RICHARD MARINS DA SILVA

EXTRAÇÃO DE GLOBULINAS DA FARINHA DE SEMENTES DE TREMOÇO: PURIFICAÇÃO DA PROTEÍNA γ-CONGLUTINA E APLICAÇÕES TECNOLÓGICAS DO ISOLADO PROTEICO

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, para obtenção do título de *Doctor Scientiae*.

Orientador: Luis Antônio Minim

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RESUMO

SILVA, Richard Marins da, D.Sc., Universidade Federal de Viçosa, julho de 2022. **Extração de globulinas da farinha de sementes de tremoço: purificação da proteína γ-conglutina e aplicações tecnológicas do isolado proteico**. Orientador: Luis Antônio Minim. Coorientadoras: Valéria Paula Rodrigues Minim, Valéria Monteze Guimarães e Márcia Cristina Teixeira Ribeiro Vidigal.

Algumas proteínas alimentares, além das suas propriedades nutricionais, podem apresentar propriedades nutracêutica, como ação antidiabética, e tecnológicas, como estabilizante interfacial, propriedades essas que são de interesse tanto para o consumidor quanto para a indústria de alimentos. As proteínas extraídas de sementes de cereais e leguminosas têm recebido muita atenção de pesquisadores e da indústria de alimentos devido à sua composição e sustentabilidade ambiental, bem como a sua grande disponibilidade em comparação com as proteinas animais. Entretanto, estudos sobre purificação e aplicações técnicas ainda são poucos comparados as proteínas animais, assim, estudos sobre as proteínas vegetais, em especial as proteinas de tremoço é necessário. O objetivo desta tese foi desenvolver uma metodologia de extração de proteínas do tremoço para produção de isolado proteico de globulinas e purificação da proteína γ – conglutina e também estudar as propriedades interfaciais (adsorção interfacial e cinética de adsorção) do isolado proteico. A purificação da γ – conglutina foi realizada através de uma combinação de técnicas de extração (alcalina – salina e precipitação isoelétrica) e uma etapa cromatográfica, utilizando uma coluna de criogel de troca catiônica. Após sua purificação, a espectrometria de massa foi utilizada para confirmar sua identidade. A γ – conglutina purificada apresenta folhas betas como estrutura secundária principal, ponto isoeletrico de 7,51 e temperatura de desnaturação de 68° C. Através dessa metodologia foi possível obter essa proteína com alto grau de pureza. Posteriormente, as propriedades interfaciais do isolado proteico em um sistema água – ar através do tensiometro de gota pendente, além de sua solubilidade e potencial zeta, foram estudadas em pH 3,4 e 6,8 e com adição de NaCl e $CaCl_2$. A solubilidade e potencial do isolado proteico foram dependentes das condições do meio. A redução da tensão interfacial, analisada por 2.5 h até o equilíbrio, foi maior em pH 3,4 e ausência de sais. O pH e os tipos de sais afetaram a taxa de difusão e adsorção interfacial do isolado. A partir dos resultados obtidos, foi possível entender como o ambiente afeta as propriedades tecnológicas das proteínas do tremoço, principalmente a capacidade do isolado proteico de tremoço reduzir a tensão interfacial do sistema estudado. Esses resultados são relevantes mediante o crescimento do uso das proteinas vegetais e traz informações de como separar e aplicar essas proteínas no âmbito da Ciência e Tecnologia de Alimentos.

Palavras-chave: Purificação. Proteinas vegetais. Propriedades interfaciais. Tremoço.

ABSTRACT

SILVA, Richard Marins da, D.Sc., Universidade Federal de Viçosa, July 2022. **Extraction of globulins from lupin seed meal: purification of γ-conglutin protein and technological applications of protein isolate**. Adviser: Luis Antônio Minim. Co-advisers: Valeria Paula Rodrigues Minim, Valéria Monteze Guimarães and Marcia Cristina Teixeira Ribeiro Vidigal.

Some food proteins, in addition to their nutritional properties, may have nutraceutical properties, such as antidiabetic action, and technological properties, such as an interfacial stabilizer, properties that are of interest to both the consumer and the food industry. Proteins extracted from cereal and legume seeds have received much attention from researchers and the food industry due to their composition and environmental sustainability, as well as their wide availability compared to animal proteins. However, studies on purification and technical applications are still few compared to animal proteins, so studies on plant proteins, especially lupin proteins, are necessary. The objective of this thesis was to develop a methodology for extracting proteins from lupin to produce globulin protein isolate and purification of the γ-conglutin protein and to study the interfacial properties (interfacial adsorption and adsorption kinetics) of the protein isolate. The purification of γ – conglutin was carried out through a combination of extraction techniques (alkaline – saline and isoelectric precipitation) and a chromatographic step, using a cryogel cation exchange column. After its purification, mass spectrometry was used to confirm its identity. Purified γ – conglutin has beta sheets as the main secondary structure, an isoelectric point of 7.51 and a denaturing temperature of 68° C. Through this methodology it was possible to obtain this protein with a high degree of purity. Subsequently, the interfacial properties of the protein isolate in a water-air system through the pendent drop tensiometer, in addition to its solubility and zeta potential, were studied at pH 3.4 and 6.8 and with the addition of NaCl and $CaCl_2$. The solubility and potential of the protein isolate were dependent on the conditions of the medium. The reduction in interfacial tension, analyzed for 2.5 h until equilibrium, was greater at pH 3.4 and in the absence of salts. The pH and types of salts affected the rate of diffusion and interfacial adsorption of the isolate. From the results obtained, it was possible to understand how the environment affects the technological properties of lupin proteins, mainly the ability of the lupine protein isolates to reduce the interfacial tension of the studied system. These results are relevant in view of the growth in the use of plant proteins and provide information on how to separate and apply these proteins in the field of Food Science and Technology.

Keywords: Purification. Vegetable proteins. Interfacial properties. Lupin.

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1. INTRODUÇÃO GERAL

A proteína desempenha um papel crucial na manutenção da saúde humana e faz parte de todos os órgãos ou tecidos do corpo. Atualmente, as proteínas vegetais estão ganhando importância sobre as proteínas animais devido sua disponibilidade, aumentado pelas preocupações das pessoas e organizações com o bem-estar animal e o aumento da carga de emissões de gases de efeito estufa em função da grande quantidade de animais utilizados para produção de alimentos, além do aumento populacional e consequentemente, maior demanda por alimentos e também a preocupação com a saúde. Componentes nutricionais, composição de aminoácidos, peso molecular, sequência, forma, tamanho, ponto isoelétrico, carga líquida, estabilidade ao calor, solubilidade, emulsificação, aeração, capacidade de absorção de água/óleo e hidrofobicidade são algumas das propriedades características de proteínas considerados importantes para aplicação em matrizes alimentícias (KUMAR et al., 2022; ONWEZEN et al., 2021).

Todas essas características são influenciadas pela fonte e pelo tipo de proteína vegetal. De forma geral, as proteinas vegetais são classificadas e nomeadas com base na solubilidade de cada fração: albuminas, solúveis em água; globulinas, insolúveis em água mas solúveis em soluções salinas; prolaminas, solúveis em soluções alcoólicas relativamente fortes (60 – 70% de álcool) mas não são solúveis em água ou soluções salinas; e as glutelinas, insolúveis em soluções aquosas neutras, alcoólicas ou salinas, mas podem ser extraídas por álcalis (OSBORNE, 1924).

Na maioria dos casos, a proteína vegetal é encontrada nas sementes e grãos que armazenam a maioria das fontes de nitrogênio. No contexto da nutrição proteica humana, os grupos de plantas mais importantes são grãos de cereais e leguminosas, incluindo oleaginosas, consumidas como parte de componentes de grãos (por exemplo, farinhas de grãos) ou como ingredientes proteicos enriquecidos como co-produtos da extração de óleo ou produção de amido (por exemplo, proteína de soja e glúten). Existem várias razões pelas quais as proteínas vegetais ainda são subutilizadas para a alimentação humana: seus valores nutricionais mais baixos (em uma única base de fonte) em comparação com as proteínas animais; dificuldades em maximizar a sua funcionalidade física devido ao seu grande peso molecular e/ou tamanho e fraca solubilidade em água; e o custo econômico associado ao isolamento e recuperação de frações proteicas (DAY, 2013).

Apesar da falta de um ou dois aminoácidos essenciais na concentração necessária, as proteínas à base de plantas geralmente vêm com quantidades maiores de aminoácidos que estão fora do grupo de aminoácidos essenciais. Especificamente, as proteínas vegetais são ricas em aminoácidos não-essenciais e seus benefícios funcionais são críticos para o metabolismo humano. A proteína de soja, por exemplo, é uma boa fonte de arginina e glicina, que são nutrientes essenciais no ciclo da uréia e na síntese de colágeno (QIN; WANG; LUO, 2022).

Atualmente, a soja é a leguminosa mais cultivada mundialmente, sendo o Brasil um dos maiores produtores. Entretanto, diferentes culturas estão ganhando espaço como fonte alternativa de proteinas como o tremoço, devido a sua falta de modificação genética no cultivo comercial, maior sustentabilidade e menor custo de produção (SHRESTHA et al., 2021).

O tremoço é uma planta herbácea da família das leguminosas, pertencente ao gênero *Lupinus*, que inclui em torno de 450 espécies, existindo quatro diferentes espécies de interesse agrícola: o tremoço branco (*Lupinus albus*), tremoço azul (*Lupinus angustifolius*), tremoço amarelo (*Lupinus luteus*) e tremoço andino (*Lupinus mutabilis*) (FÆSTE; PLASSEN, 2008). O principal interesse no tremoço está ligado à sua alta concentração de proteína, que é considerado uma boa fonte de lisina e reduzida fonte de aminoácidos contendo enxofre. O tremoço é uma fonte de proteínas de alta qualidade, que têm efeito benéfico sobre o metabolismo lipídico e glicídico, sendo a fração de proteína γ-conglutina, de interesse para controlar à insulina e diabetes e uma boa fonte de ferro (LUCAS et al., 2015).

Sementes de tremoço, após a retirada da casca, contêm cerca de 39-44% em peso de proteínas, cerca de 7-10% em peso de óleo e são ainda compostas de fibras alimentares, contendo até 39% de fibra, composta por 75-80% de fibra solúvel, 18-25% de fibra insolúvel e 5-9% de hemicelulose, açúcares, minerais e água (BÄHR et al., 2014). Uma consideração relevante que deve ser feita sobre o consumo do tremoço é a presença de alcaloides, que funcionam como agroquímicos naturais, entretanto, Calabrò et al. (2015), ao caracterizarem o tremoço branco, constatou níveis de alcaloides inferiores ao limite tóxico para a saúde (20 mg/100g) em sementes de tremoço branco (*Lupinus albus* L.), sendo esta semente indicada para o consumo humano.

As principais proteínas de armazenamento das sementes de tremoço são as globulinas (~87% da proteína total do tremoço), que também são denominadas conglutinas (RITTHAUSEN, 1882). As albuminas compõem ~13% do total de proteínas, enquanto as glutelinas e prolaminas são encontradas apenas em quantidades muito pequenas (DURANTI et al., 1990). As globulinas consistem principalmente de conglutinas α e β (conglutinas principais), conglutinas γ e δ (conglutinas de menor concentração) (BLAGROVE; GILLESPIE, 1975).

As proteínas do tremoço já foram estudadadas quanto as propriedades para aplicações na indústria de alimentos (propriedades espumantes, emulsificantes e gelificantes) e farmacêutica (BURGOS-DÍAZ et al., 2016; KHAN et al., 2015). Völp; Seitz; Willenbacher (2021) avaliaram a estrutura e a reologia de espumas feitas de isolado de proteína de tremoço e relataram que as espumas ficaram estáveis por pelo menos 1 hora. Além disso, a capacidade de formação de espuma aumentou fortemente com o aumento do pH ou força iônica. Em outro estudo, realizado por Wong et al. (2013), a capacidade de formação de espuma da fração proteica de tremoço que é mais rica em γ-conglutina foi comparável à da clara de ovo, entretanto um estudo aprofundado sobre as propriedades interfaciais ainda não foi realizado.

