RAIANE RODRIGUES DA SILVA

MIXED GELS FORMED BY CASEIN AND PEA PROTEIN: STRUCTURE AND INTERACTIONS

Dissertation submitted to the Food Science and Technology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

Adviser: Antônio Fernandes de Carvalho

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Author

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ABSTRACT

DA SILVA, Raiane Rodrigues, M.Sc., Universidade Federal de Viçosa, July, 2023. **Mixed gels formed by casein and pea protein: structure and interactions.** Adviser: Antônio Fernandes de Carvalho.

Protein hydrogels are responsible for the structure of various types of food products. The wide variety of products available is made possible by the ability to modify proteins, facilitated by factors such as pH, temperature, and ionic strength. These modifications allow proteins to interact with themselves or with proteins of different origins. However, many times the association of different types of proteins may not occur in synergism, modifying the structure of the gel. Due to this, it is necessary to study to understand the interactions responsible for the structuring of the different protein gels and the structural differences caused by the incorporation of different proteins in the same system. Thus, this dissertation aimed to understand the structure and interaction of mixed gels formed from casein and pea protein. When initially analyzing the suspensions containing different proportions of casein and pea protein, it was observed that the proteins have a preference for forming isolated systems. However, when applying heat treatment, the interactions between proteins increased, but only among those from the same source. In the formation of hydrogels through the acidification of the system, it was observed that the interactions between proteins of the same origin were more evident compared to the interactions between proteins of different origins. With the application of heat treatment before the acidification process, pea proteins were more affected than caseins, making the gels with higher amounts of pea protein even stronger due to pea-pea interactions. Through analyses using temperature variation, the gels formed by acidification were studied concerning their structure and interactions. It was possible to conclude that gels consisting mostly of casein are more sensitive to temperature changes, presenting less strong interactions. On the other hand, gels formed by pea protein are less sensitive to temperature changes and have strong interactions. Additionally, it was observed that the substitution of 20% casein can result in several changes in the gel structure, such as the approximation of protein chains, favoring interactions, and conferring new characteristics to mixed gels. However, proteins of different origins still have a preference for interacting with proteins of the same origin, forming independent systems. Therefore, future studies are necessary to increase the interaction between

these proteins and promote the formation of new systems, enabling the application of these gels in the formulation of new products.

Keywords: Casein. Pea protein. USAXS. Chemical interactions. Mixed gels.

RESUMO

DA SILVA, Raiane Rodrigues, M.Sc., Universidade Federal de Viçosa, julho de 2023. **Géis mistos formados por caseína e proteínas de ervilha: estrutura e interações.** Orientador: Antônio Fernandes de Carvalho.

Hidrogéis proteicos são responsáveis pela estrutura de diversos tipos de produtos alimentícios. A ampla variedade de produtos disponíveis é viabilizada pela capacidade de modificar as proteínas, facilitada por fatores como pH, temperatura e força iônica. Essas modificações permitem que as proteínas interajam consigo mesmas ou com proteínas de diferentes origens. Contudo, muita das vezes a associação de diferentes tipos de proteínas pode não ocorrer em sinergismo, modificando a estrutura do gel. Devido a isso, são necessários estudos para entender as interações responsáveis pela estruturação dos diferentes géis proteicos e as diferenças estruturais causadas pela incorporação de diferentes proteínas em um mesmo sistema. Sendo assim, esta dissertação teve como objetivo entender a estruturação e a interação de géis mistos formados a partir de caseína e proteína de ervilha. Ao analisar inicialmente as suspensões contendo diferentes proporções de caseína e proteína de ervilha, foi observado que as proteínas têm uma preferência por formar sistemas isolados. No entanto, ao aplicar um tratamento térmico, as interações entre as proteínas aumentaram, mas apenas entre aquelas provenientes da mesma fonte. Na formação de hidrogéis por meio da acidificação do sistema, foi observado que as interações entre as proteínas da mesma origem foram mais evidentes em comparação às interações entre proteínas de origens diferentes. Com a aplicação do tratamento térmico antes do processo de acidificação, as proteínas de ervilha foram mais afetadas que as caseínas, tornando os géis com maior quantidade de proteína de ervilha ainda mais forte devido as interações ervilha-ervilha. Através de análises utilizando variação de temperatura, os géis formados por acidificação foram estudados em relação a sua estrutura e interações. Foi possível concluir que géis constituídos majoritariamente por caseína são mais sensíveis às mudanças de temperatura, apresentando interações menos fortes. Por outro lado, géis formados por proteína de ervilha são menos sensíveis a mudanças de temperatura e apresentam fortes interações. Adicionalmente, observou-se que a substituição de 20% de caseína pode resultar em várias alterações na estrutura do gel, como a aproximação das cadeias de proteínas, favorecendo interações e conferindo novas características aos géis

mistos. No entanto, as proteínas de diferentes origens ainda têm preferência por interagir com proteínas de mesma origem, formando sistemas independentes. Portanto, estudos futuros são necessários para aumentar a interação entre essas proteínas e promover a formação de novos sistemas, possibilitando a aplicação desses géis na formulação de novos produtos.

Palavras-chave: Caseína. Proteína de ervilha. USAXS. Interações químicas. Géis mistos.

ILLUSTRATIONS LIST

Chapter I: Literature review

Chapter II: Impact of protein ratio and thermal treatment on the rheological properties of high-concentrated casein micelles: pea protein suspensions

Chapter IV: Molecular interactions involved in acid-gelation of mixed systems between pea protein and casein protein

SUMMARY

General introduction

1. Introduction

Hydrogels are tridimensional structures capable of incorporating a huge amount of water in its structure (Zha et al., 2021). In a phisical aproach, hydrogels can also be defined as a dispersion (solid phase) formed by the interaction between the biopolymer chain, such as carbohydrates and proteins, forming a tridmensional structure wich has intertices where the water (liquid phase) can be housed (Khalesi et al., 2020).

The hidrogels in food industry are present in different products, such as, yogurts, jelly, cheese and other (Zha et al., 2021). Over the years, most of hydrogels are formulated utilizing proteins, mainly animal-sources protein. The amphiphilic nature of the proteins allow them to conect and form the gels (Alves & Tavares, 2019). Concerned about climate change and population growth, the production of food based on animal proteins is becoming a concern, due to the difficult to increase the productive scale and the contribution of this chain to the emission of greenhouse gases (Day et al., 2022; Nascimento et al., 2023; Zha et al., 2021).

Between the different proteins groups, the plant protein as reliable substitutes for their animal-based and is useful to building different food systems. Compared with animal protein, plant protein are considered low cost and low greenhouse effect, besides that, present less allergenic properties what stimulates its consumption (Nascimento et al., 2023).

Plant proteins are obtained from several sources, *e.g.* cereals, legumes, oilseeds, nuts, and due theses different sources they have structural differences and thus allow the formation of different types of systems (Day et al., 2022; Nascimento et al., 2023). Therefore, their application on food industry becomes difficult due several factor like the low solubility and the unpleasant taste, being necessary the application of some strategies to improve their use in food industry (Day et al., 2022).

The association of plant protein in products that are already accepted can positively impact the environment by reducing the amount of animal protein, while improving the technical, functional and sensory characteristics of plant proteins (Nascimento et al., 2023).

The mix between proteins from different sources often causes structural changes in hydrogels. With this, several studies have been developed in order to verify the effect of this mixture (Chihi et al., 2018; Roesch et al., 2004), the type of mechanism

(Ben-Harb et al., 2018) used, and also its application for example as in the controlled release of actives (Liu et al., 2023).

2. General objective

Understand the structure and interaction that exist in a mixed systems formed by casein and pea protein and verify the ratio effect on these systems.

2.2. Specific objectives

- Verify the effect of thermal treatment, concentration, and ratio on the physicochemical properties of mixed casein micelles and pea protein systems.
- Formulate high-concentrated mixed hydrogels formed by casein and pea protein by acidification and evaluate the ratio and the thermal treatment effect.
- Investigate the molecular interactions existing in a mixed hydrogel formed by casein and pea protein.

3. Scientific valorization

Impact of protein ratio and thermal treatment on the rheological properties of high-concentrated casein micelles: pea protein suspensions. Nascimento, Luis Gustavo Lima., da Silva, Raiane Rodrigues., Descamps, Amandine., Trivelli, Xavier., Casanova, Federico., Marie, Rodolphe., Martins, Evandro., de Carvalho, Antônio Fernandes., Delaplace, Guillaume., Junior, Paulo Peres de Sá Peixoto. Food Hydrocolloids.

Acid gelation of high-concentrated casein micelles: pea protein mixed systems. Nascimento, Luis Gustavo Lima., da Silva, Raiane Rodrigues., Doumert, Bertrand., Martins, Evandro., Casanova, Federico., Marie, Rodolphe., de Carvalho, Antônio Fernandes., Delaplace, Guillaume., Junior, Paulo Peres de Sá Peixoto. Food Hydrocolloids.

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Chapter I: Literature review

Abstract

Regarding the climate changes that has happened in the last few years, it becomes increasingly necessary to rethink habits, especially when it comes to food. One of these habits is the consumption of products of animal origin, mainly animal proteins, due to its contribution to environmental problems, such as greenhouse effect and water availability. Therefore, the change of this proteins to proteins that causes less impact, *e.g.* vegetable proteins and edible insects proteins, are advised. Vegetable proteins are available in various legumes and vegetables, and unlike animal proteins, they can be produced in a smaller area and do not contribute to the greenhouse effect. Despite the advantages, its flavor is often not pleasant, which makes it less acceptable. With this, the association of these two proteins in systems that are well acceptable, *e.g.* dairy products, becomes as a way to change the off flavor and reduce environmental impacts. Therefore, the objective of this article is to understand the formation of mixed gels, formed by casein and vegetable protein, through a literature review and understand the gaps for the elaboration of new studies to make possible the elaboration of these mixed systems for a future commercialization.

Keywords: Vegetable protein; Animal protein; Hydrogels;

1. Introduction

For many centuries, protein has already been an indispensable part of human food, whether in fresh food or as a new product, *e.g.* cheese and tofu. According to United Nations (2017), in 2050 the world will reach 9 billion people, however, with the increasing population, the amount of protein, especially animal protein also need to increase. Although, this expansion becomes difficult due to the impacts generated by livestock on the environment, *e.g.,* water demand, greenhouse gas emission, land demand (Ismail et al., 2020). Thus, one way to reduce the environmental problems is modifying the food habits, changing for example the protein sources to a protein with less impact, such as, plant protein, wich has less demand for area, water and does not contribute to the greenhouse effect (Hertzler et al., 2020).

Nevertheless, there are some problems associated with this change, one of them which has more impact on the consumer is the sensorial characteristics, such as the flavor that reminds beans (Nascimento et al., 2023; Oliveira et al., 2022). An alternative to improve the acceptability of plant protein is masking its original flavor with something already well-accepted by consumers. One of the options is to mix plant protein with dairy products, which are very well disseminated and accepted by the population due to the wide variety of products with different aromas and flavors.

The production of dairy products is already well understood, however, with the incorporation of new protein sources, the structure and other attributes can be modified. Therefore, there is a need for further studies focusing on understanding the modifications that can be caused by the incorporation of different proteins in the same system and besides that the viability of this kind of production.

2. Milk protein

Milk is one of the most protein sources that are available for humans, along with meat, seafood and eggs (Day et al., 2022). According FAO (Food and Agriculture Organization) (2021), the consumption of processed and fresh dairy products during the period of 2015 to 2020 is around 15 kg/per capita.

Regarding nutrition, milk is a “complete food”. Bovine milk normally consists of 85%–87% water, 3.8–5.5% fat, 5% carbohydrates, and 2.9–3.5% proteins, at the

macronutrient level. Bovine milk is also rich in various bioactive substances at the micronutrient level, such as vitamins, minerals, biogenic amines, organic acids, nucleotides, oligosaccharides, and immunoglobulins (Foroutan et al., 2019).

The milk versability is possible due to the facility of modifying the proteins that are present, through, for example, the use of enzymes, pH modification, ionic strength and the application of different technologies. Among the proteins present in milk, casein is the most abundant being 80% of them and it is recognized as the class of proteins obtained by recovering the precipitate formed after acidification of milk at pH 4.6 at 20 °C (N. N. Silva et al., 2019). The other 20% of protein is considered whey protein, and in the industries is normally obtained as a co-product of cheese-making. Nowadays, whey protein is as important as milk and has been a promising product to dairy industries due to its nutritional value (Zhao et al., 2022). A more thorough description of proteins and their significance will be provided in the topics that follow.

2.1 Casein

Casein micelles (CMs) is a protein present in the milk of all mammal species. They are a family of phosphoproteins. Their biological functions are related to their actions as calcium vectors, which pass from mammalian females to neonates, and as chaperones, which prevent the formation of insoluble protein aggregates, known as amyloid fibrils, preventing the onset of various diseases (N. N. Silva et al., 2019; Thompson et al., 2019).

Casein is considered an intrinsically unstructured protein, but also a stable micellar structure composed by the association of four sub-fractions, α_{S1}-, α_{S2}-, β- and κ-casein, with calcium phosphate, water, and minerals as present in Figure 1 (Huppertz et al., 2017). Among the casein fractions, there are those considered sensitive to calcium, α_{S1}-, α_{S2}-, β-casein. On the micelles, theses fractions are located inside, and are associated by electrostatic interactions with colloidal calcium phosphate (CCP). The fractions assemble through weaker connections such as hydrogen and ionic bonding, van der Waals interactions, and hydrophobic interactions (Runthala et al., 2023). Inversely, κ-casein is placed on the surface of the micelles, creating a “brush”, responsible to maintain the electrostatic and steric repulsion between the micelles, stabilizing them.

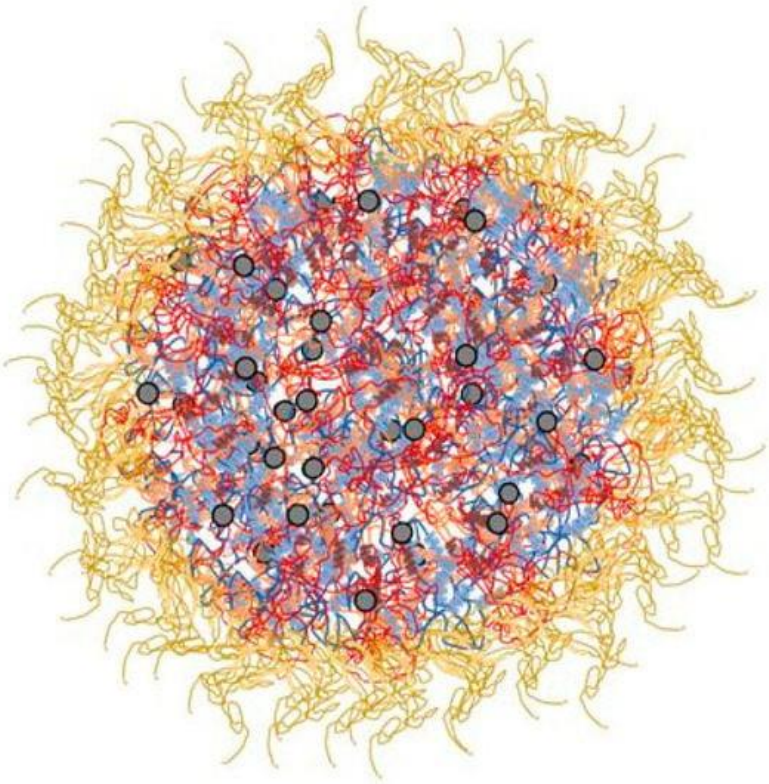

Figure 1. Casein micelle structure and its components: κ-casein (yellow); α-casein (blue); β-casein (red); calcium phosphate nanoclusters (grey dots).

