cromatografia de troca iônica é eficiente para a obtenção da proteína SLPI, porém, ainda é necessário muito estudo e aperfeiçoamento para que seja obtida uma proteína pura, livre de contaminantes. Estão sendo feitos diversos estudos e análises para o desenvolvimento de um protocolo de purificação eficaz na obtenção da proteína recombinante humana SLPI pura. Após a padronização do roteiro experimental, poderá ser iniciada a etapa de testes de atividade com a proteína obtida no laboratório, para certificação de que a proteína rhSLPI produzida em microalga é biologicamente ativa.

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