Este grão também é conhecido por ajudar no controle do diabetes tipo II, que foi creditado a proteína γ – conglutina (MUÑOZ et al., 2018). No que diz respeito a ensaios *in vivo*, uma redução dos níveis glicêmicos em ratos hiperglicêmicos foi induzida por tratamento oral com a proteína. O efeito redutor da γ - conglutina na dose mais elevada (120 mg/kg de peso corporal) foi comparável ao obtido com aproximadamente metade da dose de metformina (50 mg/kg de peso corporal) (MAGNI et al., 2004). Tapadia et al. (2021) estudando os efeitos antidiabéticos e os mecanismos de ação da γ – conglutina, concluiram que a proteína trabalha como um mimético a insulina, ativando vias celulares associadas à sinalização da insulina, aumentando o transporte de glicose para as células.

O desenvolvimento de técnicas e métodos para a separação e purificação de macromoléculas biológicas, tais como proteínas tem sido um pré-requisito importante para muitos dos avanços feitos em biociência e biotecnologia nas últimas cinco décadas. A farinha de tremoço tem um nível de proteína relativamente alto, mas contém grandes quantidades de compostos não proteicos, como fibras e gordura. Como resultado, para ser considerado um ingrediente de alto valor, sua proteína precisa ser concentrada por meio da separação dos principais compostos não proteicos (LO; KASAPIS; FARAHNAKY, 2021).

As proteínas totais podem ser extraídas da farinha desengordurada usando diferentes condições de extração, tais como protocolos aquosos, alcalinos ou salinos. Alguns processos envolvem adicionalmente a filtração por membranas para remover aditivos de extração, tais como sais e materiais não proteicos de baixo peso molecular. As proteínas totais são ainda purificadas e fracionadas em conglutinas individuais utilizando uma combinação de técnicas de purificação cromatográfica dependendo da necessidade ou aplicação especificada (MANE et al., 2018).

A maioria dos relatos sobre extração dessas proteínas usa extração alcalina seguida de precipitação isoelétrica em pH ácido, sedimentando as principais frações de conglutina, enquanto a γ-conglutina permanece na fração solúvel em ácido (MANE et al., 2018). Entretanto, os autores relatam o enriquecimento do resíduo de fibra das sementes com γ-conglutina após a extração alcalina convencional, indicando potencial ligação da γ-conglutina à fibra. Portanto, a abordagem convencional de extração alcalina seguida de precipitação ácida não é uma abordagem viável para a extração seletiva de γ-conglutina. Esta suposição é corroborada por Muranyi et al., (2016), pelas baixas quantidades de γ - conglutina em isolados proteicos extraídos em solução alcalina (2,9 – 3,4%) em comparação com isolados extraídos em solução salina (3,6 - 4,4%) ou solução salina-alcalina (4,1 – 4,7%).

Sironi; Sessa; Duranti, (2005) desenvolveram um método de separação das quatros frações de conglutinas, onde a farinha de tremoço desengordurada foi suspensa em água (1:20 m/v) e mantida em pH 8,0-8,5 com uma solução diluída de NaOH, sob agitação por 2 a 3 horas à temperatura ambiente. A suspensão foi centrifugada a 10000 x *g* durante 30 min. Após centrifugação, foi obtido um sedimento (fração A) contendo as conglutinas α, β e δ e um sobrenadante, contendo γ – conglutina bruta. A γ - conglutina bruta foi posteriormente purificada por precipitação seletiva com Zn^{2+}, entretanto, a interação da γ - conglutina com o Zn^{2+} pode ser um problema para posterior utilização da proteína.

Diferentes aspectos sobre a purificação de proteínas, em especial a γ – conglutina, por métodos cromatográficos são apresentados pela literatura. As informações apresentadas fornecem orientações a fim de aperfeiçoar os métodos já existentes (aumento de rendimento e pureza) e desenvolver novos métodos de purificação (novas fases estacionarias).

A cromatografia de troca iônica por exemplo, é um método amplamente aplicado na purificação de proteínas. Está bem estabelecido, eficiente e aplicável à purificação em larga escala. A ligação de proteínas é determinada principalmente por interações eletrostáticas entre a carga da proteína e a fase estacionária carregada. Como consequência, os valores ótimos de pH para ligação ou eluição de uma coluna de troca iônica podem ser previstos pelo ponto isoelétrico para muitas proteínas (WIDMANN; TRODLER; PLEISS, 2010). Moléculas carregadas positivamente ligam-se a adsorventes de troca iônica carregados negativamente (troca catiônica), enquanto moléculas carregadas negativamente se ligam a um adsorvente de troca iônica carregado positivamente (troca aniônica) (DERBYSHIRE; WRIGHT; BOULTER, 1976).

Técnicas de otimização cromatográfica, para purificar biocompostos com alta pureza e rendimento usando o menor número de etapas possível têm impulsionado a demanda pelo desenvolvimento de novos adsorventes. Dentre os materiais que podem ser utilizados como suportes sólidos em processos de separação, os criogéis macroporosos têm uma posição de destaque. São compostos por grandes poros estruturais e baixo custo de produção, possibilitando sua utilização na captura direta de biomoléculas de extratos brutos, propiciando alta transferência de massa e baixa queda de pressão durante a separação (NEVES et al., 2020). Os criogéis podem ser produzidos com grupos reativos adequados apenas escolhendo reagentes apropriados ao formar os géis. A superfície pode, além disso, ser quimicamente modificada por acoplamento de diferentes ligantes. A gelificação criotrópica também permite a preparação de vários criogeis com diferentes morfologias, assim a combinação única de alta porosidade, alta resistência mecânica, estabilidade e interações não específicas mínimas devido à natureza hidrofílica dos polímeros são as principais características desses materiais poliméricos (ERTÜRK; MATTIASSON, 2014).

Na última década, tem se verificado um grande número de trabalhos relacionados ao estudo de extração de proteínas vegetais e avaliação de suas propriedades tecnológicas (propriedades gelificantes, emulsificantes e espumantes) (LUO et al., 2021; MA et al., 2018; MILETI et al., 2022). A extração de proteínas vegetais através do uso de metodologias de baixo custo, pode levar a extratos de proteínas vegetais com baixa quantidade de contaminantes obtendo diferentes proteínas com funcionalidades específicas. A substituição de proteínas de origem animal para vegetal é uma tendencia com grandes desafios para a indústria de alimentos, que exigirá a disponibilidade de proteínas de origem vegetal com alta funcionalidade no que diz respeito à estabilização de sistemas multifásicos, como espumas (por exemplo, chantilly e sorvete). Sendo assim, compreender o comportamento interfacial das proteínas vegetais é crucial para desenvolver sistemas macroscópicos estáveis, porque seu comportamento é muitas vezes muito mais complexo do que no caso de proteinas animais (YANG; SAGIS, 2021).

A capacidade das proteinas de adsorverem em uma interface e estabilizar o sistema alimentício resulta da sua natureza anfifílica, formando misturas multifásicas, se ligando a uma região hidrofílica e a uma segunda região, hidrofóbica. Assim, proteínas estabilizam a interface por meio de sua capacidade de diminuir a tensão superficial (interfacial) e formar camadas viscoelásticas, ou também podem fornecer estabilidade às dispersões de alimentos por meio de efeitos eletrostáticos e estéricos ou combinação deles (HINDERINK et al., 2021; MILETI et al., 2022; YANG; SAGIS, 2021).

O mecanismo de adsorção e a viscoelasticidade do filme interfacial são determinados pela conformação da proteína. Geralmente, as proteínas globulares rígidas são mais elásticas do que as proteínas flexíveis de bobina aleatória (random coils). No entanto, em comparação com as proteínas globulares, as proteínas flexíveis têm taxas de adsorção mais rápidas e podem mudar de conformação com mais facilidade (CAO et al., 2022). Como as globulinas do tremoço são caracterizadas como proteinas globulares, é necessário estudos sobre como essas proteinas adsorvem em diferentes interfaces (água – ar e/ou água – óleo).

Considerando as informações disponíveis na literatura e a necessidade de novos métodos de extração, purificação e estudos sobre as propriedades tecnológicas de proteínas vegetais, as globulinas do tremoço serão extraídas e a partir do isolado proteico, extrair a proteína γ – conglutina. Para a purificação da γ – conglutina, será utilizado inicialmente a extração salina – alcalina, precipitação isoelétrica e cromatografia de troca catiônica, utilizando um criogel supermacroporoso de troca catiônica, sendo que o criogel será caracterizado quanto suas propriedades morfológicas e hidrodinâmicas. Posteriormente, identificação e caracterização da γ – conglutina (ponto isoelétrico, estrutura secundária e propriedades térmicas). O isolado proteico constituído de globulinas será analisado quanto a sua solubilidade, potencial zeta e às suas propriedades interfaciais, utilizando um tensiometro de gota pendente, em que métodos estáticos e dinâmicos serão usados para avaliar a capacidade desse isolado em formar um filme e assim reduzir a tensão interfacial na condição água – ar em pH 3,4 e 6,8 na ausência e/ou presença de NaCl ou $CaCl_2$.

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3. CAPÍTULO 1

Extraction, purification by cation exchange supermacroporous cryogel and physico-chemical characterization of γ-conglutin from lupin seeds (*Lupinus albus* L.)

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Extraction, purification by cation exchange supermacroporous cryogel and physico-chemical characterization of γ-conglutin from lupin seeds (*Lupinus albus* L.)

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This study focused on the extraction, purification, and physicochemical characterization of γ-conglutin, a protein present in lupin seeds with properties of reducing blood glucose levels. Total protein was extracted with an alkaline-saline solvent, followed by isoelectric precipitation. Chromatographic purification of the precipitated fraction was performed using a cation exchange supermacroporous cryogel column. Electrophoresis of the eluted fraction from chromatography presented a single band of ~48 kDa under non-reducing conditions (two bands of ~30 and ~17 kDa, under reducing conditions) confirming the success of the purification protocol. Liquid chromatography-tandem mass spectrometry analysis confirmed the identity of the protein as γ-conglutin. The purified γ-conglutin had an isoelectric point of 7.51, β-sheets prevailing as a secondary structure, and denaturation temperature close to 68°C. The outcome of this work showed that γ-conglutin was obtained with a high degree of purity. The proposed purification protocol is simple and can be easily scaled up.

KEYWORDS
mass spectrometry, supermacroporous cryogel, white lupin, γ-conglutin purification

1 | INTRODUCTION

Plant-derived proteins are increasingly being used as a substitute for animal proteins in the human diet for several reasons, such as increasing vegan and vegetarianism trends, environmental concerns, and lower costs than animal sources. These increased the interest of food manufacturers to incorporate functional plant proteins as ingredients in their products [1]. Lupin is a plant of the Leguminosae family, belonging to the genus Lupinus and presents four species of agricultural interest: the white lupin (Lupinus albus L.) is extensively grown in Europe, blue lupin (Lupinus angustifolius L.) is a major rotation crop in Western Australia, yellow lupin (Lupinus luteus L.) is native to the Mediterranean region and the Andean lupin (Lupinus mutabilis Sweet) is grown mostly in South America [2,3]. Lupin proteins offer potential health benefits and contribute to the sustainability of cropping systems [4].