Source: Lima Nascimento et al., (2023)

Almost 40% of the total casein fractions in milk is α_{S1}-casein. This casein fraction has 199 amino acids and among these 8 residues are phosphorylated (N. N. Silva et al., 2019). Due to phosphorylation, the molecular mass can go from ~23 KDa to ~23.6 KDa. Based on the primary sequence, the isoelectric point (pI) predicted is 4.9, although this can decrease by 0.5 pH units due to phosphorylation of the 8 serine residues, which is consistent with its empirically observed pI (4.4-4.8) (Huppertz et al., 2017).

α_{S2}-casein corresponds to 10% of caseins fractions in milk and it is also a phosphorylated fraction. The reference protein, α_{s2}-Casein A-11P, has 207 amino acids, 11 of which are phosphorylated (Thompson et al., 2019; Walstra, M Wouters, et al., 2006). Due to this phosphorylation, the α_{s2} casein is the fraction that has more sensibility to calcium presence, only 2 mM of $CaCl_2$ causes precipitation of 90% of α_{s2}-caseins at pH 7.0. The molar mass of α_{S2}-casein has ~24.3 kDa for the non-phosphorylated protein and ~25.2 kDa for the phosphorylated protein and the pI varies

from 4,91 to 4,42 depending on the phosphorylation (Runthala et al., 2023; Thompson et al., 2019; Walstra, M Wouters, et al., 2006).

β-casein (β-CN), is in milk at about 35% of casein content (N. N. Silva et al., 2019). The reference protein is the β-CN A2-5P which has a molecular mass of ~23.6 KDa, however, with the phosphorylation of 5 serine residues, this value can increase to ~24 KDa. The pI is estimated at 5.1, decreasing to ~4.7 because of the phosphorylation, which is somewhat lower than the experimental value of 4.8–5.0 (Huppertz et al., 2017). The β-CN is the most amphiphilic casein fraction due to the C-terminal section (136–209 residues), which contains most of the non-polar residues and is characterized by little charge and high hydrophobicity (Walstra, M Wouters, et al., 2006). This amphiphilic character is also important to create the micelles aggreggates when the casein is in a solution (N. N. Silva et al., 2019).

κ-casein (k-CN) is represented by 15% of caseins fractions (N. N. Silva et al., 2019). Unlike the other casein fractions, it is the only one that may be glycosylated, having just 36%, of the total amount that are non-glycosylated (N. N. Silva et al., 2019). The reference protein to k-CN is κ-CN A 1P which has 169 amino acids and two cysteine groups between them (Walstra, M Wouters, et al., 2006). Without posttranslational modification, the molecular mass of κ-casein A is ~19.0 kDa, and a pI of ~5.9 is expected. The molecular mass increases and the pI decreases as a result of phosphorylation and glycosylation, reaching as low as ~3.5 (Huppertz et al., 2017). Due to the low phosphorylation, the k-CN solubility is not impacted by the presence of calcium in the medium (N. N. Silva et al., 2019).

2.2 Whey proteins

Obtained by the preparation of cheese, whey protein (WPI), is a rich source of essential and branched-chain amino acids, composed of β-lactoglobulin (~50%), α-lactalbumin (~25%), with minor fractions including bovine serum albumin (BSA), immunoglobulins (Igs), and lactoferrin (LF) (Zhao et al., 2022).

β-lactoglobulin is the major constituent of whey proteins. It has 162 amino acids, a molecular mass of 18.3 kDa, and a quaternary structure that is easily modified by pH and ionic strength, however, they do not precipitate during milk acidification (Thompson et al., 2019). Due to their high hydrophobicity, this protein tends to bind some apolar

compounds, such as vitamin D, fatty acids, cholesterol or carotenoids (Broersen, 2020).

α-lactalbumin, biologically, is a regulatory protein of the lactose synthase enzyme (Brew, 2013). It is a small protein with a molecular mass of around ~14 kDa with a tertiary structure that is stabilized by Ca^{2+} (Thompson et al., 2019; Walstra, M Wouters, et al., 2006). The Ca^{2+} remotion or the reduction of pH to 4 can cause the loss of calcium from the structure, causing the loss of protein stability and making this protein can be denatured at relatively low temperatures (Walstra, M Wouters, et al., 2006).

Bovine Serum Albumin (BSA) is a protein present in the blood plasma. Normally, is a single polypeptide chain composed of ~585 amino acid residues with a molecular mass of ~66.8 kDa. The structure of this protein is stabilized by the cross-linked 17 disulfide bridges of cysteine (Cys) amino acid residues (Jahanban-Esfahlan et al., 2019).

The proteins immunoglobulins (antibodies) are also present in the human blood and are responsible for the equilibrium of the immune system. The immunoglobulins present in milk, are responsible for protecting the gut mucosa of the newborn against pathogens. They are present in milk in the concentration of 0.6–1 $g.L^{-1}$ but the colostrum contains up to 100 $g.L^{-1}$, this level drops quickly in the postpartum period (Fox et al., 2015; Thompson et al., 2019). There are several types of Igs in milk, however in bovine milk, the most common is IgG. All monomeric Igs molecules have the same fundamental structure, which is made up of four subunits polypeptides, two identical heavy chains, and two identical light chains, with a total molecular mass of 160 kDa (Hurley & Theil, 2013).

Lactoferrin is a protein with a tertiary structure with iron on its structure, that is an inhibitor to some bacteria, due to its ability to sequestrate the Fe^{3+} from the milk serum, reducing the disponibility and preventing bacteria from using this mineral for their growth (Walstra, M Wouters, et al., 2006). LF is a single-chain protein formed by two globular lobes which are linked by an extended α -helix, with a molecular mass of around 80 kDa (Lönnerdal & Suzuki, 2013).

Besides the high level of nutritional value which makes the application of whey in foods and beverages promising. Several studies have been highlighting its effectiveness as a health promoter, acting in the prevention or assisting in the treatment of various diseases, *e.g.* cancer (Cereda et al., 2019), diabetes (Pal & Ellis, 2010),

cardiovascular diseases (Ballard et al., 2013), which makes this product important as milk.

3. Plant protein

Environmental issues are harmful consequences of human activity in the natural world and these ecological problems impact the community in decreasing the quality of life. Over the years, the population is becoming more aware of the consequences of their acts on the planet and start to change the habits as a way to reduce the environmental damage, caused for example by animal proteins consumption. Nowadays, the number of vegans and vegetarians are around 10% of the population, but consumers who consider themselves flexitarian or who want to reduce their meat intake account for 30-40% of the population (Aschemann-Witzel et al., 2020), causing less environmental impact.

However, it has some barriers to increasing, even more, the consumption of plant protein while reducing the consumption of animal protein. These barriers are related to the difficulty to prepare products with a vegetal base, the off-flavor related to these products, the difficulty to find products in the supermarket, due to lack of varieties and the normal habits, especially traditions, that are commonly associated with meat (Aschemann-Witzel et al., 2020).

Edible plant proteins are from several sources such as legumes (soybean, pea, bean, chickpea, lupin, faba bean, cowpea), cereals (rice, wheat, millet, sorghum, maize, and barley), pseudocereals (amaranth, quinoa, and buckwheat), seeds (chia, flaxseed, sesame, pumpkin, and sunflower), almonds and nuts (Sá et al., 2020).

The plant proteins are normally classified into four different groups depending on their solubility in various aqueous-based media and susceptibility to heat-induced denaturation (Table 1).

Group	*Solubility*	*Heat-induced denaturation*
Albumin	Water	Heat sensible
Globulin	Diluted salt solutions	Heat sensible
Prolamin	Alcoholic solution with 50 e 70% ethanol/water mixture or diluted acidic media	Heat resistant
Glutelin	Diluted alkaline solutions	Heat resistant

Table 1. Classification of proteins by Osborne (1923)

Adapted from (Hinderink et al., 2021)

One of the ways to get around the disadvantages of consuming vegetable proteins is to associate them with animal proteins through already known dairy products. One of the most common colloidal systems in food today are hydrogels.

4. Hydrogels

Hydrogels are a tridimensional structure capable to incorporate a huge concentration of water (hydrogels), air (aerogels), and oil (oleogels) (Cao & Mezzenga, 2020). In a rheological approach, the gels are characterized by a material that has an elastic modulus (G') greater than the viscous modulus (G'') (Panahi & Baghban-Salehi, 2019).

The structure of hydrogels is mainly formed by hydrophilic functional groups such as amino, carboxyl, and hydroxyl groups, which are responsible to allow water absorption in gel networks. Besides that, the presence of cross-links between polymer chains (physical or chemical links) helps to maintain the gels networks. Therefore, the gel will be influenced by the nature of the polymer used and how they are associated within the gel, influencing structural and rheological characteristics, such as viscoelasticity and water retention capacity. (Khalesi et al., 2020; Zhang et al., 2023).

Hydrogels can be also categorized regarding the polymers present in the system, which can be a single (when just one polymer are present on the matrix), mixed (when more the one polymer are present on the matrix) or filled system (when filler particles are physically encapsulated in the matrix). (Zha et al., 2021).

The hydrogel formation can happen by different mechanisms: thermal, acid, enzymatic, and applying some technologies such as ultrasound (Panahi & Baghban-Salehi, 2019; Zha et al., 2021; Zhang et al., 2023). In the thermal-induced approach, the gels are normally formed by electrostatic interaction, hydrogen bonding, and hydrophobic interaction (Zhang et al., 2023). These interactions are formed in two steps: a) the unfolding or dissociation of protein molecules caused by heat; b) the association and aggregation reactions resulting in a gel system (Totosaus et al., 2002). The effect of heat on the gelation of micellar casein and plant proteins (soy protein and pea protein) was explored by Silva et al., (2019). They observed that even after the heat treatment, the CMs do not aggregate with plant protein to form separate systems.

Cold-induced gels are another eco-friendly and secure way for creating protein hydrogels. The gels are first heated in temperatures low than the denaturation temperature and subsequentially reduce the electrostatic repulsion after cooling. However, it frequently needs certain cross-linking agents, salt ions, acids, or other chemicals in comparison to heat induction, that with be responsible to the interaction between the chains (Zhang et al., 2023). Several studies have been developed as a way to understand this type of gelation (Beghdadi et al., 2022; Chihi et al., 2018; Liu et al., 2023; Mession et al., 2017b; Oliveira et al., 2022). Different types of protocols can be applied to improve the interactions between plant proteins and CMs. Beghdadi et al., (2022) verified the effect of pre-treatments on the network between proteins. They applied two pre-treatments, the first one was a heat treatment (85°C for 1 h) of CMs-Pea protein and CMs-Whey protein mixtures, and in the second one, CMs, pea protein, and whey protein suspensions passed by a heating (85 °C for 1 h) individually. After these treatments, the suspensions were acidified with glucono-δ-lactone (GDL). As a result, when the proteins are pre-treated individually, there was a favoring of hydrophobic interactions between pea protein and CMs, increasing texture parameters such as stiffness and firmness, compared to CMs-WPI gel.

The mixed gels normally present different characteristics when compared to pure casein gels, such as the formation of a weakest gel (Shand et al., 2008), which may be a result of the non-interaction between different protein sources (Mession et al., 2017a). Because of this, different techniques have been studied to improve the interactions between casein and plant protein, such as enzymes, ultrasound, pH shift, and high hydrostatic pressure, among others.

Transglutaminase is one of the enzymes that can be applied to increase the interaction between animal and plant proteins. Transglutaminase has the ability to generate ε-(γ-glutamyl)-lysine (Gln-Lys) isopeptide bonds by cross-linking inter- and intra-molecular reactions between Lys-ε-amino and Gln-γ-amide groups of proteins (Cui et al., 2020; Tang et al., 2023). The application of transglutaminase aiming for the improvement of interactions between lentil protein and casein micelles, resulted in a mixed gel with the same characteristics as pure casein gel, showing that the replacement of animal proteins by vegetable proteins, in a certain amount, can occur without changes in parameters such as rheology and water retention (Tang et al., 2023).

The high ultrasound treatment is effective in improving protein interactions. On this process, the sample are submitted to acoustic waves that are capable to create cavitation due the formation and collapse of the bubble. This process can increase hydrophobicity and solubility improving the functional properties of the colloidal systems (Akharume et al., 2021). Nascimento et al. (2023) concluded that the application of ultrasound treatment (495 W/15 min) can improve the rheology properties of the systems formed by the mix between pea protein and casein due to the reduction of the size of the aggregates and due to the ability of the ultrasound to increase the surface hydrophobicity, being capable to create more interactions between proteins.

5. Conclusion and perspectives

The impact of animal protein consumption on the environment changes is a reality, such as global warming. The decrease in the number of animal protein production makes a difference in the release of greenhouse gases. This is possible due to new technologies to insert easier the plant-based proteins in the market. One of the strategies to overcome the resistance of the consumers is utilizing mixed gels systems formed by milk and plant proteins.

Most studies have shown that it is possible to replace a certain part of the milk proteins with vegetable proteins, however, these alterations may cause changes in the systems, either during their formation and/or in their final structure of the gel. Therefore, more research is required to optimize the interactions between the components of

mixed gel aiming for similar characteristics with the gels already consumed by the population.

The application of green technologies, such as ultrasound, high hydrostatic pressure, pH shift, and pulsed electric field, among others, can be presented as a future solution. Furthermore, the study of different crosslink agents and the formulation of gels with different proportions between the two proteins may have promising results.

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Chapter II: Impact of protein ratio and thermal treatment on the rheological properties of high-concentrated casein micelles: pea protein suspensions

Impact of protein ratio and thermal treatment on the rheological properties of high-concentrated casein micelles: pea protein suspensions.

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Luis Gustavo Lima Nascimento, Raiane Rodrigues da Silva, Amandine Descamps, Xavier Trivelli, Federico Casanova, Rodolphe Marie, Evandro Martins, Antônio Fernandes de Carvalho, Guillaume Delaplace, Paulo Peres de Sá Peixoto Junior

Abstract

Currently, there is an increase in the consumption of plant-based products for healthy and sustainable reasons, which increases the demand for plant proteins, such as pea. However, the low techno-functionality and undesirable flavor make their incorporation into food systems challenging. Thus, the association of these proteins with a more consumer-accepted product, such as milk, is an alternative. However, it is necessary to understand the properties of such mixed systems. This study aimed to investigate the physicochemical properties of mixed casein micelles (CMs): pea protein systems at different protein ratios (80:20, 60:40, 40:60, 20:80 w/w (CMs: pea protein)) in high-concentrated protein systems (12%, 14% and 16% ($w.w^{-1}$) of protein), before and after thermal treatment. Thus, rheological assays, dynamic light scattering, electrophoresis, and nuclear magnetic resonance were applied as analytical tools. A synergistic effect in the elasticity and viscosity results at the 20:80 ratio was observed. The increase in temperature and concentration further accentuates this phenomenon. This effect has been explained by volume excluded arguments which are commonly found in binary systems composed of particles displaying very different sizes. These results bring a new insight of the mechanisms underlying the physical properties of composite plant/animal protein systems.

Keywords: Casein micelles; Pea Protein; Mixed Gels; Heat-treatment; Aggregates; NMR.

1. Introduction

The world population is expected to reach 9.7 billion people by 2050 (United Nations, 2015) and, to sustain the upcoming demand, the industry needs to increase food production. However, the food industry should propose solutions that englobe sustainability, economic and environmental questions, as well as to attend the consumer desire (Liu et al., 2022). Among the food constituents, the proteins are essential macromolecules that present several physiological roles such as in the construction of tissues, muscle contraction, catalyzing reactions, immune system, and nitrogen storing, among others (Damodaran, 2008). For these reasons, this nutrient needs to be daily consumed through the intake of meats, eggs, milk, and vegetables. Thus, in the near future, it is expected to be a higher demand for protein sources and, consequently, an intensification of animal handling. However, the expansion of animal handling is accomplished by increasing deforested areas, greenhouse gas emissions, water consummation, and energy costs (Ismail et al., 2020). One possible way to get around these environmental problems consists in incorporating more plant proteins into the human diet. The demand for pea proteins in the world market tends to grow by about 12% per year in the coming years (Grandviewresearch, 2019). It is partly explained by its low allergenicity and high nutritional value with a good balance of amino acids. In contrast, pea proteins have a beany taste and low solubility, factors that make their application in the food industry challenging (Boukid, Rosell, & Castellari, 2021). Therefore, for pea proteins to be effectively incorporated into food formulations, some technological strategies must be applied to mitigate these inconveniences. A strategy to increase the consumption of pea is mixing them with other protein sources that possess more consumer acceptability.

It is clear the change in consumption habits to a more sustainable and plant-based. However, this transition may take time due to lower the acceptability of pea. Currently, milk is one of the most important sources of animal protein and can be used as a raw material in the production of various processed foods such as UHT milk, cheese, yogurt, powdered milk, cream, butter, and ice cream (Walstra et al., 2005). The wide variety of dairy products is possible due to the functionalities of milk proteins. Therefore, the creation of mixed systems using the combination of pea proteins and milk proteins could be an alternative to increase the consumption of vegetable protein, as well as to develop dairy products with totally new sensorial and techno-functional

characteristics (Chihi et al., 2018). However, the incorporation of another protein source significantly changes the final product, and how the two types of proteins interact in mixed systems is not well elucidated, and studies in the area are still scarce (Alves & Tavares, 2019). Thus, the impact of this association as well as the optimization of protein interactions must be better understood for the development of innovative products.

Studies involving the formation of casein micelles (CMs) -pea protein thermal and acid gels, in general, demonstrate an absence of synergism in the rheological properties of these protein sources. It is probably due to the existence of a thermodynamic impartibility between these proteins causing the formation of independent systems, microphase separation, and gels with less elastic structure and greater syneresis (Ben-Harb et al., 2018; Mession, Roustel, & Saurel, 2017). However, there is an absence of studies of mixed systems in higher protein concentrations, which can possibly favor the interactions between the protein sources.

This study aims to create highly concentrated mixed casein micelle: pea gels and understand the effect ratio and concentration of proteins on the rheological properties of the systems. Also, a thermal treatment was applied to follow the changes caused in the systems after the application of a common unit operation in the food industry.

2. Materials and methods

2.1. Materials

The micellar casein (CMs) powder (Promilk 85B) was kindly provided by Ingredia SA (Arras, France) and pea protein powder (F85S) was provided by Roquette SA (Lestrem, France). All other reagents were of analytical grade.

2.2. Sample preparation

Both protein powders were separately rehydrated in deionized water at 12, 14, and 16% $w.w^{-1}$ concentrations. These concentrations were defined in the pre-test and chosen based on the highest concentration that the pea protein could be solubilized. The hydration process occurred overnight at 25 ℃ using a magnetic stirring plate at 600 rpm. After complete rehydration, the proteins were mixed in four ratios of CMs: Pea, being 80:20, 60:40, 40:60, and 20:80, and systems formed only with CMs or pea

proteins were also analyzed. After the mixture, the mixed protein systems were stirred at 30 ℃ for 2 hours at 600 rpm.

2.3. Effect of thermal treatment in the mixed systems

Each protein mixture produced as described in section 2.2 was placed in centrifuge tubes and put in a water bath at 85 ℃ for 1 h. After that, the samples were immersed in an ice bath to decrease rapidly the temperature to 30 ℃. The long thermal treatment time was chosen to produce denaturation in the pea proteins aiming to potentialize possible protein interactions.

2.4. Apparent viscosity and flow curve

Before the thermal treatment, the rheological behavior and apparent viscosity of the samples were evaluated regarding their flow behavior in a strain-controlled rheometer (ARES, TA Instruments, USA) using a cone-plate geometry with an angle of 0.04 rad and 0.0457 mm gap. The analysis was performed at 30 ℃ by varying the shear stress from 0.1 until 300 s^{-1} in three cycles (to detect possible thixotropic behavior), being the first and third upward and the second downward. The third curve was used to determine the flow behavior and consistency index using the power-law model (equation 1).

$$\sigma = k.y^n \quad (1)$$

Where $\sigma\ (Pa)$ is the shear stress, k ($Pa.s^n$) is the consistency index, $y\ (s^{-1})$ is the shear rate, and n (dimensionless) is the behavior index.

2.5. Dynamic light scattering (DLS)

The particle size distribution analysis for each sample was performed according to Silva et al. (2018) with some modifications. A DLS apparatus (DynaPro Nanostar, Wyatt, CA, USA) at a 90° scattering angle and 658 nm wavelength excitation was used. Briefly, before and after the thermal treatment, the protein suspensions were diluted 100 times, in deionized water. After dilution, the samples were put in a polystyrene cuvette. The analysis was performed at 30℃.

To verify the forces that are responsible to stabilize the pea protein aggregates, the same procedure to determine the particle size distribution was applied. However, in this analysis, the pea protein suspension was diluted to the final concentration of 8 µg/mL and after was added in the same solutions utilized on electrophoresis to native, non-reduced, and reduced conditions in the final concentration of 0.8 µg/mL.

2.6. Electrophoresis

The electrophoresis was carried out to gather information about the formation of disulfide bonds in the samples. The suspensions were analyzed in polyacrylamide gels in reduced and non-reduced conditions, as described by Veloso, Teixeira, and Ferreira (2020), with slight modifications. The samples were diluted with deionized water at 8 mg/mL. For the reducing condition, 2-mercaptoethanol buffer solution (1:4) was added. Then, 10 µm of samples were put on the top of the polyacrylamide gel composed of a stacking gel of 4% and a separation gel of 12% acrylamide. The protein migration was performed in a SE 600 Series Vertical Slab Gel unit (Hoefer Scientific instrument, San Francisco, US), using a Tris-glycerin buffer solution at pH 8.8. A voltage of 30 V was applied in the first 30 min of analysis, followed by the application of 90 V until the last band reach approximately 4/5 of the gel. After the protein migration, the gels were dyed by immersion in a solution of 0.3% of Coomassie® Brilliant Blue R-250 dissolved in acetic acid: methanol: water (1: 4.5: 4.5) for 4 h at 25 ℃. After, the gel was immersed in methanol: acetic acid solution to decolorate and promote a contrast between the protein bands and the background. The gels were digitalized, and the images were analyzed using ImageJ software (Hermanto, Sholaikah, & Mulyani, 2016).

2.7 Nuclear magnetic resonance (NMR)

NMR was applied to gather information about the water dynamics in the samples. The NMR experiments were recorded on a Bruker AVANCE NEO 900 spectrometer equipped with a 5 mm TCI cryo-probe at 85 °C. Two types of experiments were run: standard 1D-^{1}H, and 2D-^{1}H-diffusion (*ledbpgp2s* pulse program, recycling delay=10 s, d20=120 ms, p30=1,5 ms, 8 scans, 16 gradients values from 5 to 95 % with a linear variation, duration 24 minutes). The samples were prepared according to section 2.2 with the replacement of water for deuterium oxide and transferred into standard 5 mm NMR tubes. 70 µL of mineral oil was then added to avoid solvent evaporation (Wieruszeski et al., 2006).

2.8 Small amplitude oscillatory shear (SAOS) test

SAOS test was carried out to get the elastic and viscous properties of the pure and mixed protein systems. The effect in the sample structure during and after the thermal was analyzed by SAOS according to Costa et al. (2021) with slight

modifications. The suspensions were carefully placed in the rheometer (ARES, TA Instruments, USA) equipped with cone-plate geometry (60 mm, 2°) using a gap of 0.54 mm. The sample excess was removed with a spatula and paraffin oil was put on the edges of the geometry to avoid water evaporation during the experiments. The time and temperature sweeps were performed within the linear viscoelastic region, at 1 Hz frequency and 0.1% amplitude. The sample was allowed to equilibrate for 5 min. Then, a temperature sweep test was performed from 30 ℃ until 85 ℃ with a heating rate of 8 ℃/ min (the same rate achieved in the water bath). As soon as the temperature reached 85 ℃, a time sweep test was performed for 1h. After, a second temperature sweep is applied from 85 ℃ until 30 ℃ at a rate of 15 ℃/min. After, a time sweep for 5 min at 30 °C was applied to verify complete gel formation. In the end, a frequency sweep test (0.1 to 50 Hz, at 0.1% amplitude) was applied to the samples.

2.9 Statistical analysis

The samples were compared regarding ratio, concentration, and thermal treatment by variance analysis (ANOVA). When a significant difference ($p < 0.05$) was found, the Tukey HSD test with 5% significance was applied to differentiate means. All the experiments were performed at least in duplicate, and the data was evaluated utilizing SAS software student edition.

3. Results and discussion

3.1. Apparent viscosity and flow behavior

The apparent viscosity for all the studied suspensions decreased when the shear rate applied increased (Figure 1A, 1B, 1C). The shear-thinning behavior was more evidenced in systems with a higher amount of pea proteins and higher protein concentrations. The shear-thinning is usually explained by the increase in the molecular orientation when the shear rate increases, which decreases the degree of molecular entanglement (Varesano, Aluigi, Vineis, & Tonin, 2008). The apparent viscosities at 200 s^{-1} were chosen as the parameter for a sample comparison, once this is a common shear rate applied in the food industry and encountered in the mouth (Hubbe et al., 2017). Comparing the protein ratios at the same protein concentration, the 100:0 suspension presented the lowest apparent viscosity for all the concentrations studied. This suggests that pea proteins display a higher voluminosity than casein proteins in this condition. So, the gradual replacement of casein micelles for the, more

voluminous, pea protein increased the gel G* in the samples. Indeed, between the ratio 100:0 to 20:80, the apparent viscosity increased continuously, from pure CMs to pure pea suspensions, thus the 20:80 presented the highest apparent viscosity (Figure 1A). However, between 20:80 and 0:100 no increase is observed at 12% (w.w^{-1}) and 14% (w.w^{-1}), (Figure 1B). At 16% (w.w^{-1}), the apparent viscosity at 20:80 was higher than in pure pea suspensions (0:100) (Figure 1C). This remarkable non-monotonous curb indicates that there is a synergism in the protein ratio of 20:80, in higher protein concentration suspensions.

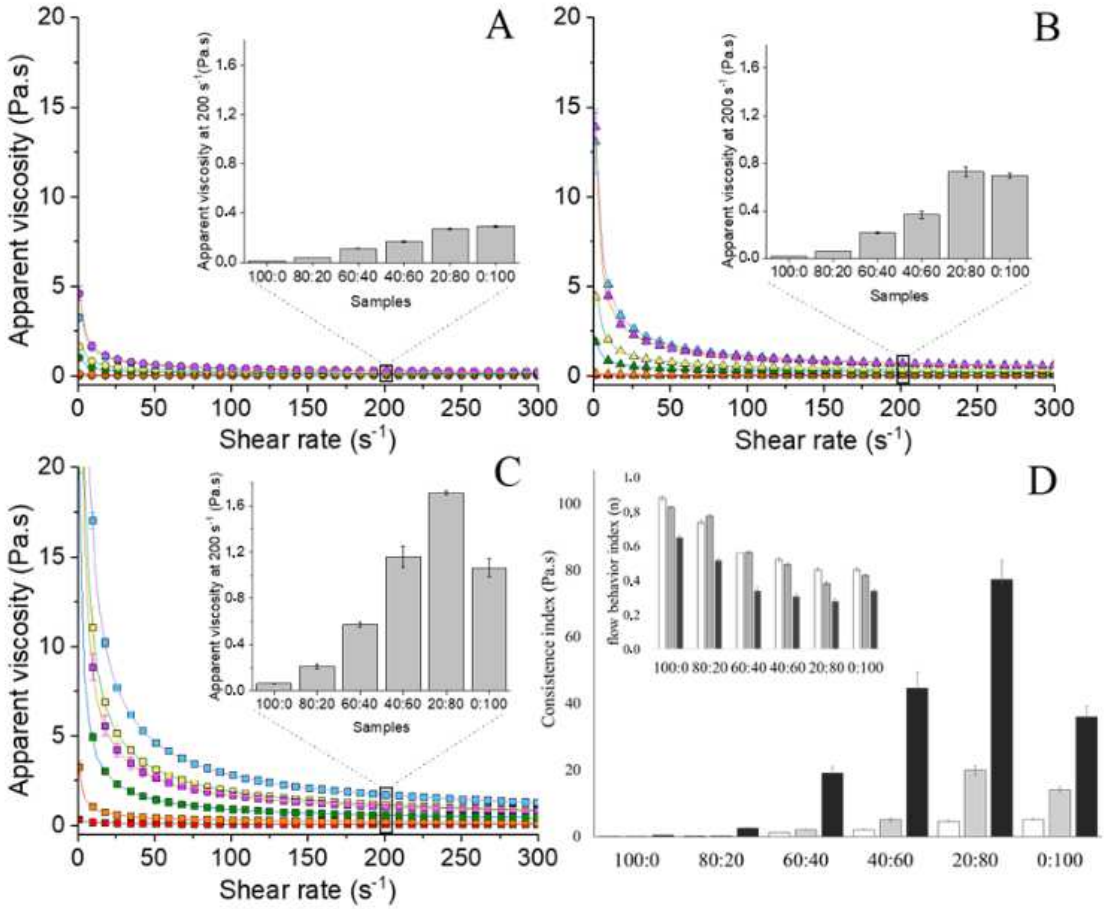

Figure 1. Apparent viscosity as a function of shear rate and apparent viscosity at 200 s^{-1} shear rate (inserted graphs) for protein suspensions before thermal treatment at 12 (○) (A), 14 (△) (B), and 16% (w.w^{-1}) (□) (C) total protein concentration. CMs: pea protein ratios: (— red) 100:0; (— orange) 80:20; (— green) 60:40; (— yellow) 40:60; (— blue) 20:80; (— purple) 0:100. (D) consistency index and flow index (inserted graph) for 12 (white), 14 (light gray), and 16% (w.w^{-1}) (dark gray) protein suspensions

The flow behavior of the suspensions is depicted in Figure 1D, where the power law was used to fit the shear strain/shear rate curves. All the samples were classified as pseudoplastic fluids, for the range with r^2 higher than 0.999, as the flow index (*n*) was found to be lower than 1 (inserted graphics in Figure 1D). The consistent index of the suspensions followed the same behavior found for the apparent viscosity. In the same way, the *n* decreased following the increase of pea protein until the minimum value in the 20:80 ratio at 16% ($w.w^1$) protein concentration. Showing the reinforcement of pseudoplastic nature at this specific ratio and concentration.