Article Related Abbreviations: AAm, acrylamide; AMPSA, 2-acrylamido-2-methyl-1-propanesulfonic acid; CV, column volume; FTIR, Fourier-transform infrared; HETP, height equivalent to a theoretical plate; MBAAm, *N*,*N*'-methylenebisacrylamide

It has been reported that lupin proteins present technological properties (foaming, emulsifying, and gelling properties) for applications in the food and pharmaceutical industries [5,6]. The total protein content corresponds to ca. 30–40% w/w) and the globulin γ-conglutin, also called 7S, corresponds to approximately 5% w/w of the total proteins [7].

Recently, the 3-D structure of γ-conglutin of *Lupinus angustifolius* L. was presented (Protein Data Bank: 4PPH) [8], revealing a fold rich in disulfide bridges and β-structures, but also containing several α-helix structures. Studies show their resistance to proteolysis, strongly suggesting a non-storage role, although their classification is as seed storage proteins [8,9]. There is a lack of information on the technological properties of that fraction. However, it is associated with the promotion of health benefits controlling type II diabetes because this protein can modulate the metabolism of muscle glucose by exhibiting insulin-mimetic activity [10–12].

Despite its nutritional and proven health benefits and technological properties of interest to the food industry, lupin is still underused and does not play an important role in the human diet. Extraction and purification of lupine proteins with suitable technological properties would make it attractive, as a protein-rich and functional ingredient for food development [1]. In the case of γ-conglutin to be used as a functional ingredient, purification and physicochemical characterization would be fundamental.

Different analytical chromatographic methods of protein purification, in particular γ-conglutin, are presented in the literature (metal affinity, insulin affinity, and anion exchange with molecular-exclusion) [13–15]. Although conventional chromatographic supports are very efficient, some drawbacks are involved such as high backpressure and the requirement of non-particulate and diluted feed solution. Polymeric gels produced under freezing conditions are known as cryogels and have become an interesting material in the field of bioseparation [16]. They are, already consolidated matrices, presenting a highly porous structure with a low resistance to flow. Furthermore, these materials have a versatility regarding their surface functionalization, which allows customizing the chromatographic process depending on the biomolecule of interest [17–19].

While some lupine protein extraction studies have already been reported, as far as we know no studies have reported the extraction and application of preparative chromatography techniques to purify γ-conglutin. This work proposes a simple and inexpensive methodology for γ-conglutin extraction and purification by alkaline-saline extraction, isoelectric precipitation, and cation exchange chromatographic steps using supermacroporous cryogel column alternative to conventional columns. Furthermore, the purified protein was identified by MS (LC-MS/MS) and characterized as to its pI, secondary structure, and denaturation temperature (T_d). The results obtained in this work can expand the possibilities of extraction and use of this intriguing protein present in lupine.

2 | MATERIAL AND METHODS

2.1 | Material

Lupin seeds (*L. Albus*) were purchased in a local market and were the seeds of a single production batch from the state of Paraná, Brazil. Note that, 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPSA, 99%), *N,N*-tetramethylethylenediamine (99%), acrylamide (AAm, 99%), *N,N′*-methylenebisacrylamide (MBAAm, 99%), ammonium persulfate (98%) and trypsin (T-6567) were from Sigma-Aldrich (St. Louis, MO, USA). Other chemical reagents were purchased from Vetec (São Paulo, Brazil). All reagents used were of analytical grade. Deionized water was used in all experiments.

2.2 | Methods

2.2.1 | Alkaline-saline extraction

Lupin seeds were peeled and the cotyledons milled in a disk mill. The flour between 20 and 25 mesh was used for the experiments. The flour was defatted in a soxhlet apparatus by hexane extraction at 37°C for 6 h. After extraction, the solvent was removed by evaporation (40°C and 12 h). The defatted flour had the following characteristics: moisture: 4.23% w/w (Association of Official Analytical Chemists n° 925.45b), ash: 2.24% w/w (Association of Official Analytical Chemists n° 923.03) and total protein (Kjeldahl method): 37.05% w/w.

Lupin protein concentrate was obtained by alkaline-saline extraction with modifications [20]. The defatted flour was suspended in a sodium phosphate buffer (0.1 mol/L and pH 8.5) in a ratio of 1:20 w/v, in the presence of NaCl (0.5 mol/L) under constant stirring for 12 h, at room temperature (25 ± 2°C). The suspension was then filtered through an 80 μm membrane and the protein filtrate was dialyzed against water to remove NaCl and to precipitate globulins, for 4 days at a temperature of 4°C. The aqueous protein extract was centrifuged for 10 min at 10 000 x g at 4°C for separation of the albumin. The precipitate composed of globulins was then freeze-dried (−40°C and 0.12 mbar) and stored under refrigeration for later use.

2.2.2 | Acid precipitation

The freeze-dried Lupin protein concentrate was suspended in a sodium acetate buffer (0.025 mol/L, pH 4.5) and kept under stirring for 1 h. The suspension was centrifuged for 15 min at 3200 x g and 4°C. In this condition, γ-conglutin remained soluble and the supernatant was used in the chromatographic process.

2.2.3 | Cation exchange chromatography in supermacroporous cryogel

The capture of γ-conglutin was performed by preparative chromatography using an AKTA pure system (Model 25 M1; GE Healthcare, Sweden). Polyacrylamide cryogel was produced by polymerization of the monomers Aam (4.7% m/v) and MBAAm (1.3% m/v) catalyzed by *N,N*-tetramethylethylenediamine (23.8 μL) and ammonium persulfate (100 μL) [21]. The monomeric mixture was poured into 1 cm diameter glass tubes (15 cm length) and polymerization took place under cryogenic conditions (–12°C) for 24 h. After thawing and extensive washing with water, the gel matrix was dried at 60°C and stored in a dry state.

The prepared dry cryogel was functionalized with AMPSA, a cationic exchanger [22]. A solution of potassium diperiodate cuprate (15 mL) and NaOH 1 mol/L (5 mL) was recirculated through a glass column (Tricorn 10/150; GE Healthcare, Sweden) containing the cryogel at a flow rate of 0.5 mL/min for 30 min, at a constant temperature of 40°C. Next, 15 mL of AMPSA solution (2 mol/L in NaOH 1 mol/L) was recirculated through the column at a flow rate of 0.5 mL/min for 1 h and 40°C. Finally, the column was washed with 200 mL of HCl (0.1 mol/L), followed by 800 mL of water at a flow rate of 0.5 mL/min for the removal of unbounded AMPSA. The cryogel was characterized according to its microporous structure where a small sample taken from the center of the dry cryogel was coated with a gold layer for SEM (LEO EVO 40 XVP; Zeiss, Germany), hydraulic permeability (K_W) with pressure drop in the flow velocity range of $(2.12 \times 10^{-4}–1.06 \times 10^{-3})$ m/s (Mól et al.), porosity (E_p) and height equivalent to a theoretical plate (HETP) by the measurements of residence time distribution using ultra-pure water in a velocity range of $(2.12 \times 10^{-4}–1.06 \times 10^{-3})$ m/s and 5% v/v acetone solution as a tracer. The signal at the column exit was monitored by a UV detector (280 nm).

Mean residence time (t_R) and variance (σ_t^2) was determined by the calculation of the first and second statistical moment of the recorded signal, respectively. Porosity and HETP were determined according to Equations (1) [23] and (2), respectively.

$$t_R = \frac{L}{U} \in_t \tag{1}$$

$$\mathrm{HETP} = \mathrm{L}\frac{\sigma_t^2}{t_R^2} \tag{2}$$

The supernatant containing γ-conglutin from the acid precipitation was purified in a cation exchange cryogel column (glass column Tricorn 10/150; GE Healthcare, Sweden) at a flow rate of 2 ml/min, at room temperature. Initially, the AMPSA activated cryogel was equilibrated with three column volumes (CVs) of sodium acetate buffer (0.025 mol/L, pH 4.5). Subsequently, the supernatant containing γ-conglutin was injected into the column (~5 CVs). The column was washed with the equilibration buffer and subsequently, the adsorbed proteins were eluted with the same equilibration buffer containing NaCl (1 mol/L). The adsorption, washing, and elution profiles were monitored at 280 nm. The obtained peaks were collected for further analysis.

2.3 | Analytical procedures

2.3.1 | Quantification of total protein

Total proteins were estimated using the biuret method [24], at 540 nm, and using BSA as the reference protein.

The degree of recovery (%) was calculated as the ratio between the protein mass (mg/L) recovered at each extraction step and the protein mass (mg/L) in the feed solution.

2.3.2 | Sodium dodecyl sulfate–PAGE

Protein fractions were analyzed by sodium dodecyl sulfate (SDS)–PAGE with 10% separating gel and 5% stacking gel containing SDS [25]. Aliquots of 10 μL were loaded into the gel wells and electrophoresis was carried out at 80 V in stacking gel and 100 V in separating gel. The analysis was performed on the reductive and non-reductive conditions of the proteins. The protein bands were stained with Coomassie Brilliant Blue G-250.

2.3.3 | LC-MS/MS analysis

Enzymatic digestion

After separation and detection of the proteins by electrophoresis through a gel matrix, the formed bands around 50 kDa in non-reducing condition and 30 and 17 kDa in reducing conditions were excised, perforated, and subjected to enzymatic digestion using trypsin from porcine pancreas indicated for use in proteomics, treated with trypsin from porcine pancreas (reference T-6567; Sigma-Aldrich, USA) [26]. The trypsin solution was prepared at the final concentration of 25 mg/L.

Mass spectrometry

The tryptic peptides from enzymatic digestions were resuspended in 100 µL of 0.1% formic acid aqueous solution, centrifuged at 20 000 x g for 20 min, with 90 µl of this solution being transferred to sampler flasks, total recovery model (Waters, USA). Tryptic peptides from each sample were applied to an LC-MS/MS system, composed of a nanoAcquity UPLC (Waters, USA) and a micrOTOF-QII–QTOF mass spectrometer (Bruker Daltonics, Germany). The chromatographic analysis of the samples occurred in a trap column and a capillary column ProteCol C18 GHQ303 3.0–300 µm × 150 mm, operating at a flow rate of 4.5 µL/min. The peptides were automatically eluted and injected into the mass spectrometer, acting in online mode, with the aid of a microESI needle. In this step, solutions of water and 0.1% formic acid v/v and ACN and 0.1% formic acid v/v were used as mobile phases A and B, respectively. The programming started with a desalination step with the maintenance of 5% of mobile phase B v/v for 14 min. Then, a gradient consisting of a linear ascent ramp starting from 5 to 50% of mobile phase B v/v for 30 min was performed, remaining at 50% for 5 min, linear ascent ramp starting from 50 to 90% of mobile phase B v/v for 3 min, remaining at 90% for 2 min, linear descent ramp starting from 90 to 10% of mobile phase B v/v for 3 min, followed by maintenance at 10% for 3 min. Data acquisition lasted approximately 60 min. The ion scanning occurred in the positive mode for the MS^1 spectra in the mass range between 100 and 2000 m/z and MS^2 between 70 and 2000 m/z. The quadrupole TOF (QTOF) mass spectrometer was operated in the auto-MSn mode, which made the acquisition of the MS^2 spectra for the most intense ions in each complete scanning spectrum, excluding single charge ions [27].

The data acquisition in the QTOF was managed by the Hystar application, version 3.2 (Bruker Daltonics). The spectra were processed using Data Analysis, version 4.0 (Bruker Daltonics), and CompassXport, version 3.0 (Bruker Daltonics), using the standard configurations for proteomics.

Protein identification

Protein identification was performed by comparing the mass lists generated and the protein sequences of the taxonomic group of green plants–Viridiplantae, annotated and reviewed in the SwissProt group, deposited in the database UniProt Knowledgebase [28].