The synergism in the apparent viscosity in the 20:80 protein ratio may be related to the specific organization of the proteins in the systems at higher protein concentrations. It has been already reported a synergism effect in protein binary systems at a ratio where one of the proteins is much more abundant. Wong, Vasanthan, & Ozimek (2013) observed a synergistic effect between pea protein and whey protein at 20:80 blend ratio in the elastic modulus (G'), hardness, and viscosity parameters, which varies depending on protein concentration and pH. Tomé, Pires, Batista, Sousa, and Raymundo (2014) observed synergism in the G' of a protein mixed emulsion formed by the mixture of pea protein with hake protein at 20:80 ratio at pH 7.0. The authors argue that it may be caused by the optimum balance between repulsive and attractive forces in the system and at this specific ratio the protein-protein interactions are optimized. However, in the present case, a straighter forward explanation is the fact that a binary mixture of different size particles will not display a monotonous steric hindrance based on volume exclude arguments (Meng et al., 2014). Besides that, the presence of small particles, such as caseins, are capable to modify the structure of systems formed by large particles, such as pea protein, being capable to form more interactions between them.

The heat treatment is commonly applied to modify proteins in the food industry seeking the increase proteins' techno-functional properties. Thus, how the protein systems pure or mixed behave after heat treatment was studied as the next step.

3.2. Particles size distributions

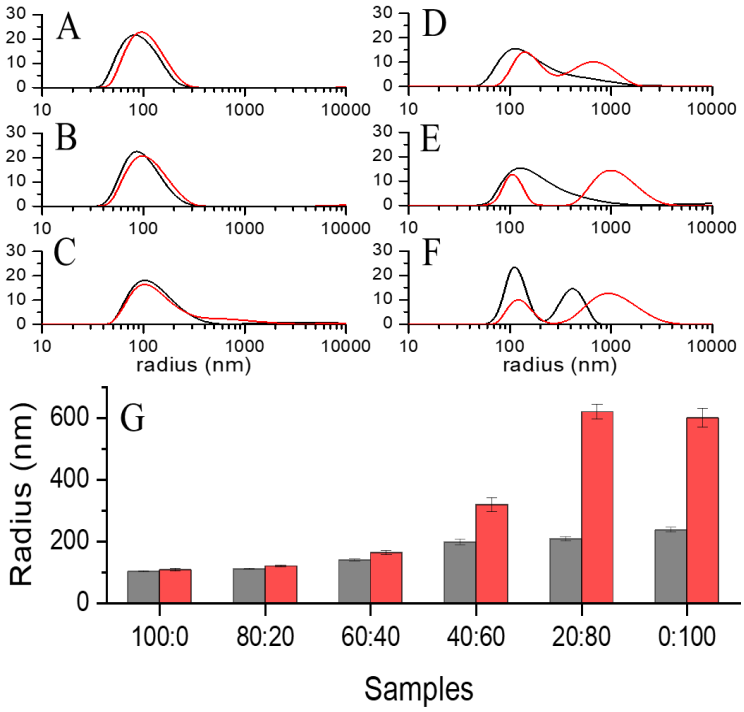

Figure 2. Particle size distribution of mixed protein suspensions before (black line) and after (red line) heat treatment at 12% (w.w^{-1}), (A) 100:0, (B) 80:20, (C) 60:40, (D) 40:60, (E)20:80, and (F) 0:100. (G) Average particle size before (dark gray) and after (red) heat treatment at 12% (w.w^{-1}).

The particle size of the suspensions by diluting a small amount of solution taken from samples at 12% (w.w^{-1}) was analyzed by DLS. It was observed one particle population in the casein suspensions with a hydrodynamic diameter of 213.95 ± 9.5 nm (Figure 2A). This diameter is in accordance with the results generally found for casein micelles in the pH condition studied (Nascimento et al., 2020; Silva et al., 2018). The thermal treatment applied did not cause any change in the casein micelle size, which explains the absence of change observed in the SAOS analysis. Contrarily, the pea proteins presented two particle sizes population, the first around 180 nm and the second around 690 nm (Figure 2F). It has been reported that pea proteins' native complexes have sizes of around 15 nm (Wu, Wang, Ma, Cai, Wang, 2020), thus, the particle sizes found in this study are much bigger than the expected values suggesting that the majority of pea proteins are aggregated. One cause of that could be the harsh production process applied in the extraction of the pea protein (Adebiyi & Aluko, 2011), which impacts their functional properties. Kornet et al. (2021) also found large protein aggregates in the commercial pea protein, compatible with the aggregates profile found in this study. The authors also observed the absence of a denaturation peak in

the commercial pea protein, showing that all the proteins were already denatured. However, the mixture of the two types of proteins, caseins and pea, did not significantly impact the sizes of the aggregates. This result further supports the assumption that there is no strong interaction between the two types of proteins.

The thermal treatment led to an additional increase in the pea population sizes, mainly in the largest particle population. The pea proteins are globular proteins that unfold and aggregate by different interactions such as hydrophobic, electrostatic, and disulfide bonds (Sun, & Arntfield, 2012). Thus, the increase in the G* observed in the gels where pea protein was present is likely to be linked to the increase in the aggregates' voluminosity.

To understand the nature of the aggregates in the system, a electrophorese analysis in reduced and non-reduced conditions was performed.

3.3. Electrophoresis

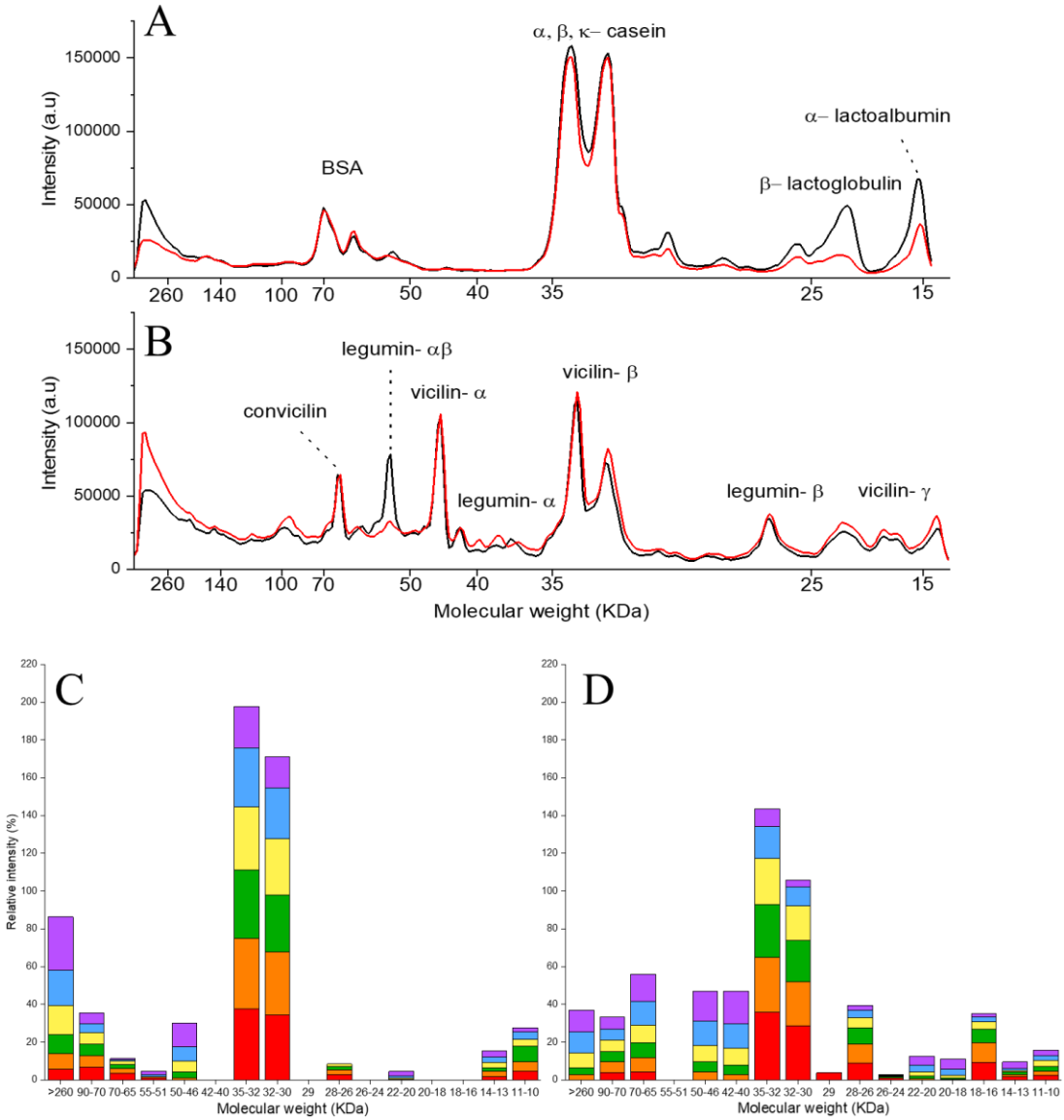

Figure 3. (A) 100:0 and (B) 0:100 protein band intensities before (black line) and after (red line) heat treatment at 12% (w.w^{-1}) protein concentration. Relative protein band intensities in non-reduced (C) and reduced (D) conditions. CMs: pea protein ratios: (— red) 100:0; (— orange) 80:20; (— green) 60:40; (— yellow) 40:60; (— blue) 20:80; (— purple) 0:100. Pea protein structure PDB ref: 3KSC and 7U1J.

The electrophorese peak intensities profile for the suspension at 12% (w.w^{-1}) protein concentration is shown in Figure 3. The protein band profile in non-reducing conditions was analyzed for pure casein and pea system before and after thermal treatment as can be observed in Figures 3A and 3B. The casein powder used in the experiment presented a small amount of whey proteins, as can be seen in the peaks between 18 and 10 KDa, which correspond to β-lactoglobulin and α-lactalbumin (Tarhan, & Kaya, 2021). In the sample, the casein band appeared at 35 – 32 KDa, also it was visualized as a small amount of BSA at 50 – 72 KDa molecular weight (Figure

2A). It was opted to use a commercial casein powder in the experiment to facilitate the transference of the results found here to applications in the food industry. Thus, it was admitted a small amount of serum proteins in the sample, which did not interfere with the overall sample physicochemical properties as can be seen by the results reported in section 3.5. The thermal treatment did not cause changes detectable by electrophoresis in the casein fractions; however, the serum proteins peak decreased after heating, probably due to the formation of new interactions with k-casein by thiol-disulfide interchange reactions (Anema, 2021).

The pea protein powder presented several protein fractions, which include convicilin, legumin- αβ, vicilin-α, legumin-α, vicilin-β, legumin-β, and vicilin-γ, which can be visualized in Figure 3B. The larger aggregates seen by electrophoresis make about 250 KD. It seems that there is a difference in size between the aggregates seen by electrophoresis (which “dissolves” all non-covalent linked aggregates) and the ones seen in DLS. The larger aggregates seen in electrophoresis could correspond to the hydrodynamic radius of the smaller, 100 nm population seen in DLS, if, in solution, these aggregates adopt a very loose “dendritic” conformation. However, is more unlikely that such aggregates would correspond to the larger, 600 nm of hydrodynamic radius, seen in DLS which would mean that those last larger aggregates are not formed by covalent links. Since it is also possible that larger covalent linked aggregates did not enter the electrophoresis gel, another DLS analysis has been done.

Analyzing the DLS results after being treated with the electrophoresis solutions, the pea protein aggregates in the suspension show a reduction in particle size. After the treatment with β-mercaptoethanol is possible to see a reduction of the hydrodynamic diameter from around 231 nm to 121 nm (Figure 4). This result shows that the pea aggregates can be stabilized by sulfhydryl and disulfide interactions, however, there are also non-covalently bound pea globulins responsible for maintaining these aggregates (Kornet et al., 2021). To better understand the nature of the aggregates in the system, a electrophorese analysis in reduced and non-reduced conditions was performed.

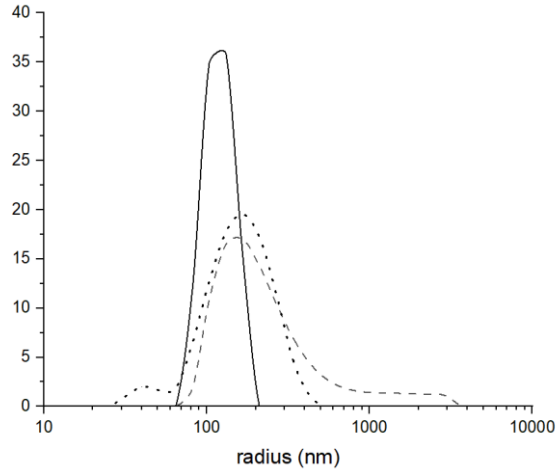

Figure 4. Particle size distribution of pea protein suspension before heat treatment at 12% (w.w-1) after being treated with reagents used in electrophoresis. Native condition (solid line), non-reduced condition (dot line), and reduced condition (dash line).