The mass lists generated by the QTOF were converted into the mgf format and were, then, compared to the database through the MASCOT Server software (Matrix Science, USA). The search was performed using the MS/MS ion search form at http://www.matrixscience.com/search_form_select.html, accessed on May 23, 2019. The parameters used for the research were: enzymatic digestion by the trypsin with a lost cleavage, carbamidomethylation of cysteine as fixed modification and oxidation of methionine as a variable modification, error tolerance of 0.3 Da for the parent ion, and 0.6 Da for the fragments. Proteins were identified by the presence of at least two peptides aligned in their sequence.

2.3.4 | Zeta-potential for pI determination

The zeta-potential of γ-conglutin was determined as a function of pH, using the instrument Zetasizer Nano Series (Malvern Instruments, Worcestershire, UK), following the Smoluchowski approximation for electrophoretic mobility [29]. The purified γ – conglutin was dispersed in water (0.45 mg/mL). The pH of this solution was automatically adjusted in the range 11.0–2.0, using HCl or NaOH 0.1 mol/L and 25°C.

2.3.5 | Attenuated total reflection–Fourier-transform infrared and secondary structure

Infrared spectra of γ-conglutin were recorded using a Fourier-transform infrared (FTIR) spectrometer equipped with an attenuated total reflectance cell (Varian 640-IR) [30]. A dried protein sample was placed on the attenuated total reflectance crystal and the spectra were recorded in the range of 4000–400 cm^{-1} (16 spectra at 4 cm^{-1} resolution) and referenced against the empty cell. The spectrum was subjected to the second derivative analysis and curve-fitting procedures to locate overlapping peaks in amide–I (1700–1600 cm^{-1}). The relative proportions of different secondary structures were determined by computing the areas of spectral components (Gaussian peaks) attributed to a particular substructure in the region of amide–I [31].

2.3.6 | Nano differential scanning calorimetry

The thermal denaturation of γ-conglutin was evaluated using a NanoDSC calorimeter (TA Instruments, USA). A stock solution of γ-conglutin was prepared in sodium acetate buffer (0.025 mol/L, pH 4.5) at a concentration of 1 mg/mL. This solution was degassed for 10 min and filled into the nano differential scanning calorimetry sample cell. The same buffer solution without protein was

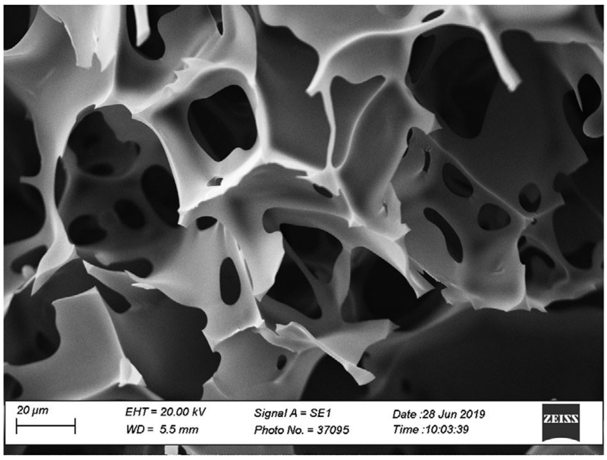

FIGURE 1 Scanning electron micrograph of the cation exchange cryogel produced

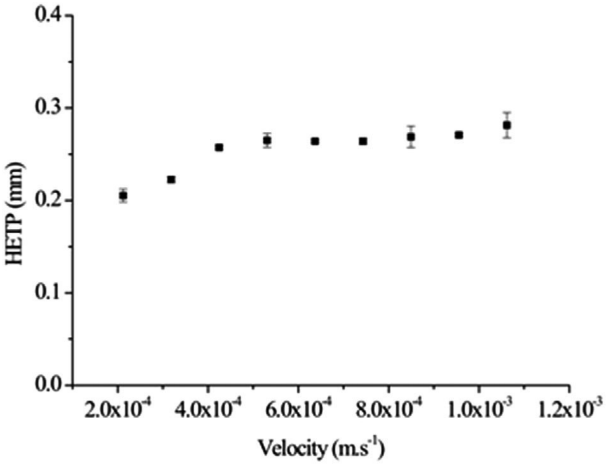

FIGURE 2 Height equivalent to a theoretical plate (HETP) at different flow velocities (2.12×10^{-4} to 1.06×10^{-3} m/s)

placed in the reference cell, in order to obtain a sample versus buffer scan. The temperature of the cells was increased from 30 to 110°C, at a rate of 1°C/min, and the heat flow necessary to keep the two cells in thermal equilibrium was recorded. A scan was also performed with the buffer solution of the sample. The thermogram was prepared for analysis by subtracting the buffer.

3 | RESULTS AND DISCUSSION

3.1 | Cryogel characterization

The cryogel was synthesized by the polymerization of the monomers AAm and MBAAm in a semi-frozen liquid media in which the formed ice crystals acted as porogen and thus, as a template for the shape and size of the highly interconnected pores. This basic cryogel matrix has low or no ability to interact with proteins due to the absence of functional binding groups on the pore walls. In this study, cryogels were activated with AMPSA, providing sulfonic groups to the cryogel matrix [22].

The produced cryogel (Figure 1; 10.5 cm column length) presented large pores (10–100 µm in diameter) continuously interconnected and distributed along the monolith, which provide channels for the mobile phase to flow. The hydraulic permeability (K_W) was determined as 5.154×10^{-13} m^2. This result was comparable to those reported in the literature 8.27×10^{-13} and 5.9×10^{-13} m^2 [21,32]. The porosity of the monolith under study was 84.12% ($R^2 = 0.999$), which agreed with the results previously reported [22,33]. The low resistance caused by the highly interconnected pores allowed the use of higher flows with low backpressure, besides the possibility of use with concentrated or particulate solutions.

HETP (Figure 2) was in the range of 0.2–0.3 mm. In the cation exchange cryogel developed in another study [22], the results of HETP were in the range of 0.8–1.3 cm, that is, a smaller number of equilibrium stages compared to results obtained in our study.

3.2 | γ-conglutin extraction and purification

Seed storage proteins consist of complex groups of biomolecules that are often difficult to purify due to their heterogeneous nature and the lack of unique functionalities. In addition, the major storage globulins, namely 7S and 11S globulins, are oligomeric proteins and exhibit dissociation/association behavior, depending on the pH and ionic strength of the medium [34]. Lupin globulins are the major storage proteins (~ 87% total protein) followed by albumin (~13% total protein). Glutelins and prolamins are found in very small quantities [3].

Total soluble proteins (36.5 ± 1.5% w/w) composed of globulins and albumins were extracted from lupin seed meal by alkaline-saline extraction. The globulins were subsequently fractionated by acid precipitation at pH 4.5. After precipitation of most of the globulins at isoelectric pH (pH 4.5), the soluble fraction contained in the supernatant was estimated to be 9.6% of the total proteins obtained by alkaline-saline extraction. This fraction was used in the chromatographic steps.

A typical elution profile is shown (Figure 3). The first breakthrough occurred at one CV and is due to anionic proteins present in the extract that cannot adsorb (probably α- and β-conglutins) with pI in the range of 5.0– 6.0 [35] and remained soluble after acid precipitation. The second breakthrough occurred around two CVs, after column

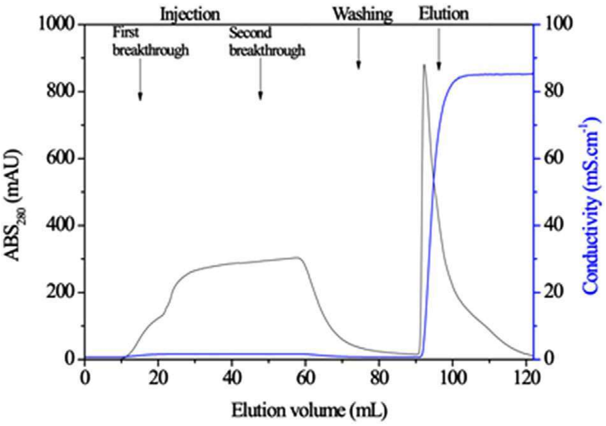

FIGURE 3 Purification profile of γ-conglutin by cation exchange chromatography. The column was equilibrated with 0.025 mol/L sodium acetate (pH 4.5) and eluted with the same equilibration buffer containing 1 mol/L NaCl. The flow rate was 2 ml/min in ambient room temperature

saturation. Dynamic binding capacity was calculated as shown in the equation 5 [36], using the second breakthrough at 10% of rupture and the delay volume correction, giving rise to a 100.72 ± 1.10 mg γ-conglutin protein/g of cryogel, similar to other results reported in the literature [36]. The protein adsorbed corresponded to 68.02 ± 8.05% of the total proteins present in the pre-purified extract obtained from the acid extraction step.

The SDS–PAGE of the injected sample and then eluted from the column is presented (Figure 4A). A band close to 66 kDa is observed in lane 1 (pre–purified extract after alkaline-saline extraction), indicating the presence of the α- and β-conglutin fractions. In lane 4 (eluted from the column) there is a single band indicating the presence of a single protein. This unique band in the non-reducing condition has an approximate molar mass of 48 kDa. In the reductive condition (Figure 4B), two bands (lane 4) are presented: one with an approximate molar mass of 30 kDa and another with an approximate molar mass of 18 kDa. These results indicate that only one protein was adsorbed on the column, suggesting, in principle, to be the γ-conglutin. The presence of two bands in the reducing condition is due to the breaking of the SS–bonds. The electrophoretic profile presented in this study is in accordance with that presented in the literature [37].

3.3 | Protein identification and characterization

3.3.1 | Mass spectrometry

Results of MS enable the identification of two conglutins in the sample obtained in the non-reducing condition (Table 1). In the results obtained by Mascot software (Matrix Science, London, UK), score values above 30 indicate extensive identity or homology ($p \leq 0.05$). The best identification protein score was 445, for the γ-conglutin 1 protein, which was very different from the others, with nine peptides identified and 30% coverage

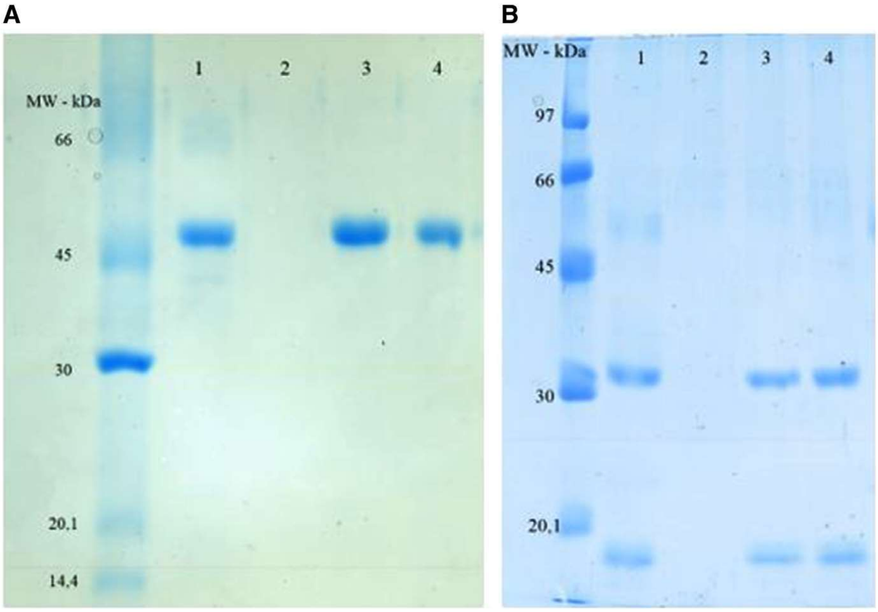

FIGURE 4 Sodium dodecyl sulfate (SDS)–PAGE of the molecular maker (Lane 0), pre-purified extract (Lane 1), washing (Lane 2), second breakthrough (Lane 3), and sample eluted from cation exchange cryogel column (Lane 4). (A) Non-reducing condition and (B) Reducing condition

TABLE 1 Proteins identified by the LC-MS/MS strategy and compared to sequences deposited in SwissProt by MASCOT online

					Theoretical			
Sample*	**Access number**	**Protein name**	**Coverage**	**Protein score**	**MW (kDa)**	**pI**	**Sequence of peptides identified**	**Score**
1	Q9FSH9	γ-conglutin 1	30%	445	49.8	8.39	LHWGNILKR	48
							TPLMQVPVLLDLNGK	58
							IPQFLFSCAPTFLTQK	43
							QGEYFIQVSAIR	54
							AVGPFGLCYDTK	84
							KISGGVPSVDLIMDK	36
							ISGENLMVQAQDGVSCL-GFVDGGVHTR	34
							AGIALGTHQLEENLVVFDLAR	68
							VGFNTNSLK	57
1	Q9FEX1	γ-conglutin 2	6%	160	50.2	6.61	RTPLMQVPVLLDLNGK	64
							SRVEFNSNSLK	40
2	Q9FSH9	γ-conglutin 1–α chain	8%	149	29.2	8.73	LHWGNILKR	38
							TPLMQVPVLLDLNGK	55
							IPQFLFSCAPTFLTQK	47
3	Q9FSH9	γ-conglutin 1–β chain	15%	223	16.4	6.16	HSIFEVFTQVFANNVPK	47
							AVGPFGLCYDTKK	76
							ISGGVPSVDLIMDKSDVVWR	31
							SRVGFNTNSLK	35
							SCSNLFDLNNP	53

*Sample 1: Non-reducing condition; Samples 2 and 3: Reducing condition.

(Viridiplantae (Green plants – 39 930 sequences) database accessed on May 23, 2019). However, the presence of γ-conglutin 2 isoform (score 160) cannot be disregarded due to the presence of the two peptides, found as unique to it.

The peptide sequence found for the γ-conglutin 1 isoform (VGFNTNSLK) differed in two amino acids for the γ-conglutin 2 isoform (SRVEFNSNSLK), presenting a glycine and a threonine for isoform 1 while isoform 2 presented a glutamic acid and one serine for the same positions in the peptide analyzed. The γ-conglutin is divided into two classes in almost all varieties of lupin: γ_1 and γ_2, with the closest homology, found within the species *L. angustifolius* and *L. albus* [38]. In relation to the peptides identified in the bands referring to the reducing condition, they were identified as being of the γ-conglutin.

3.3.2 | Isoelectric point

Unlike most plant proteins having pI around 4.5, the γ-conglutin purified in this study has a pI of 7.51 (Figure S1). The sequence and interactions of the amino acids that determine the distribution of electric charges and their side chains, as well as the 3-D conformation of the protein, are associated with its basic characteristic. In addition, this characteristic can be influenced by the low proportion of glutamic acid (pI = 3.22), around 2–4 times lower than that of α-, β-, and δ-fractions [39,40]. The obtained pI value is similar to those reported in the literature, which is in the range from 7.66 to 8.39 [35,41]. Variations in the pI are expected, considering the analysis methodology used and the variety of lupin seed used, cultivated under different soil and climatic conditions.

3.3.3 | Secondary structure

FTIR spectrum was obtained to study the secondary structure of the purified γ-conglutin. The amide-I band (1700–1600 cm^{-1}) is highly sensitive to small variations in molecular geometry and hydrogen bonding patterns, such that each type of secondary structure gives rise to a different C=O stretching frequency. It is therefore especially useful for analysis of the secondary structure composition and conformational changes of proteins [42]. The self-second

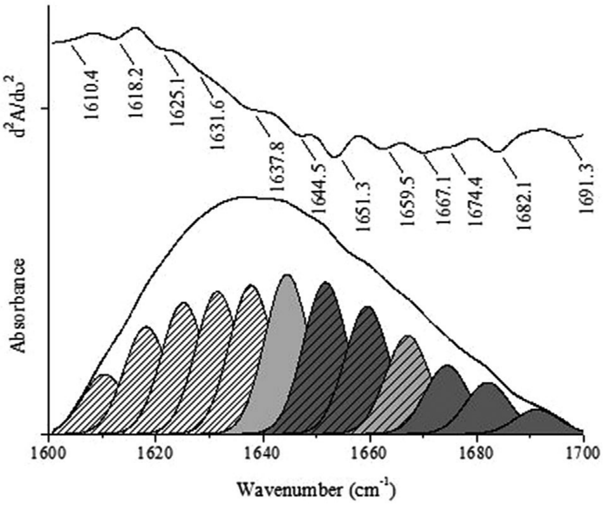

FIGURE 5 Second derivative spectra and Gaussian curve fitted of γ-conglutin protein. Component bands assigned to β-sheet structures are shaded white and striped. Random coils structures are shaded light grey. A-helix structures are shaded dark grey and striped. Turns structures are shaded light grey and striped. β-sheets/turns are shaded dark grey

derivative spectrum and Gauss curve-fitting procedure are presented where several peaks were obtained, which usually considers that structures can be identified by bands of wavenumbers (Figure 5).

The region between 1610 and 1640 cm^{-1} is characteristic of β-sheet structures while the bands above 1673 cm^{-1} may be due to turns/β-sheets. α-Helix structures have a band peak at wavenumbers of 1650–1658 cm^{-1}. Random coil structures occur at around 1645 cm^{-1} and turn structures, at around 1667 cm^{-1} [31]. The secondary structure composition was found to be 46.34% β-sheets, 21.96% α-helix, 12.56% random coils, and 11.35% β-sheets/turns, and 7.75% turns. The purified γ-conglutin is very rich in β-structures, but there is also a high α-helix content. Therefore, the γ-conglutin should be a polypeptide with a highly ordered and stable conformation. These results are in good accordance with previous data obtained with *L. albus* γ-conglutin analyzed by FTIR [43] and data obtained with *L. angustifolius* γ-conglutin crystal analysis [8].

3.3.4 | Thermal characteristic of γ–conglutin

The secondary protein structure is organized by the presence of non-covalent interactions between the lateral chains of amino acids, as well as the presence of disulfide bonds. At temperatures from 25 to 90°C, the α-helix and the β-sheet structure unfold into the disordered structure and turns, with a major conformational transition occurring at 55°C [44].

Differential scanning calorimetry is used to provide essential information about the thermal properties of proteins. Heat-induced changes in protein structure are accompanied by changes in the thermogram, characterized by the presence of an endothermic peak. The thermal characteristics of γ-conglutin were determined and the thermogram is shown (Figure S2), where the T_d was 68.31°C. The thermal transition was irreversible, as confirmed by the second scan performed after cooling the solution (data not shown). This result is in accordance with the T_d of the γ-conglutin found, which was between 64.4 and 67.3°C [45]. However, this result differs from another reported result, where the mean T_d was 56.5°C in the monomeric condition of the gamma conglutin (pH 4.5) [46]. The author further explains that during exposure to temperature, various types of interactions between monomers can occur resulting in the formation of temporary/permanent complexes with a different number of protomers, thus influencing the T_d, which explains the results found in the different works cited with this study.

4 | CONCLUDING REMARKS

In this study, a protocol for the purification of γ-conglutin, present in lupin seeds was developed. The use of the saline-alkaline extraction, acid precipitation, and cation exchange chromatography steps led to obtaining high purity γ-conglutin, as confirmed in SDS–PAGE electrophoresis by the presence of a single band. The identity of this single band was confirmed by MS as γ-conglutin. This protein, considered a basic protein, was confirmed by its pI and has a highly ordered secondary structure rich in β-sheets. These results, combined with the knowledge of its T_d, can thus provide useful information for the possible use of this globulin in the food processing industry and the direction of protein engineering research to improve its physicochemical properties.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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Supporting Information

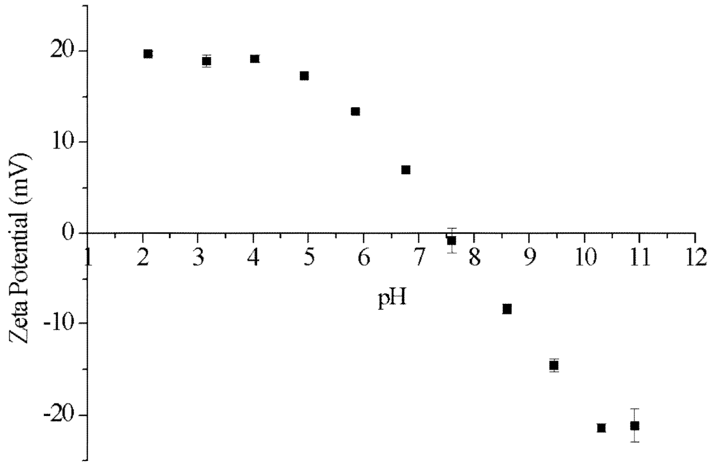

Figure S1 – ζ-Potential of purified γ–conglutin at different pH values.

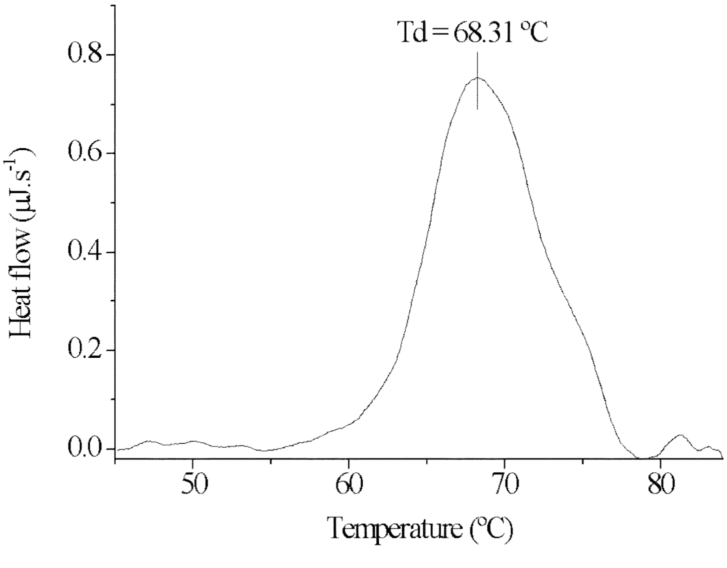

Figure S2 – NanoDSC thermogram of 1 mg/mL γ – conglutin solution at pH 4.5.

4. CAPÍTULO 2

Evaluation of pH, NaCl and $CaCl_2$ salts on solubility, zeta potential and air - water interfacial properties of the protein isolate from lupin seeds

Este capítulo será submetido ao periódico Food Hydrocolloids.

Evaluation of pH, NaCl and $CaCl_2$ salts on solubility, zeta potential and air - water interfacial properties of the protein isolate from lupin seeds

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ABSTRACT – Due to the large population increase and the consequent need for greater food production capacity, plant proteins have been gaining great attention. Aspects of health and environmental sustainability increase interest in the use of these kinds of foods. However, literature is still scarce in relation to the knowledge of its technological properties. Lupin is a legume seed rich in proteins and presents high nutritional and health benefits but is little used in the human diet. In this work, lupin proteins were extracted using an alkaline-saline solution followed by dialysis. Then, globulins were separated by isoelectric precipitation to give lupin protein isolate (LPI). The solubility, zeta potential and interfacial properties of LPI were evaluated at pH of 3.4 and 6.8 in the presence of NaCl or $CaCl_2$. The solubility of the LPI was found to be dependent on the pH and the type of salt studied. Besides, this study showed that the ability of LPI to reduce interfacial tension is mainly due to electrostatic interactions. In addition, the type of salt changes the electrostatic shielding of the LPI electrical double layer, which leads to a decrease in electrostatic potential at the surface of LPI. The rate of diffusion is highest at pH 6.8 under conditions without salts or in the presence of NaCl. When the salt was changed to $CaCl_2$ diffusion was decreased, probably due to protein-protein interactions. On the other hand, the protein adsorption rate is lower at pH 6.8 under all conditions studied. Apparently, it is influenced by the type of surface electrical charge of the proteins and the rate of rearrangement is greater than the rate of adsorption. The results obtained demonstrate that the lupin protein isolate can be used in multiphase food systems, such as foams, due to its interfacial properties.