After heat treatment, it was observed a decrease in the intensity of legumin- αβ. It is explained by the unfolding, followed by aggregation of the legumin fraction, which also resulted in the increase of protein aggregates with molecular weight higher than 260 KDa, probably linked by disulfide bonds (Mession, Roustel, Saurel, 2017). It was observed the absence of differences in the other protein fractions. However, the increase in G* after thermal treatment may not be completely explained by the s-s linkages, once the amount of legumin- αβ corresponded only to 16% ($w.w^{-1}$) of pea protein bands. Thus, part of the gel structuration also maybe led by non-covalent interactions.

In the mixed suspensions, the electrophoresis was made only after heat treatment, once it is unlike the formation of any covalent interaction before the heat treatment. The samples were analyzed in non-reduced and in reduced conditions to have a better idea of the interactions in the gels. The proteins share similar protein molecular weights, and the relative band intensities were calculated and plotted in Figures 3C and 3D.

The comparison between the protein bands in the two different reducing conditions brings light to the type of interactions formed in the samples. Most of the high molecular weight aggregates disappeared in the reducing conditions, which confirms the formation of aggregates by disulfide bonds after heat treatment. The remaining aggregates are probably insoluble and enable to go through the gel and are in agreement with the results shown in Figure 4. The increase in the intensity of band 70-65 KDa is probably due to the convicilin fraction that can also interact via disulfide

bond (Lam, Karaca, Tyler, & Nickerson, 2016) and was probably in the high-molecular-weight aggregates. The increase and arise in the protein bands with molecular weight around 50-40 KDa is due to the legumin- α, which comes from the breakdown of the disulfide bond that kept the legumin fractions α and β together. Thus, the legumin-β can be visualized by the increase in the band 22-18 KDa. For casein, the most remarkable difference is the increase in the peak bands 28-26 and 18-15 KDa, which is related to κ- casein, β-lactoglobulin, and α-lactalbumin, confirming the formation of S-S linkages in these proteins after heat treatment. Despite the link between the proteins with their counterparts, the bands in the mixture systems seem to be resulted only from the simple addition of the protein, since they share protein bands with the molecular weights previously observed in the non-mixed system: no new band corresponding to the molecular weight of casein ($\alpha 1$, β, κ or $\alpha 2$) and pea protein dimer have been found. Thus, it is reasonable to attest that there is no important amount of covalent or physical interaction between pea proteins and casein micelles, as proposed by other authors in less concentrated protein suspensions (Silva et al., 2019; Mession, Roustel, & Saurel, 2017).

3.4. Nuclear Magnetic Resonance (NMR)

The water peak in the samples at 12% ($w.w^{-1}$) before and after heat treatment is shown in Figures 5A and 5B. The width of the peak is a good indication of the water dynamics; the shaper the peak, the stronger the water dynamics. In Figure 4 can observe that the dynamics decreased when more pea protein was added to the suspensions whatever before or after heat treatment. This indicates that, at least in the present conditions, pea form gels with “pores” smaller than the ones of a casein network at the same concentration. These smaller pores limits the water molecules’ freedom as confirmed in the microstructure analysis after heat treatment (Júnior et al., 2015; Peters, Vergeldt, Boom & Goot, 2017) (Figure 7F). This is true for most of the ratios, at the exception of the 20:80 one; when only 20% of the casein have been added to a dispersion of 80% pea dispersion, the water dynamics was quite the same of the pure pea protein sample (0:100). Thus, the 20% of casein that replaced by the same mass, 20%, of pea proteins (in the 0:100 ratio), does not strongly change the width of the water peak between the 20:80 and 0:100 (a small before or no difference after thermal treatment) (Figure 4B). This suggests that the 20% casein micelles

present in 20:80 dispersions change the “porosity” of protein network as strong as the pea’s proteins, in these ratios.

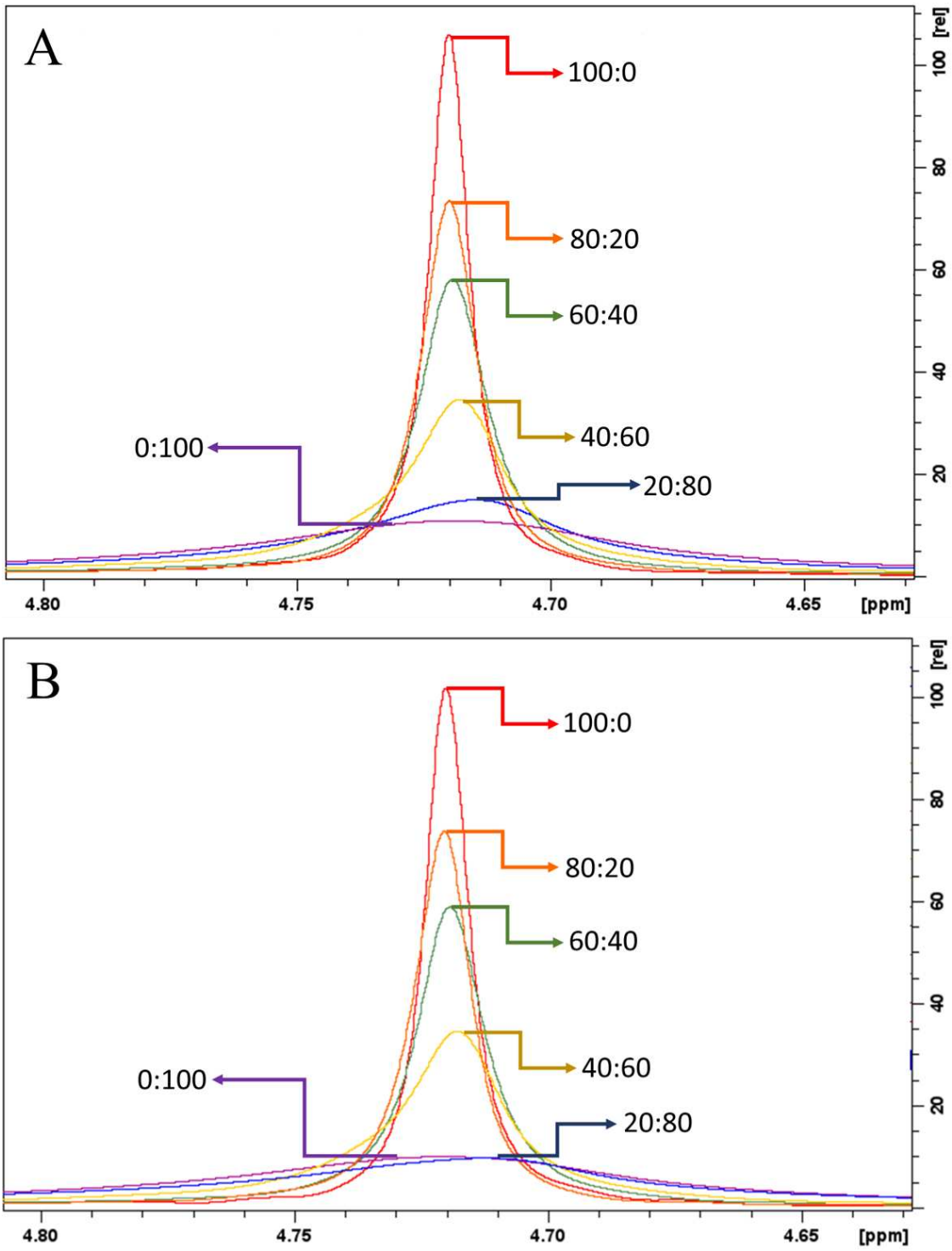

Figure 5. Water peak measured by liquid NMR analysis at 12% ($w.w^{-1}$) protein concentration before (A) and after (B) thermal treatment. CMs: pea protein ratios: (— red) 100:0; (— orange) 80:20; (— green) 60:40; (— yellow) 40:60; (— blue) 20:80; (— purple) 0:100.

3.5. Small amplitude oscillatory shear test

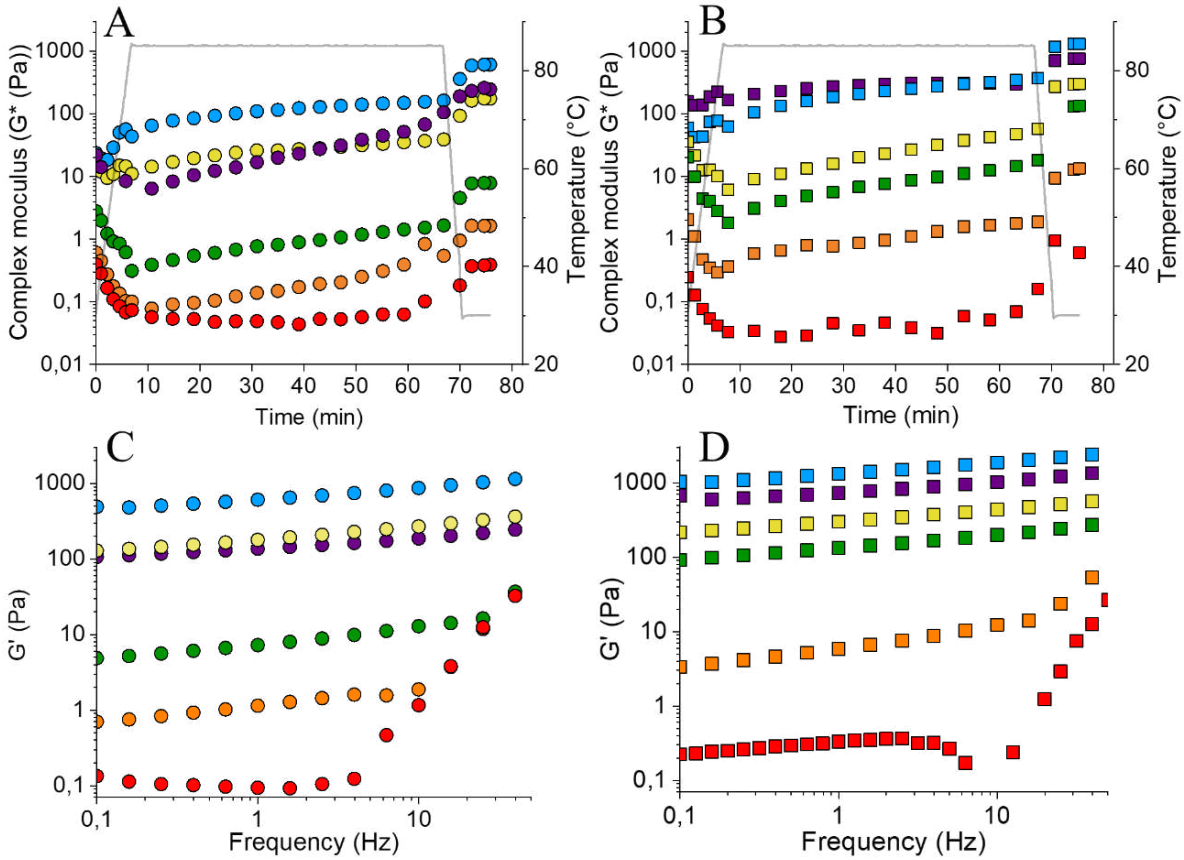

Figure 6. G* as a function of thermal treatment for casein micelle: pea protein suspensions at 12 (○A) and 16% (w.w-1) (□B). Frequency dependence of mixed casein micelle: pea protein suspensions at 12 (○C) and 16% (□D). CMs: pea protein ratios: (— red) 100:0; (— orange) 80:20; (— green) 60:40; (— yellow) 40:60; (— blue) 20:80; (— purple) 0:100.

SAOS rheology was performed to follow the thermal treatment employed in the suspensions of lower and higher concentrations, 12 and 16% (w.w^{-1}) total protein (Figure 6). The analysis can be divided into 4 regions, marked by the changes in temperature. The first is the increase in temperature from 30 ℃ to 85 ℃, which happened in the first 8 minutes. The second region is characterized by keeping the temperature constant at 85 ℃ for one hour. After, the temperature is decreased until the initial temperature (region iii) followed by maintaining the temperature at 30 ℃ (region iv) for 5 minutes. The results are presented in terms of complex modulus (G*). It was chosen as a parameter to compare the samples because it takes into consideration both elastic (G') and viscous (G'') modulus and makes easier the analysis.

The casein micelle suspension (0:100) presented the lowest G* among all samples. During the heat ramp, it was observed a decrease in G* in both 12 and 16% ($w.w^{-1}$) suspensions. It is explained by the expansion of the samples in the rheometer during the fast increase in temperature caused by the increase in the molecular motion, which disrupt hydrogen bonds (Tomé, Pires, Batista, Sousa, and Raymundo, 2014). After reaching the treatment temperature, the 100:0 G* did not change during the treatment time. It was expected since the absence of tertiary structure in the casein micelles makes it stable for heat treatment (Schäfer et al. 2017). Schäfer et al (2017) were able to detect a sol-gel transition in casein suspensions only at a pH lower than 5.4. Thus, in the studied conditions of pH and calcium content, it is unlike to observe significant changes detectable by SAOS in casein micelles upon heat. In the cooling step, it was observed an increase in G* probably due to the shrink in the system caused by the reduction of molecular movements, which increases the proximity of proteins. In addition, the formation of hydrogen bonds can take place as well (Yi et al., 2016). Thus, after the heat treatment, the difference in G* for the sample composed only of casein micelle is negligible.

The opposite behavior was observed for suspensions formed only by pea protein (0:100). The G* varies during the heat ramp in a higher degree for 12% ($w.w^{-1}$) suspensions and a lower degree in 16% ($w.w^{-1}$). The increase in molecular movements also was present in 0:100, but the beginning of pea protein unfold creates new interactions between the protein in the system, which balance the changes caused by swelling. During the heat treatment, the G* increased gradually. Pea proteins are globular proteins that are heat sensitive and, after achieving a certain temperature, some amino acids buried in the protein's natural conformation are exposed (Clark, Kavanagh, & Ross-Murphy, 2001). The exposure of hydrophobic amino acids leads to the formation of new interactions, mainly hydrophobic, but also hydrogen and disulfate bonds occur, depending on the pea protein fraction involved (Sun & Arntfield, 2012). If the newly formed interactions are sufficient to form a network and a continuous phase, a gel is established (Shand, Yaa, Pietrasika, & Wanasundaraab, 2007).

Comparing the studied concentration at the same protein ratio, it was observed an increase in the G* when the concentration increased from 12% ($w.w^{-1}$) to 16% ($w.w^{-1}$) (Figure 6C, 6D). It shows that the higher concentration of proteins makes easier their interactions, which increases the system G*. However, the changes compared before and after heat treatment were more pronounced in 12% ($w.w^{-1}$), probably

because in less concentrated suspension there is more space for protein unfolding before interactions, thus more new interactions between the proteins counterparts are formed.