1. Introduction

The rising of the world's population and the harmful effect of livestock on the environment has required an increased supply of sustainable sources of protein. An important source of gas emissions is ruminant – based animal production. Also, high intake of animal proteins has been associated with increased risk of type 2 diabetes, cardiovascular disease, colorectal cancer, and early death (Adeva-Andany et al., 2022). The growing demand for vegetable proteins is based on their low cost of production, high availability in nature and greater consumer preference for healthy ingredients. Plant protein isolates or concentrates can therefore be used to replace meat or milk proteins in many processed foods (López et al., 2019; Timilsena et al., 2016). Thus, the food industry is interested in the use of vegetable proteins as an alternative to milk or egg proteins for the development of foams or emulsions.

Legume seeds are an abundant source of protein, and lupin is a starch-free leguminous seed with a high protein content, almost as high as that of soybeans (30% - 40%), and a relatively low oil content (Duranti et al., 2008; Shrestha et al., 2021). The main lupin proteins are globulins (around 87% of total proteins), followed by albumins (around 13% of total proteins), while gluttelins and prolamines are found in very low quantities (Mane et al., 2018). Globulins are soluble in saline solutions and are divided into four main fractions known as α–, β–, δ– conglutin (acid nature) e γ – conglutin (basic nature) (Muranyi et al., 2016). Many studies have shown that lupin proteins have several beneficial health effects on chronic disease control (Arnoldi et al., 2015; Cabello-Hurtado et al., 2016).

Some technological properties of lupin proteins have already been studied. Wong et al. (2013) demonstrated the high foaming capacity of these proteins, comparable with egg white. Burgos-Díaz et al. (2016) reported that the different fractions (albumin and globulins) influence the emulsifying and foaming properties and concluded that, in general, lupin proteins have great potential for technological use in the food industry. Völp et al., (2021), in a study on the interfacial activity of lupin proteins, reported the equilibrium interfacial tension ranged between 42 and 44 mN/m. The foams produced stayed stable for at least 1 h and the foaming capacity increased as pH or ionic strength increased.

It is known that the surface charge of proteins depends on the ionic strength and/or the pH value of the bulk solution. The presence of salts causes charge shielding by interaction or actual binding of salt ions to certain functional groups in the amino acid sequence. Thus, the intermolecular interactions of proteins are modified (Dombrowski et al., 2018a). Values of pH in the neutral or slightly acidic region are common in food systems and may contain salts such as NaCl and $CaCl_2$. The impact of pH and these salts on the structure and functionality of proteins are critical factors for surface film formation and stabilization.

Studying the dynamic process of interfacial adsorption of LPI at the air-water interface is vital to understand and regulate its foaming properties and expand the possible applications of LPI from a colloidal interface perspective. Furthermore, the measurement of the diffusion, penetration and rearrangement rate constants of LPI at the colloidal interface provides support for the precise control of its macroscopic properties. Thus, the general objective of this study was to evaluate the solubility, zeta potential and interfacial properties of lupin globulins (LPI) as a function of pH and salt type (NaCl or $CaCl_2$). The results obtained will contribute to the development of colloidal food systems based on lupin globulins.

2. Material and Methods

2.1. Globulin Extraction

Lupin globulins were extracted according to Silva et al. (2021). Briefly, the lupin seeds were peeled and the cotyledons were milled to obtain flour (24 mesh). The flour was defatted in a Soxhlet apparatus by hexane extraction at 37°C for 6 h. After extraction, the solvent was removed by evaporation (40 ºC and 12 h).

The defatted lupin flour was suspended in a sodium phosphate buffer, pH 8.5 (1:20 m/v) in the presence of NaCl 0.5 mol.L^{-1} under stirring for 12 h at room temperature (25° C). The suspension was then filtered through an 0.80 µm nylon membrane and the filtrate was dialyzed against water to remove salt for 4 days at a temperature of 4°C. The aqueous protein extract was centrifuged for 10 min at 10,000 x g at 4 °C for removal of the albumins. The precipitate composed of globulins was then freeze dried (- 40 ºC and 0.12 mBar) and stored under refrigeration for later use.

2.2. Zeta Potential and Solubility

The zeta-potential of the LPI was determined using a dynamic light scattering (DLS) method as a function of pH, using the instrument Zetasizer Nano Series (Malvern instruments, Worcestershire, UK). The dried LPI was dispersed in water (1 mg.mL^{-1}). The pH of the solution was automatically adjusted in the range of 11.0 – 2.0, using HCl or NaOH 0.1 mol.L^{-1} at 25 ºC. The same procedure was performed to evaluate zeta potential in the presence of NaCl or $CaCl_2$ with ionic strength (IS) set at 0.1 mol.L^{-1}.

Protein solubility was determined in the pH range of 11.0 – 2.0. The LPI dried was dispersed in distilled water (1 mg.mL^{-1}) and the pH was adjusted with NaOH or HCl (1 mol.L^{-1}). The samples were stirred for 1 h and then centrifuged for 15 min at 10,000 × g. The concentration of soluble protein in the supernatant was determined by the biuret method.

Protein solubility was expressed as a percentage of the total protein. The same procedure was performed to evaluate protein solubility in the presence of NaCl and $CaCl_2$ (IS of 0.1 $mol.L^{-1}$).

2.3. Surface Tension

The experiments were carried out using LPI (1 $mg.mL^{-1}$) under different conditions, being: pH 3.4 or 6.8 in the presence or absence of 0.1 $mol.L^{-1}$ of NaCl or $CaCl_2$.

2.3.1. Determination of superficial tension at equilibrium

The superficial tension was monitored by an automatic drop tensiometer (PAT-1M, Sinterface Technologies e.K., Berlin, Germany) at 25 °C for 2.5 h. Briefly, a drop of the LPI solution at a defined condition (20 mm^2 area) was automatically formed at the tip of a capillary (2 mm diameter) inserted in an air space. The drop profile captured by a CCD camera and the surface tension was calculated by fitting the Laplace equation, using the device-specific software (Sinterface Tensiometer PAT 1M v. 8.01). The equilibrium surface tension was determined by adjusting an exponential model (Equation 1) to the experimental data (Neves et al., 2018):

$$\gamma(t) = \gamma_{eq} + Aexp\left(-B\sqrt{t}\right) \qquad (1)$$

where γ is the surface tension ($mN.m^{-1}$) at time t (s), γ_{eq} is the surface tension at equilibrium ($mN.m^{-1}$), A and B are parameters of the model.

2.3.2 Dilatational viscoelasticity analysis

After equilibrium was reached, sinusoidal oscillations were applied to the drop volume and the interfacial tension response was recorded. The amplitude of the oscillations was set to 10% to keep within the linear viscoelastic region and the frequency ranged from 0.01 to 0.2 Hz. The dilatational parameters elastic modulus (E') and viscous modulus (E") and the phase angle ($\tan \phi = E'/ E''$), were calculated using the Fourier transform algorithm implemented in the software package. All experiments were performed under constant temperature (25 °C), with three repetitions and the results were presented as mean and standard deviation.

3. Results and Discussion

3.1. Effects of pH and cation type on zeta potential and solubility of LPI

The solubility and the zeta potential of the LPI are influenced by the pH (Figure 1A) and by the type of salt (Figure 1B and 1C). The zeta potential is described by a sigmoidal curve, with plateau formation around two pH units outside the isoelectric region. However, the magnitude of the charge distribution is different for each condition presented (absence or presence and the type of salts). These conditions are reflected in the solubility profile as well as in the isoelectric points presented in Table 1, where in the absence of salt the IP was 5.19 while in the presence of NaCl, 4.41 and $CaCl_2$, 4.98.

The zeta potential results are also presented in Table 1. In the absence of salt, it ranged from -30.0 to +30.0 mV, while in the presence of NaCl, it was from -10.0 to +10.0 mV and with $CaCl_2$, -5.0 to +15.0 mV, indicating an electrostatic shielding effect of salt ions that leads to a decrease in electrostatic potential at the surface of LPI, which in turn can form molecular aggregates. These effects induce proteins to present different adsorption behaviors and interfacial denaturation, thus exhibiting different interfacial properties (Xiong et al., 2019).

The globulins in the presence of salt are surrounded by counter-ion and co-ion, leading to a phenomenon known as shielding of the surface charge of proteins. $CaCl_2$ provides more counter-ions in the basic pH range, maintaining a lower solubility of proteins when compared to the proteins in the absence of salt or in the presence of NaCl. As observed by the alteration of the zeta potential and solubility of the protein isolate, other alterations such as changes in the hydrodynamic radius, net charge, surface hydrophobicity and molecular flexibility may occur, and these modifications may alter the formation and stabilization of the interfacial film.

The minimum solubility of globulins in the absence and presence of salt in the isoelectric region is expected (pH 5 – 6, ~55%). Salts alter the solubility profile of proteins as they interact with the surface charges of proteins promoting the attraction and aggregation of protein particles. The divalent salt $CaCl_2$ is more effective in screening protein charges, thus inducing greater protein aggregation as seen in Figure 1C, mainly in the pH range of 5 to 10, because the proteins assume a net negative charge.

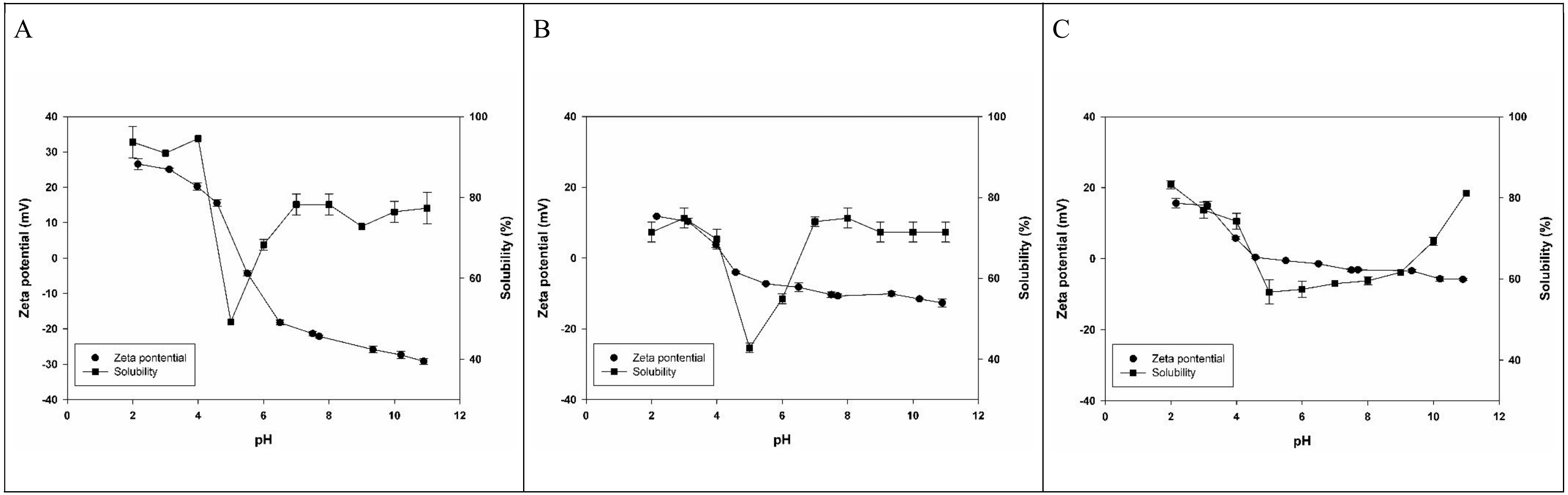

Figure 1 – Zeta potential and solubility of LPI in the absence of salt (A), in the presence of 0.1 ionic strength NaCl (B) and 0.1 ionic strength $CaCl_2$ (C).

3.2. Protein adsorption to air – water interface

The surface activity of LPI in the absence or presence of salts was explored at the air – water interface using the pendant drop method and it is presented in Figure 2. The surface tension was characterized by a rapid reduction, which is subsequently moderate, until a constant value is reached. This profile is similar in all conditions studied, where this decrease in the surface tension is associated with the adsorption of the globulins at the interface. This adsorption is attributed to a two-stage process, as a result of the initial rapid diffusion of proteins to the interface, followed by a slower and delayed adsorption by electrostatic and steric hindrance (Noskov, 2014).

The ability of LPI to reduce interfacial tension (Table 1) was greater in the absence of salts and pH 3.4, where the four protein fractions that compose these globulins have a net positive charge. These results suggest that, at pH 3.4 and in the absence of salts, proteins have greater mobility due to a lower protein-protein interaction, being able to diffuse and adsorb at the water/air interface faster at acidic than at neutral pH. Therefore, greater reduction in interfacial tension is achieved. In addition, although $CaCl_2$ is more effective than NaCl at equimolar concentration for screening electrostatic forces (Dombrowski et al., 2018), maintaining the same ionic strength, the surface tension was similar at pH 3.4 for both salts. These results agree with the theory of a decreasing adsorption barrier with increasing shielding of the surface charge of the protein (Dombrowski et al., 2018), where protein-protein interactions may prevail.

According to (Dombrowski et al., 2018), proteins carry a high surface charge and must overcome a relatively high energetic barrier to adsorption, which ends up decreasing their adsorption rate. The presence of salts alters the size of the electrical double layer, thus reducing the protein-protein repulsion and increasing its interaction, which leads to a less interfacial coverage. This effect is clear by observing the results at pH 6.8, where the proteins are mostly negatively charged. When $CaCl_2$ is added, the Ca^{2+} ions interact with the negative charges of the proteins, thus reducing their double electrical layer, which is in accordance with the results of zeta potential.

The equilibrium surface tension results of this study in the absence of salts are similar to the results obtained by Völp et al. (2021) studying lupin protein isolate of *Lupinus angustifolius*, where at pH 6.8 they obtained values between 42 and 44 $mN.m^{-1}$. The same authors also performed an experiment at pH 4.0, where the equilibrium tension remained at 47 mN/m. This result suggests a lower ability to reduce surface tension when compared to the value found in this study at pH 3.4 (~42 $mN.m^{-1}$). Thus, we can verify that the use of pH outside the isoelectric range of the LPI favors a greater reduction of interfacial tension.

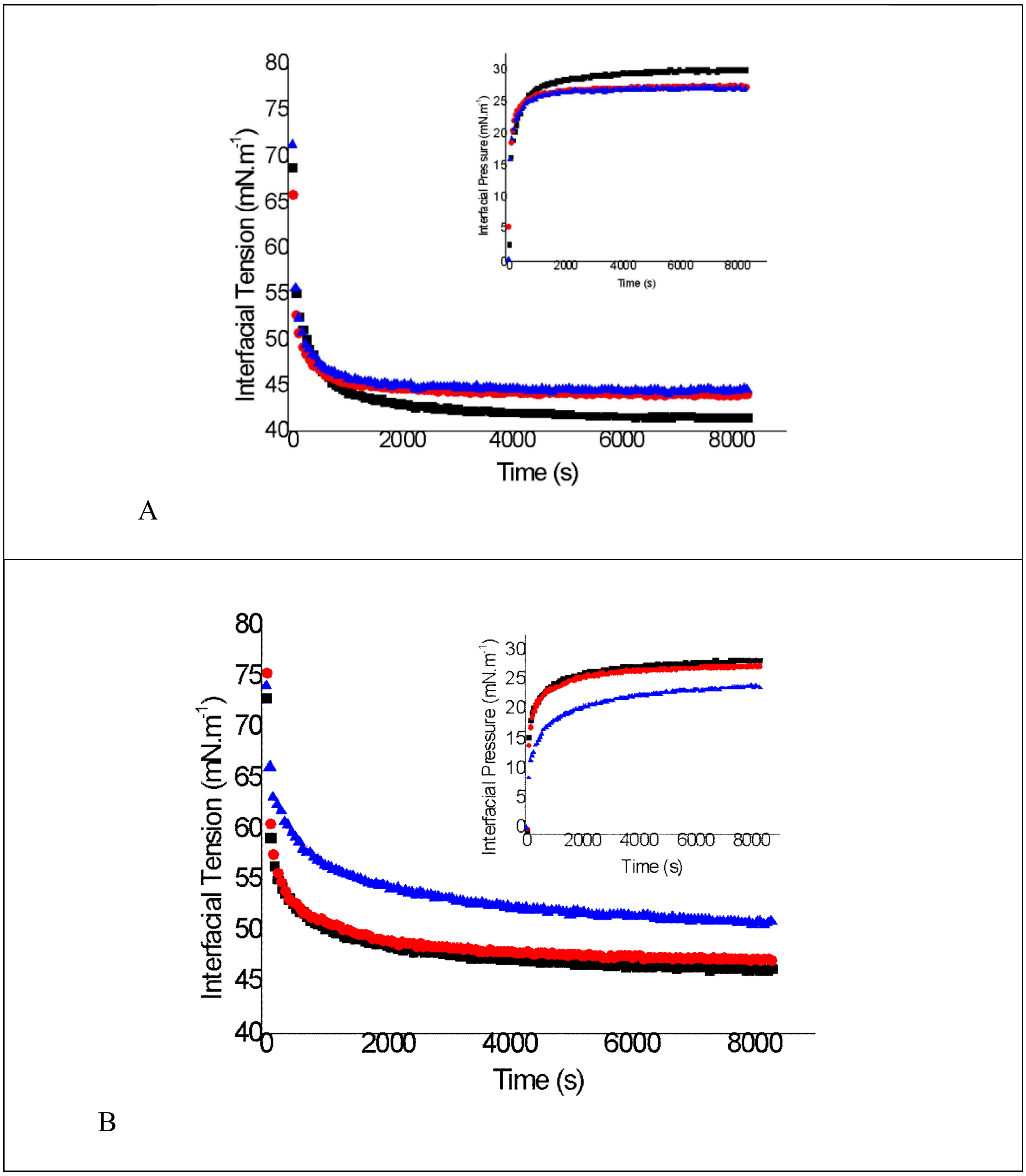

Figure 2 – Reduction of surface tension and interfacial pressure for air – water interfaces stabilized with globulins at pH 3.4 (A) and 6.8 (B) in the absence of salt (■), in the presence of 0.1 ionic strength NaCl (●) and in the presence of 0.1 ionic strength $CaCl_2$ (▲).

Table 1 – Values of surface tension at equilibrium (γ_e), zeta potential (mV) and isoeletric point for globulins at different systems.

Sample	γ_e (mN.m^{-1})		Zeta Potential (mV)		Isoelectric Point
	pH 3.4	pH 6.8	pH 3.4	pH 6.8	
Globulin	41.79 ± 0.63	46.73 ± 0.14	22.63 ± 0.43	-19.80 ± 0.39	5.14 ± 0.07
Globulin + NaCl	44.22 ± 0.34	47.61 ± 0.28	7.07 ± 0.29	-9.30 ± 0.27	4.41 ± 0.08
Globulin + $CaCl_2$	44.51 ± 0.21	50.54 ± 0.23	10.37 ± 0.78	-2.32 ± 0.34	4.98 ± 0.13

*SD = standard deviation.

3.3. Dynamic surface viscoelasticity

According to Lucassen & Van Den Tempel (1972), the interfacial dilational modulus ε is a measure of the interfacial resistance to changes in the surface area of the drop and is defined as the change in interfacial tension γ when subjected to a relatively small change in the interfacial area A ($\varepsilon = d\gamma/dA$). The dilational modulus can also be defined as $\varepsilon = \varepsilon' + \varepsilon''$, ε' being the real part (storage modulus or dilational elasticity) and ε'' being the imaginary part (the interfacial dilatational viscosity modulus related to the dissipated energy in the relaxation process) (Cao et al., 2017). The relationship between storage modulus and viscosity modulus results in $\tan\theta = \varepsilon''/\varepsilon'$, where θ is known as the phase angle. Lower values of θ (<45°) indicate a predominantly elastic character of the interface and greater θ values (>45°) a predominantly viscous interface (Simiqueli et al., 2019). The interfacial elasticity value can be used to predict the stability of the interface against Ostwald Ripening using the Gibbs Index (G_i), which states that interfaces are stable when $G_i = \frac{E_D}{E_q} > 0.5$ (Becher, 1982), where E_D is elastic dilatational modulus and E_q is equilibrium surface tension.

Figure 3 shows the dilatational modulus, phase angle and Gibbs Index at different conditions as a function of frequency. In general, the samples showed an elastic behavior substantially higher than the viscous modulus, in addition to presenting a phase angle <45 °.

This is attributed to the formation of a protein film at the interface, typical for solutions stabilized with proteins. Regarding pH, the elastic component has a strong dependence on the pH, where it is greater at pH 3.4. As explained before and observed by Felix et al. (2017) and Felix et al. (2019), this is related to the unfolding of proteins that can occur even before adsorption at the interface. In addition, using the Gibbs Index, the results obtained in Figure 3E and 3F indicate that protein solutions at pH 3.4 present more stable interfaces than at pH 6.8.