In the protein blends, the G* increased following the addition of pea protein and the thermal treatment applied. The difference increased when the amount of pea protein increased, showing that is the pea the main protein source involved in the modifications caused by heat. However, at the end of the thermal treatment, the 20:80 protein ratio presented the highest G* compared to the other samples independently of protein concentration in the system (Figure 6A, 6B). These results increment the finds in the apparent viscosity analysis since the synergism effect in the 20:80 ratio was observed only at 16% ($w.w^{-1}$) (Figure 1C). Thus, considering that pea protein was responsible for the system structuration and the new interactions formed after heat are probably formed among pea proteins, the results indicate that the synergism at 20:80 is derived from the pea-pea interaction with the CMs in the between pea aggregates.

After the samples rest time, a frequency sweep test was performed to gather more information about gel networks (Figure 6C, 6D). The frequency sweep test can be applied to gather information about the type of interactions in a food gel, *i.e* if the polymers are simply entangled, or if there is the participation of covalent bonds or only physical interactions (Tunick, 2011). A strong gel is characterized by low-frequency dependence, while a weak gel presents high-frequency dependence (Tunick, 2011). The 100:0 suspension presented highly frequency dependence, where the curves change drastically inclination before 4 Hz at 12% ($w.w^{-1}$) and 16% ($w.w^{-1}$). The frequency results are aligned with the previous data and explained by the inability of casein micelles to form a gel in the applied conditions. Regarding the concentration, in 12% ($w.w^{-1}$) protein concentration, a more stable curve in the frequency sweep test only appears when pea protein was in majority, *i.e*., 40:60 sample (Figure 6C). The protein system 60:40 showed stability during the entire test at 16% ($w.w^{-1}$), indicating that the higher the protein concentration, the higher the number of protein interactions. The increase in pea protein proportion decreases the sample's frequency dependence at 12 or 16% ($w.w^{-1}$), which indicates the formation of stronger interaction in the protein matrixes with more pea protein. It was observed that the 20:80 sample presented higher G*, but the 0:100 presented lower frequency dependence (Figure 6D). This suggests that both protein ratios present bounds of the same nature, evidencing that

the synergism effect comes from a specific network organization and not from newly formed pea-casein interactions.

Observing the results presented in this work, a model of the distribution of the aggregate can be hypothesized (Figure 7). In the mixed systems, with an increase in pea concentration (Figure 7B-7E), occurs a decrease in porosity due to the steric impediment caused by the higher voluminosity of pea protein aggregates and the connection between the pea-pea aggregates, the main bonds responsible for structuring the gel. In this system, the pea protein and CMs, could form a gel with CM protein incorporated in the interstices. Kornet et al. (2021), analyzed the gel microstructure formed with 15% of proteins (pea protein and whey protein) with different concentrations of whey (25%, 50% and 75%). After CLMS analyze, they realized that pea protein and whey protein could form a continuous gel network with pea protein incorporated in this system. Comparing the ratios 20:80 and 0:100 (Figure 7G), is possible to see that the replacement of 20% of CMs may not impact the gel "porosity" and viscosity (at least for 12 to 14%) because distances between pea proteins in the 20:80 may not allow the caseins to be placed in the interstices between pea aggregates.

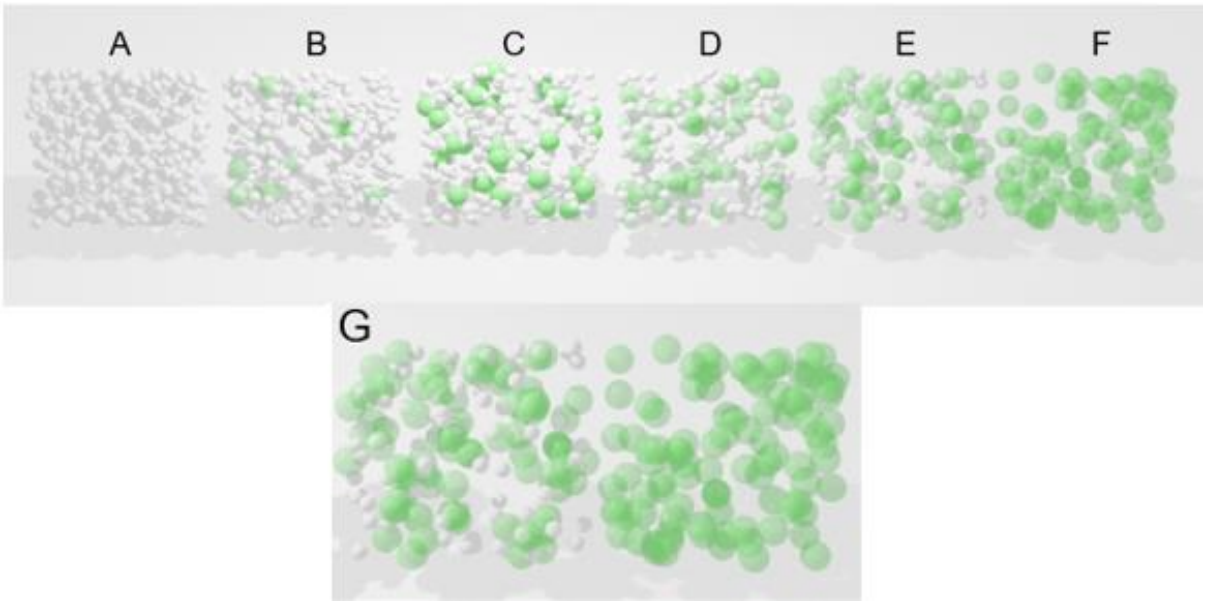

Figure 7. Schematic representation of mixed and pure systems formed by casein (white) and pea protein (green). (A) 100:0, (B) 80:20, (C) 60:40, (D) 40:60, (E) 20:80, (F) 0:100, and (G) extended version ratio 20:80 and 0:100.

4. Conclusion

In this study, rheological features of mixed casein micelles: pea proteins in high concentration suspensions were evaluated. Casein micelles and pea proteins seem to prefer interacting with themselves even in high protein suspensions. The thermal treatment increased the interaction between the pea proteins, which increased the elasticity of the systems, this effect is even more pronounced in less concentrated systems. The formation of disulfide bonds occurs, but only between milk-milk and pea-pea proteins. Despite the absence of disulfide bonds between pea and casein micelles, a synergism in the complex modulus and in the apparent viscosity of protein mixtures starting at 16% ($w.w^{-1}$) before thermal treatment and at 12% ($w.w^{-1}$) after thermal treatment was observed for 20:80 protein ratio. Thus, this study showed that the presence of a small amount of CMs interferes in the organization of the proteins and can induce more interactions in the overall system if a specific balance of forces is achieved. The next step of the investigation is the acid gelation of the mixed systems once this process is widely applied in the fabrication of fermented dairy products.

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Chapter III: Acid gelation of high-concentrated casein micelles: pea protein mixed systems

Acid gelation of high-concentrated casein micelles: pea protein mixed systems

The content of this chapter is being prepared for submission in: Food Hydrocolloids

Luis Gustavo Lima Nascimento, Raiane Rodrigues da Silva, Bertrand Doumert, Evandro Martins, Federico Casanova, Rodolphe Marie, Antônio Fernandes de Carvalho, Guillaume Delaplace, Paulo Peres de Sá Peixoto Junior

Abstract

The increased demand for plant-based products brings a new challenge to the food industry. Proteins from soy, chickpea, and pea are highly demanded. However, they still present some drawbacks such as poor techno-functional properties and remarkable beany flavor that hamper their wider application. Contrarily, milk products such as yogurt and cheeses are highly consumed and accepted worldwide. Therefore, the association of plant proteins, such as peas with milk protein is an interesting strategy to incorporate more plant-based proteins into people's diets. However, this strategy can change the gel formation and final structure. Thus, this study aim was to create mixed CMs: pea protein gel at high concentrations in four protein ratios,80:20, 60:40, 40:60, and 20:80 by acidification. The effect of a thermal treatment before gelation was also evaluated. The replacement of CMs for pea proteins disturbed the gel formation at the beginning of acidification, with an increased effect when the amount of pea protein increased. Despite this, the final gel elasticity was higher in the presence of pea proteins for the ratios 80:20 and 60:40, probably due to the formation of the pea network. It is hypothesized that pea proteins can form a network when surrounded by CMs, however, CMs restrict pea protein aggregation. Therefore, the final characteristics of mixed gels can be tailored by changing the protein ratios and applying thermal treatment before acidification, which opens the possibility for the development of new food products.

Keywords: Casein micelles; Pea protein; Acid gelation; Thermal treatment.

1. Introduction

Currently, it is a tendency toward consuming more proteins of vegetable origin. This tendency is due to higher consumer awareness about the health aspects of food and the increase in environmental concerns (Sá, Moreno, & Carciofi, 2020). Indeed, the production of animal proteins demands more land, and water and contributes more to greenhouse effects than the production of plant proteins (Thavamani, Sferra, & Sankararaman, 2020). Also, with the increased population growth, alternative sources of proteins need to be explored to achieve the demanded protein production with the minimum environmental impact. Thus, the exploration of plant protein from soy, lentils, chickpea, and pea is an alternative (Aschemann-Witzel et al., 2021).

Pea stands out among the possible legume to be explored for protein production. Pea production increased in the last 30 years, being produced in several countries. Pea presents between 20 to 24% of proteins, with starch and fiber composing the rest of the seed composition. (Ge et al., 2020; Lu et al., 2020; Burger & Zhang, 2019). In absolute value, pea presents less protein than soybean, however, the pea proteins are non-allergenic and lysine-rich (Ge et al.,2020; Senthilkumaran et al., 2022). Its digestion generates peptides bioactive, which present antioxidant properties (Duffuler et al., 2022). The main drawbacks of pea proteins concern their techno-functional properties and sensory acceptance, which are lower in comparison to milk proteins.

Milk is worldwide consumed due to its proteins' techno-functional properties and pleasant taste, with a prediction of an increase of 58% in the demand in the next 30 years (Fasolin et al., 2019). Over the course of the years, the food industry has developed technology and process to transform milk into several food products and have used milk proteins as ingredients in several other food applications (Walstra, 2005). The proteins from milk can be divided into two groups, the serum proteins, and the caseins. Caseins are the most abundant, comprising around 80% of the total proteins. There are four fractions of caseins,α-s_1, α-s_2, β, and κ, that self-assemble mainly by hydrophobic and calcium phosphate bonds, forming structures called casein micelles (CMs) (De Kruif, 2014). In the food industry, the gels formed by CMs destabilization are the base for the production of yogurt and cheeses.

Thus, the creation of products combining milk and plant proteins is an interesting strategy (Guyomarc'h et al., 2021). Since it uses the established dairy market to

increase the consumption and application of pea protein, mitigating their drawbacks, and at the same time, increasing the dairy product diversity. However, the replacement of a protein changes completely the rheological properties of the gels, and studies regarding the acidification properties of these mixed systems are scarce (Alves & Tavares, 2019). The majority of the studies focus on the impacts of the combination of CMs with pea proteins during the acidification were performed in low protein concentrations, without exploring the different protein ratio combinations (Ben-Harb et a., 2018; Mession, Roustel,& Saurel, 2017[b]; Roesch et al., 2004).

Therefore, this study aimed to investigate the gelling properties of high concentrated mixed systems formed by CMs and pea protein. It was studied different protein ratios of CMs: pea protein (80:20, 60:40, 40:60, 20:80). The effect of thermal treatment prior to acidification on the gelling properties of the systems was also evaluated.

2. Material and Methods

2.1. Materials

The Casein micelle (CMs) powder Promilk 85B was kindly provided by Ingredia SA (Arras, France). The pea protein powder (F85S) was provided by Roquette SA (Lestrem, France). All the other used reagents were analytical grade.

2.2. Sample preparation

The CMs and pea protein powders were resuspended in deionized water at 12 and 16% total concentrations. The suspensions were stirred using a magnetic stirring at 25 ℃ overnight to allow complete protein rehydration. To prevent microbial growth, sodium azide at 0.003 % ($w.w^{-1}$) was added to the suspensions. After complete rehydration, the suspensions were mixed in different CMs: pea protein ratios, which were 80:20 (80% CMs and 20% pea), 60:40 (60% CMs and 40% pea), 40:60 (40% CMs and 60% pea), and 20:80 (20% CMs and 80% pea). The pure systems of CMs (100:0) and pea protein (0:100) were also analyzed. After mixing, the samples were stirred for additional 3 hours at 25 ℃.

Then, the mixed and pure systems were submitted to thermal treatment consisting of heating the protein suspensions at 85 ℃ for 1 hour using a water bath,

followed by a fast temperature decrease until 30 ℃ using an ice bath.

Then, the samples were acidified with glucono-δ-lactone (GDL). Due to the differences in the buffer capacity of the proteins, GDL was added to the samples in different amounts aiming to standardize the time when all the systemsreach pH 4.6 (supplemented material). The acidification occurred at 30 ℃ and the samples reached pH 4.6 after 4.5 hours.

2.3. Small-amplitude oscillatory shear (SAOS) test

The formation of the acid gels was followed using the small-amplitude oscillatory shear (SAOS) test. After the addition of GDL, the samples were placed in a controlled stress rheometer (ARES, TA Instruments, USA) equipped with a 40 mm steel cone-plate geometry with an angle of 2° in a gap of 0.57 mm. The edges of the geometry were covered with silicon oil to prevent water evaporation during the test hours. After setting the gap, the suspensions were allowed to equilibrate for 2 min, and a time sweep test at 0.1% amplitude, 1 Hz, and 30 ℃ was performed. The chosen parameters were within the linear viscoelastic region (LVR), previously determined by an amplitude sweep from 0.01 to 100% at 1Hz and 30 ℃ (data not shown). After the time sweep, a frequency sweep test was performed without disturbing the formed gels. The frequency varied from 0.1 to 50 Hz at 0.1% amplitude at the same test temperature.

2.4. Water holding capacity

The water holding capacity (WHC) of the gels was performed as described by Nascimento et al. (2020). The gels were allowed to form in 15 mL centrifuge tube sat 30 ℃. After 4.5 hours, the gels were centrifugated using a g force of 4.000 *g* for 15 min at 30 ℃. After that, the released water was carefully removed, and the protein pellet was weighed. The WHC was calculated according to Equation 1.

$$WHC\ (\%) = \frac{mi - ms}{ms} \qquad (1)$$

Where mi is the suspension mass before centrifugation and ms is the mass of

removed water.

2.5. Water dynamics and free phosphate measurements by NMR

Solid-state nuclear magnetic resonance spectroscopy (NMR) was applied in the course of the acidification to access the evolution in the water dynamics and free phosphate content. ^{1}H NMR and ^{31}P NMR spectra were performed on a 9.4T AVIII Bruker spectrometer using a 4 mm probe operating at a spinning frequency of 700 Hz and temperature of 30°C. The spectra were recorded at the Larmor frequency of 400.13 MHz. Adamantane was used as an external referenced compound (δiso = 1.76ppm). The recycle time was 2 s and the 90° pulse was 2.7 µs. The spin-lattice relaxation times T_1 were calculated by saturation-recovery pulse sequence (90°-τ-90°) using a delay time in the range of 0.1 to 20 s.

2.6. Confocal laser scanner microscopy (CLSM)

The protein suspensions were prepared as described in section 2.2. Then, rhodamine B at 85g.L^{-1} was added to each sample, which was mixed for 30 s to complete the rhodamine solubilization. Then, the required amount of GDL was added to the samples to decrease the sample's pH. The samples were poured into an 8- well chamber slide (Ibidi GmbH, Germany) and put carefully in a waterbath at 30 ℃. After complete gel formation, the samples were visualized using an inverted microscope (Nikon Ti2) equipped with an sCMOS camera (Photometrics Prime95b).

2.7. Statistical analysis

The samples were compared by Analysis of variance (ANOVA). The effect of protein ratio was verified before and after heat treatment. Then, the effect of the heat treatment on each protein ratio was verified. When a significant difference ($p < 0.05$) was found, the Tukey HSD test with 5% significance was applied to differentiate means. All the experiments were performed, at least, three independent times, and the data was evaluated utilizing *SAS* software student edition.

3. Results and discussion

3.1. Time sweep analysis

The effect of acidification in the complex modulus (G*) of the samples is depicted in Figure 1. For pure systems, it can be observed a step increase in the G* modulus in the first minutes of acidification in both studied concentrations (Figure 1 A, a and F, f). It is explained by the faster acidification rates after the addition of GDL. GDL is a weak acid that dissociates slowly until reaches a plateau. However, the pH drops faster at the beginning of acidification (Zouari etal., 2018). After the first 1.5 hours of acidification, a larger difference between thepure systems was observed. While pure pea protein gels kept a slower but crescent increase in G*, the pure CMs gel presented a decrease in G* around pH 5.2, followed by a posterior increase in G* starting at pH 4.9. The differences in the curve are linked to the differences in the protein structures and mechanisms of stabilization. In suspensions, the CMs are stabilized mainly by their k-casein fraction, which is present on the micelle surface, conferring steric and electrostatic stabilization to CMs (Dalgleish, 2011). When the pH goes down, theoverall charges approach zero, and the k-casein shirk. Hence, the CMs approach each other, and aggregation takes place, the process goes on until the formation of a three-dimensional protein network. This phenomenon is perceived as the increase in the G*. Ouanezar, Guyomarc'h, and Bouchoux (2020) observed by AFM analysis the CMs morphology during acidification, where it presented a rough surface at pH 6.8, and a sphere-like form after the loose of the k-casein layer at pH 5.0. The posterior decrease is explained by internal rearrangements in the CMs trigged by the solubilization of calcium phosphate at lower pHs (Andoyo et al., 2014). When all the colloidal calcium phosphate is completely solubilized, the G* starts to increase due to the reinforcement of the three-dimensional network. This phenomenon is better visualized by plotting the loss tangent values (inserted graph), where it is observed a peak at pH 4.9 for CMs gels. On the other hand, the loss tangent keeps slowly decreasing for 0:100 samples, without any peak, explained by the absence of calcium phosphate bonds in the pea proteins. In addition, the formation of pea acid gels is due to the decrease in the surface charge of the proteins, leading to aggregation and gel formation (Klost & Drusch,2019). The final G* is another remarkable difference between the pure systems. The pea gel possessed a G* of 19,884 Pa at the end of acidification (pH 4.6), while pure CMs gel

reached 1,425 Pa, more than 10 times lower at 12% total protein concentration. Ben-Harb et al. (2018) studying mixed milk: pea acid gels at 14.8 % of protein, found that pea gels presented elastic modulus, at least, eight times higher compared to milk gels.

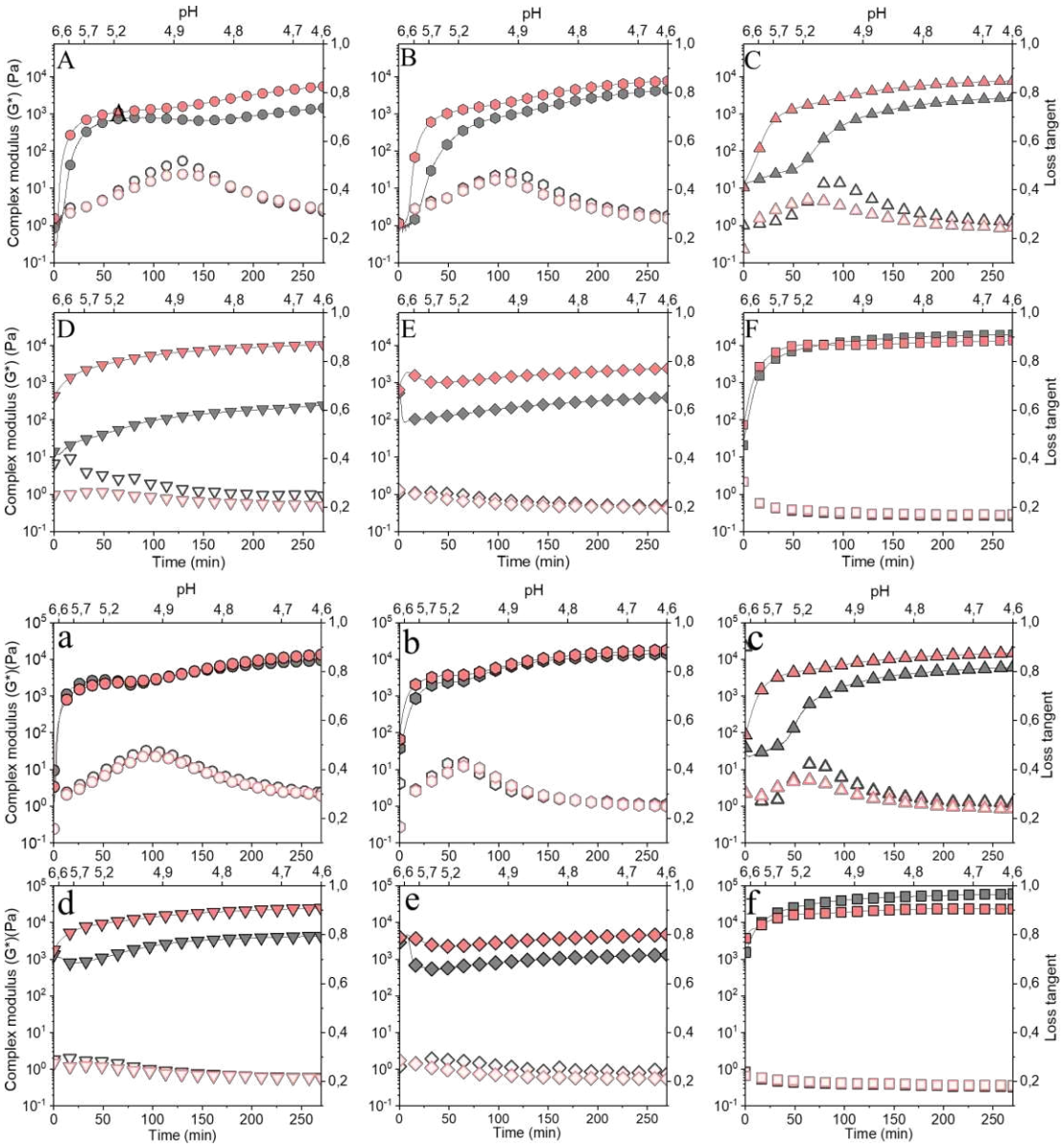

Figure 1. Complex modulus (full symbols) and loss tangent (empty symbols) as a function of acidification time for the samples without thermal treatment (gray symbols) and with thermal treatment (red symbols) prior to acidification. CMs:peaprotein ratios (Aa) 100:0, (Bb) 80:20, (Cc) 60:40, (Dd) 40:60, (Ee) 20:80, (Ff) 0:100. The high-case letters stand for 12% and lower-case letters for 16% total protein concentration.

In mixed systems, the gel formation depends on the protein ratio. Taking the 100:0 sample as a reference, when 20% of CMs are replaced by pea protein (80:20) retardation of G* increases occur (Figure 1B). After the first 30 min of acidification,

100:0 showed G* close to 295 Pa, while 80:20 showed G* around 19 Pa. However, at the end of the acidification process, the 80:20 gel possesses G* three times higher than 100:0. In resume, the replacement of CMs by a small amount of pea protein disturbs the gel formation at the beginning of acidification, but increased gel elasticity after complete gel formation. Thus, interactions formed in the middle of acidification are responsible for the final gel elasticity. The inserted graphs show that in the samples at 80:20 ratio, the peak in loss tangent occurs 16 min before and in lower intensity compared to the 100:0 sample. The further increase in the pea protein content in relation to CMs promotes an even shorter loss tangent peak time and intensity (Figure 1C - E). Thus, the pea presence seems to alter the calcium phosphate balance, which can be due to a simple dilution effect caused by the gradual replacement of CMs by pea protein, or the pea protein interferes directly in the loose of calcium phosphate by the CMs. In the further replacement of 40% CMs for pea protein (60:40 sample) (Figure 1C), the same curve behavior was observed, however with more intensity, as can be shown by the G* after 60 min of acidification. At this time, 100:0, 80:20, and 60:40 samples presented 700, 300, and 48 Pa, respectively, showing an even higher disturbance effect in the gel formation caused by the presence of pea protein. However, the final G* for 60:40 was still higher compared to 100:0, 2440, and 1440 Pa, respectively. Thus, despite the impact of pea in the CMs gel, the formation of the pea network by itself is able to promote an increase in the final gel elasticity.

The dependence of the protein ratio in the systems is clearer when the peaprotein becomes the major protein component. Until 60:40, despite the final G* being lower than G* for purely pea protein gel, it was higher compared to purely CMs protein gel. However, in 40:60 and 20:80 protein ratios, the final G* is lower compared to any pure system (Figure 1D and 1E). In the 40:60 ratio, the first G*point plotted in the graph is as high as in the 60:40 sample, however, when the pH keeps approaching the isoelectric point, the G* increases in much lower intensity compared to 60:40 or 80:20, and finished at 270 Pa. In the 20:80 sample,the first G* was higher than any other protein ratio or pure system. However, when the pH decreased, its G* also decreased, reaching a final G* of 250 Pa. Apparently, there is the formation of two independent networks as observed by other authors (Ben-Harb et al., 2018, Roesch, Juneja, Monagle, & Corredig, 2004), where a protein disturbs another's protein network formation. When CMs are the majority, the CMs gel disturbance is balanced by the

formation of the peanetwork, increasing G*. However, when the pea protein is present in a higher amount, it is hypothesized that CMs are homogeneously distributed within the pea protein network. Due to their thermodynamic incompatibility, there is a tendency for more pea-pea interactions at this ratio. As a consequence, the formation of larger pea aggregates occurs, strengthening the system elasticity before acidification. However, during acidification, the CMs formed network restricts the interactions between the pea protein aggregates, which decreases the final gel elasticity.

The increase in the total concentration in the systems promoted a general increase in the G* for all studied ratios (Figure 1a – 1f). In the pure systems, the final G* was increased proximately 6.5 and 3 times for CMs and pea protein, respectively. The lower increase in pure pea gel is probably due to system saturation. The proteins are already very close and the network is highly connected, thus the addition of more pea protein caused less effect comparing 12 to 16% total concentration. Regarding the G* curves, the profile observed for samples containing 16% protein is very similar to 12% protein. The replacement of CMs for pea protein showed the same tendency compared to 12% total concentration, *i.e.*, a disturbance of the initial aggregation stages in the CMs gelation. However, the 80:20 ratio is the only mixed gel that showed a final G* higher than the G* of pure CMs. Thus, even at 60:40 where the CMs were the majority protein present, the final G* was lower. It shows that the gel behavior depends not only on the ratio of the proteins but also on their concentrations.

The temperature is a useful tool to promote modification in the proteins and sometimes promotes interactions between two different proteins, as in the case of β-lactoglobulin and κ-casein (Andoyo, 2014). The formation of disulfide bond previous to acidification between β-lactoglobulin and κ-casein promoted by thermal treatment is applied in the yogurt production technology to increase final gel elasticity and decrease syneresis (Walstra, 2006). Despite having the required amino acids, such interactions do not take place between CMs and pea proteins (Mession, Roustel, Saurel, 2017). However, the thermal treatment still impacts the pea protein structures and pea-pea interactions. Thus, the effect of heat treatment in the suspensions prior to acidification was evaluated (Figure 1).

The effect of thermal treatment was similar in both protein concentrations studied. For CMs pure gel, it was observed a slight increase in G* in the sample treated

thermally, probably delivered by the denaturation of the small amount of serum protein present in the powder (Alting, Hamer, de Kruif, & Visschers, 2000). Interestingly, the pea protein gel with previous thermal treatment showed a final G* slightly lower compared to pea gels without thermal treatment (Figures 1F and 1f). As pointed out previously, the pea proteins in both 12 and 16% concentrations are very close to each other. The heat treatment caused protein denaturation and more interactions were formed, as can be evidenced by the higher G* at time zero. These new interactions may reduce the protein's freedom to rearrange, thus, during the acidification, fewer connections were formed, resulting in a less rigid gel.

In a mixed system, besides the increase in the final G*, the thermal treatment reduced the gel formation time mainly in the 60:40 protein ratio. Before thermal treatment, after 30 min of acidification, the G* was 40 Pa for the 60:40 gel. When the suspension was heated prior to acidification, the G* increased to 3 KPa at the same acidification time. Thus, thermal treatment is a useful tool to increase gelation properties of mixed systems, even if no strong interaction between the proteins of different sources takes place. This phenomenon is explained by the partial pea protein aggregation occurring during heat treatment, which gives additional protein connections to the formed network (Shand, Ya, Pietrasik, & Wanasundara, 2007).