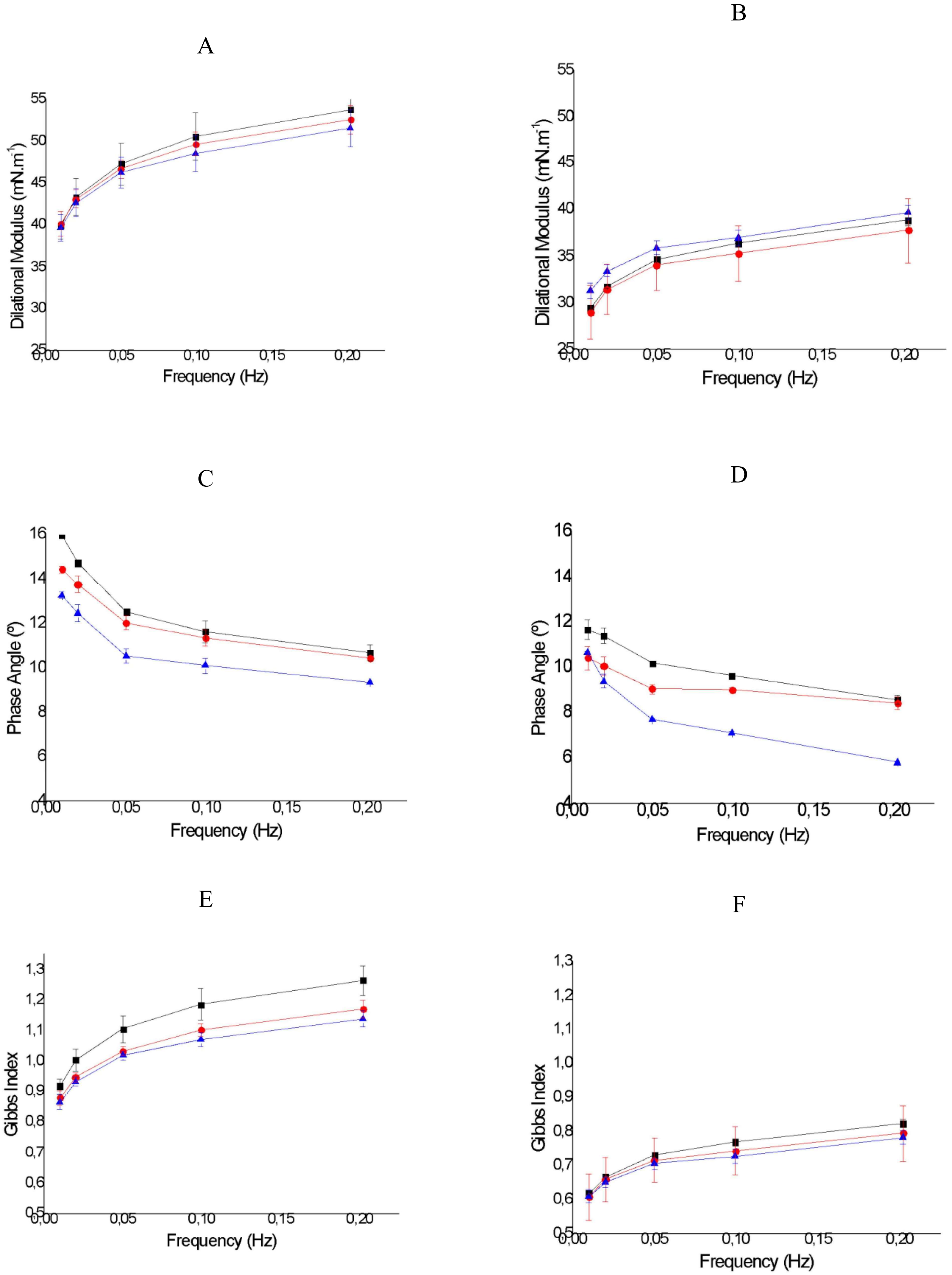

Figure 3 – Dilatational modulus and phase angle as a function of frequency for air – water interfaces stabilized with globulins at pH 3.4 (A – C – E) and pH 6.8 (B – D – F) in the absence

of salt (■), in the presence of 0.1 ionic strength NaCl (●) and in the presence of 0.1 ionic strength $CaCl_2$ (▲).

3.4. Kinect Study

To obtain more information about the interfacial behavior of the LPI, the surface tension data were analyzed using the kinetic approach, and the kinetic parameters, which characterize various surface/interfacial mechanisms, were determined and the results are presented in Table 2. Several dynamic stages of the adsorption process can be distinguished: diffusion, adsorption, penetration and unfolding, interfacial reorientation, and rearrangement and aging (Mileti et al., 2022).

During the first adsorption step, at lower interfacial pressures, diffusion is the predominant step, thus the Ward and Tordai equation 2 can be used to determine the diffusion coefficient (Ward & Tordai, 1946):

$$\pi = 2C_0K_BT\sqrt{\frac{Dt}{3.14}} \tag{2}$$

where C_0 is the protein concentration in the continuous phase, K_B is the Boltzmann constan, T is the absolute temperature, t is the time e D is the diffusion coefficient. If the adsorption process is controlled by protein diffusion, a fit of π versus $t^{0.5}$ will be linear, and the regression coefficient of the fit will be the diffusion rate (K_{diff}) (Macritchie, 1978). After the protein diffuses to the interface, interfacial adsorption and molecular rearrangement can be evaluated by the first order equation 3 below:

$$Ln\left(\frac{\pi_f - \pi_t}{\pi_f - \pi_0}\right) = -K_0t \tag{3}$$

where π_f, π_t and π_0 is the interfacial pressure at the final time, at time t and at the initial time, respectively. K_0 and t represent the first-order rate constant and the time. The slope of the first region corresponds to the interfacial adsorption rate (Ka) of the protein and the second slope represents the interfacial rearrangement rate (Kr) of the protein (Zhou et al., 2021).

In general, the diffusion was influenced by the surface charge of LPI, where the rate of increase in the interfacial tension decreases with time due to interfacial saturation and an increase in the electrostatic/steric barrier. At pH 3.4, globulins assume a net positive charge and have a diffusion rate similar to pH 6.8, where globulins have a predominantly net negative charge, suggesting that electrostatic repulsions between globulins initially favor the rate of diffusion to the interface and then interfacial saturation occurs. At pH 3.4 and in the presence of NaCl, the diffusion rate was lower than at pH 6.8, while in the presence $CaCl_2$, an opposite effect was observed. When proteins acquire a higher zeta potential, there is a greater reduction in interfacial tension and higher rates of interfacial diffusion (Table 1). Once, as the proteins are all positively charged at pH 3.4, a lesser effect of the ions was observed. However, at pH 6.8, the proteins assume a predominantly negative charge.

The reduction of interfacial tension as well as the diffusion rate were directly influenced, as Na^+ and Ca^{2+} ions drastically alter the electrostatic shielding of the electrical double layer, favoring protein-protein interaction, which results in the formation of protein clusters with lower interfacial activity. Jeyarajah & Allen, (1994) proposed that the interaction between proteins (specifically β-Lactoglobulin) and Ca^{2+} occurs through charged carboxylic groups and further induces structural changes within the protein molecule, exposing hydrophobic parts of the molecules and leading to an increase in interactions forces protein-protein, in particular hydrophobic interactions. As hydrophobic interactions are necessary in interfacial adsorption, protein-protein interaction can limit adsorption and reduce its interfacial activity. In addition, Ca^{2+} changes the hardness of the solution, which can reduce the mobility of proteins that, together with the aggregates, reduce the rates of diffusion, penetration and rearrangement.

Table 2 - Diffuse rate K_{diff} ($mN.m^{-1}.s^{-0.5}$), molecular adsorption Ka (s^{-1}) and configurational rearrangement Kr (s^{-1}) parameters for adsorption of samples with different conditions on the air-water interface.

Sample	K_{diff} (t = 480 s)		Ka $\times 10^{-3}$		Kr $\times 10^{-3}$	
	pH 3.4	pH 6.8	pH 3.4	pH 6.8	pH 3.4	pH 6.8
Globulin	0.54 ± 0.05	0.57 ± 0.01	1.19 ± 0.01	0.949 ± 0.05	5.64 ± 0.02	1.78 ± 0.06
Globulin + NaCl	0.41 ± 0.05	0.55 ± 0.05	1.66 ± 0.03	1.07 ± 0.01	3.36 ± 0.01	3.38 ± 0.03
Globulin + $CaCl_2$	0.54 ± 0.01	0.45 ± 0.03	1.88 ± 0.02	0.93 ± 0.07	3.07 ± 0.02	3.16 ± 0.02

Regarding adsorption and interfacial rearrangement steps, adsorption rates are higher at pH 3.4 than at pH 6.8 which, apparently is influenced by the type of surface electrical charge of the proteins. When the LPI presents a net positive charge (pH 3.4), the rearrangement rate is higher than when the net charge is predominantly negative (pH 6.8) for all conditions studied, suggesting that the presence of a balance between different charges can delay the interfacial rearrangement rate. The LPI has a fraction (γ - conglutin) which, despite being in small concentration, has a pI close to the pH 6.8 studied. According to Buchmann et al., (2019), this condition could increase the hydrophobic attraction between the fractions, increasing the aggregation and reducing the mobility and interfacial rearrangement or also generate a competitive adsorption between the fractions.

In the rearrangement process, proteins continue to slowly change their conformation and build a viscoelastic interfacial film (Beverung et al., 1999). Additional layers can be added at the monolayer interface when the conformational changes of the adsorbed proteins provide a favorable environment for the sublayer proteins to interact with the adsorbed molecules. This could explain higher mobility after adsorption, with higher rates of rearrangement due to its constant dynamics of conformational change.

4. Conclusion

In this work, the interfacial properties, solubility and zeta potential of the lupin protein isolate were evaluated under the effect of pH and the presence of different salts. The solubility of lupin globulins is directly affected by pH and the presence of different salts. The solubility of the lupin protein isolate is minimal in the isoelectric region. The pH changes the charge distribution of the protein, modifying the protein-solvent and protein-protein interactions. The salts provide an electrical shield, reducing the zeta potential and providing possible hydrophobic interactions between proteins. In general, lupin globulins are able to reduce the interfacial tension in the air-water condition, but this reduction is influenced by the type of salt. The reduction in interfacial tension was greater at pH 3.4 and the absence of salts indicating that the presence of predominantly positive charges leads to greater mobility towards the interface. The kinetics of diffusion, adsorption and protein rearrangement were also affected by the conditions of the medium and are directly influenced by the electrostatic distribution. This study provides supporting information about the potential use of lupin proteins in food foams.

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5. CONCLUSÕES

No Capítulo 1, foi apresentado uma nova abordagem para purificação de umas das frações proteicas que compõem o tremoço, a γ – conglutina, uma proteína de potencial uso para redução nos níveis de glicose no sangue. O método de extração de salina – alcalina foi eficaz para extrair as globulinas e posteriormente, uma etapa cromatográfica utilizando uma coluna preparada com criogel foi utilizada para purificar a γ – conglutina. A fração coletada na etapa cromatografica foi submetida a análise de eletroforese, onde na condição não – redutora apresentou uma banda de ~48 kDa, enquanto na condição redutora, duas bandas foram identificadas com tamanho aproximado de 30 e 17 kDa. Essa proteína foi identificada através de espectrometria de massa e caracterizada quanto sua estrutura secundária que é formada principalmente por folhas betas, ponto isoelétrico de 7,51 e temperatura de desnaturação de 68° C, gerando resultados relevantes para possíveis aplicações.

No Capítulo 2, foi estudada a potencialidade do isolado proteico de tremoço como agente interfacial. A estabilidade física de sistemas aerados depende da capacidade de biomoléculas adsorverem em uma interface, causando a redução da tensão interfacial. O isolado proteico apresenta solubilidade mínima na região isoeletrica (pH 5 – 6) e potencial zeta variando de -30,0 a +30,0 mV na ausência de sal, enquanto na presença de NaCl, foi de -10,0 a +10,0 mV e com$CaCl_2$, -5,0 a +15,0 mV. Através da análise da tensão interfacial foi possível verificar que o isolado proteico de tremoço é capaz de adsorver em uma interface do tipo água – ar, reduzindo a tensão interfacial e essa capacidade é influenciada diretamente pelo meio que as proteinas se encontram. Os sais utilizados e a variação do pH afetam a distribuição de cargas do isolado, influenciando as etapas de difusão, adsorção e rearranjo interfacial.

Através deste estudo, foi explorado o potencial uso das proteinas do tremoço nos diferentes âmbitos da Ciência e Tecnologia de Alimentos. O tremoço se apresenta como uma fonte alternativa de elevada concentração de proteínas em um mercado que ainda trabalha muito com a soja. O seu uso ainda é limitado pela sua disponibilidade e/ou até mesmo o conhecimento dessa semente e estudos sobre a forma de extração de suas proteinas e utilização das mesmas são necessários. O metodo de extração do isolado proteico pode ser facilmente reproduzido e escalonável, para obtenção das globulinas do tremoço. Uma única etapa cromatografica foi necessária para separar e purificar a γ – conglutina do isolado proteico, demonstrando a eficácia das colunas de criogel para purificação de proteínas. O isolado proteico de globulinas do tremoço apresenta capacidade de reduzir a tensão interfacial em sistemas água – ar, sendo que essa propriedade é influenciada pelo pH e tipo de sal. Essa propriedade é de interesse para a industria de alimentos para a formulação de sistemas alimenticios aerados. Assim, esse estudo nos apresenta o tremoço como uma fonte de proteínas alternativo as proteínas animais e nos traz novas perspectivas sobre métodos de extração e aplicações.