3.2. Frequency sweep

The gels frequency dependence varies according to the nature of the network interactions. Gels with simply entangled polymer networks present higher frequency dependence when compared to gels with covalent bonds within the network (Tunick, 2011). A frequency sweep test was performed to determine the sample's frequency dependence and construct a better understanding of the protein interactions. The slope of the G' curves in the double logarithmic plot was calculated and is depicted in Figure 2. As the samples have a predominant gel nature, *i.e.* a higher G' compared to G'', only the G' was used to characterize the samples. During the entire frequency sweep no crossover of G' and G'' was observed (data not shown), showing the solid-like nature of the sample even in small observation times (50Hz) (Stojkov, Niyazov, Picchioni, & Bose, 2021). CMs and pea protein pure gels presented the highest and lowest frequency dependence, respectively. The mixed systems decreased the frequency dependence as the amount of pea protein increased in both studied protein

concentrations. The decrease in the frequency dependence seems to be caused only by a simple additive effect, which evidences the absence of newly formed strong interactions between the CMs and pea proteins and the formation of separated protein networks. The application of thermal treatment did not cause a significant difference in the frequency dependence in the samples, probably due to the absence of new stronger interaction within the protein matrix.

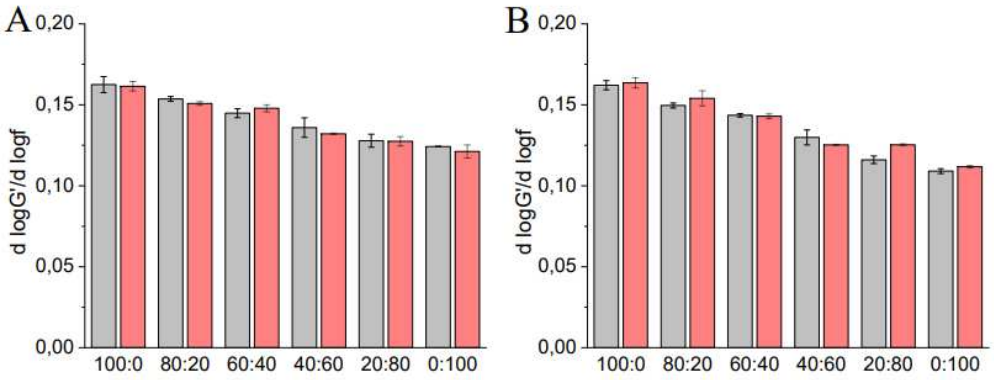

Figure 2. The slope of the double logarithmic curve in the frequency sweep testat 12% total protein concentration (A) and 16% total protein concentration (B), untreated (gray) or thermally treated (red) prior to acidification.

3.3. Water holding capacity (WHC)

The WHC of a protein gel is usually linked to the gel's microstructure. Stronger and more compact gels present high WHC, once the water diffusion becomes harder in this kind of structure (Cortez-Trejo, Gaytán-Martínez, Reyes-Vega, & Mendoza, 2021). In the pure systems, the CMs and pea gels presented 82.6 ± 1.9 % and 98.3 ± 0.8 %, respectively at 12% total protein concentration (Figure 3A). These results corroborate with the frequency sweep test, which indicates that pea protein gels possess a stronger interconnected network compared to CMs gels. As observed in the frequency dependence test, the progressive increase in the pea protein content increased the WHC of the systems, showing the role of the pea proteins in the gel network. In the 16% gels (Figure 3B), the WHC increased, however, little differences can be noted comparing the protein ratios. It is explained by the saturation of the gels caused by the high protein concentrations.

The application of heat treatment prior to acidification affected differently the WHC properties of the systems. While a WHC increase was observed for the systems

where pea proteins were present, the opposite behavior was observed for CMs gels. It was probably caused by the faster protein rearrangement at the beginning of acidification in the 100:0 sample heat treated. The same phenomenon was observed by Lucey, Munro, & Singh (1998), studying acid milk formed using GDL acidification. Despite being a practical method to infer the gel's microstructure. The WHC test presents low sensibility and evaluates the water-structure relation only after the complete gel formation. Thus, solid-state NMR was performed during the course of acidification to follow the changes in the water dynamics during the entire gelation process.

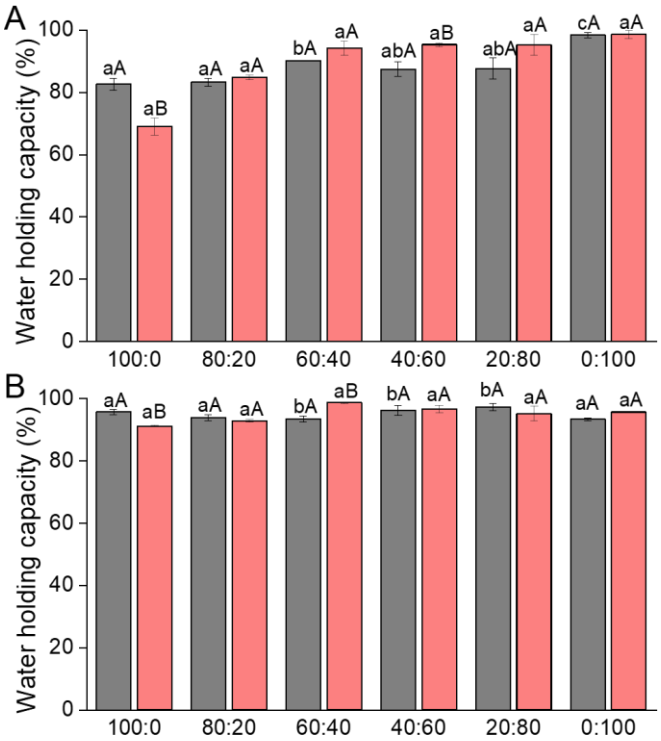

Figure 3. Water holding capacity at 12% total protein concentration (A) and 16%total protein concentration (B), untreated (gray) or thermally treated (red) prior to acidification.

3.4. Water dynamics measurement

The water dynamics for 16% of total protein gels during the acidification were determined by solid-state ^{1}H NMR (Figure 4A and 4B). The pure systems presented opposite behavior, being the final spin-lattice relaxation time T_1 for pure pea gels 73% lower when compared to CMs gels. These observations are in accordance with WHC measurements. The higher water relaxation times imply in high water dynamics within the gel structure, which is related to a coarser microstructure with larger pore sizes for

the CMs gels. On the other hand, the low water mobility in the pea gels indicates a more homogeneous microstructure with small pore sizes. Besides the final T_1, the acidification curve shape depends on the major protein fraction present. In the CMs gels, at the beginning of acidification, was observed an increase in T_1, *i.e.* an increase in the water mobility until a peak at approximately pH 4.8. After that, it was observed a decrease in T_1 values. For the pure pea gels, the T_1 kept decreasing during the entire course of acidification. The initial increase in T_1 in 100:0 samples can be explained by the CMs' behavior during acidification. As observed in the time sweep analysis, the solubilization of calcium phosphate occurs during acidification. It has been observed a positive correlation between calcium phosphate solubilization and the increase in water mobility, measured in terms of T_2 (Mariette & Marchal, 1996). After that, the CMs aggregate and form a tridimensional network, entrapping water, hence decreasing T_1. The pea proteins do not present the same salt composition and behavior, for this reason, the initial increase in T_1 was not observed. The decrease in pH decreased the overall charge of the proteins, which approach their isoelectric point causing aggregation with posterior networkformation and decreasing the water dynamics.

In the mixed system, the T_1 at the beginning of acidification followed the behavior of the prominent protein fraction. Thus, in 80:20 and 60:40 gels, it was visualized a slight decrease of T_1 in the first minutes of acidification, followed by its decrease when the acidification proceeds. In 40:60 and 20:80 samples, the curve profile is closer to pea protein gels, and the T_1 decrease during the entire acidification process. These results were also in agreement with the WHC test. Interestingly, the replacement of CMs for pea decreases the final T_1 in a non-linearway. For example, when 20% of CMs were replaced by pea protein a high drop in T_1 was observed, and the further increment of pea protein kept decreasing T_1, but to a lower degree.

The application of thermal treatment before suspension acidification also was evaluated through T_1 (Figure 4B). In general, the thermal treatment promoted a decrease in the T_1 for most studied samples, with more influence in the systems containing more pea protein. However, the opposite behavior was observed for CMs gels, which presented an increase in the final T_1 of CMs gels, indicating higher water mobility. These results agree with the WHC, with heated casein micelles presenting decreased WHC. For the other gels, besides the lower final T_1, the heat treatment increased the rate of T_1 drop, which indicates a faster network formation, agreeing with

the G* results. For 20:80 and 0:100 samples, the T_1 values kept stable after the first 2 hours of acidification, showing that the additional interactions caused by thermal treatment in the pea protein led to less protein rearranging during acidification. These results correlate with the founds in WHC and G*, where only slight differences were observed before and after heat treatment for the samples containing a high amount of pea.

As observed in G* curves, the CMs' loss of calcium phosphate has an important role during CMs gels, and the presence of pea proteins seems to anticipate this process. To better understand the relation between calcium phosphate distribution within the mixed systems, the free phosphate was evaluated at the beginning and at the end of acidification by NMR and it is discussed in the next section.

3.5. Free phosphate measurements

The ^{31}P NMR peak volume is corresponding to the quantity of free phosphate found in the samples at the beginning and the end of acidification is shown in Figures 5Cand 5D. The pure systems (only casein or only pea) present different free phosphate content at the beginning of acidification, being the amount of free phosphate in the pea protein pure gels is 33 times lower compared to CMs pure gel. Also, the pure systems present remarkable differences comparing the beginning with the end of the acidification process. In CMs pure gels, an increase of 57% in the amount of free phosphate was observed, corresponding mainly to the liberation of CM colloidal phosphate, while free phosphate content did not change for pure pea gels. The results correlate with previous pH, measurements. As expected, phosphorous and calcium solubilization occurs during sample acidification for the CMs system. Concerning the mixed systems, the replacement of 20% of CMs for pea protein (80:20 sample) led to a decrease of 71% in the free phosphate content when compared to CMs pure gels, while the further increase of pea protein content, sample 60:40, only decrease 27% in free-phosphate content comparing to 80:20 sample. This non-linear effect of phosphorous decrease as a function of the pea protein ratio increase demonstrates the chelating effect caused by pea proteins: a small quantity of pea inside a gel is enough to bind a high quantity of phosphate and, very likely, also calcium.

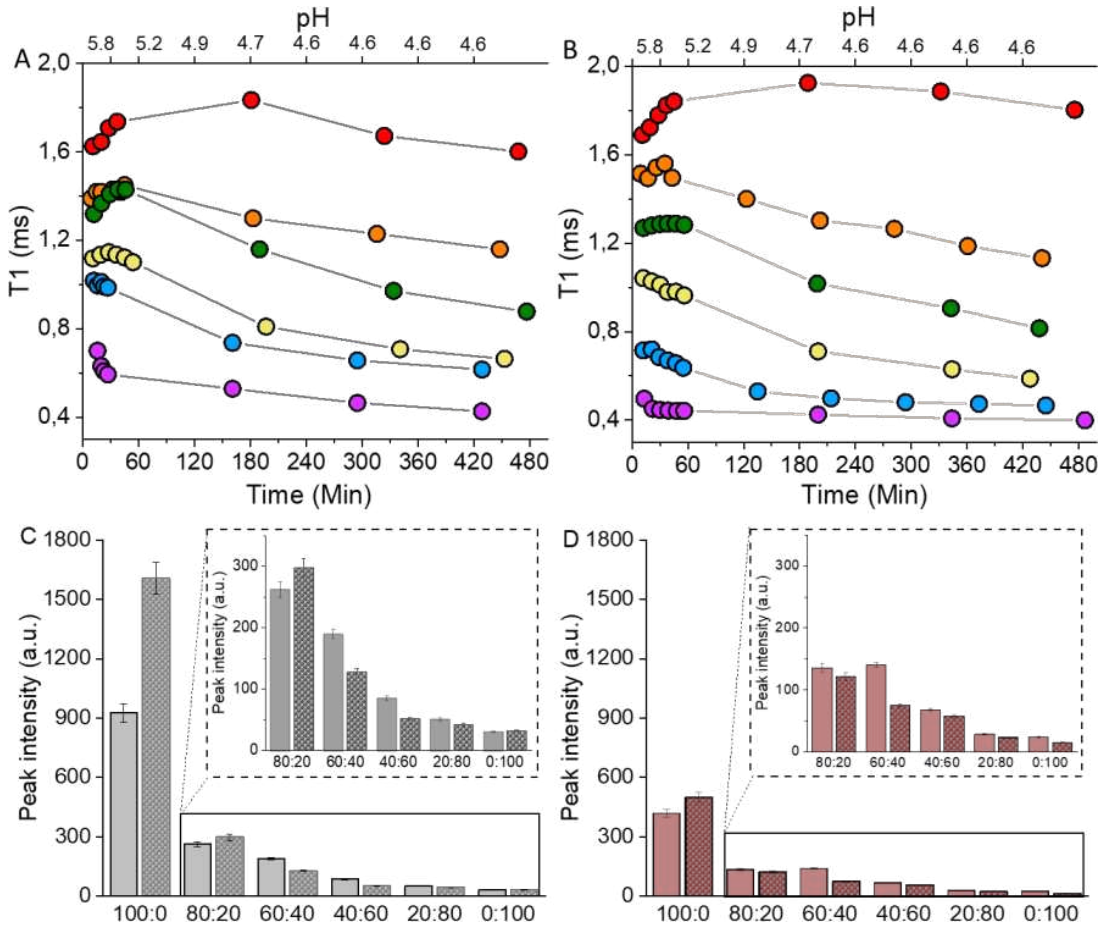

Figure 4. Spin-lattice relaxation times T_1 of water as a function of acidification time for CMs: pea ratios at 100:0 (red), 80:20 (orange), 60:40 (green), 40:60 (yellow), 20:80 (blue), 0:100 (purple) samples at 16% protein content. (A) without heat treatment and (B) with heat treatment. Free phosphate at the beginning, (smoothbars), and at the end, (rough bars), of acidification for 16 % protein content without (C) and with heat treatment (D).

The amount of free phosphate decreased for all samples, pure or in combination, after heat treatment, with a more intense effect in the CMs pure system (Figure 4D). However, the ranking in terms of free phosphate content between the beginning and the end of the acidification kept unchangeable whatever before or after heat treatment, *i.e.*, it was observed an increase in free phosphate content for CMs gels, and no effect in pea systems was reported observed. The decrease is explained by the reduction in the solubility of phosphate with the application of heat treatment. The prolonged heat treatment leads to irreversible precipitation of the salts, which interacts with the CMs surface (Broyard & Gaucheron, 2015) and very likely, with the pea protein's surface.

3.6. Confocal laser scanner microscopy (CLSM)

The gels' microstructure is shown in Figure 5. As the fluorophore attaches equally to both proteins it is impossible to differentiate them in a mixed system. However, the image of the mixed systems summed with the previous results is a valuable tool to understand the protein's interactions and arrangements. It is clear the differences between the pure gels. The CMs gels presented a microstructure similar to that found in the literature (Nascimento et al., 2020) with pores on the micro-scale. The CMs presented a coarser microstructure with larger pore sizes compared to pea gels. These results corroborate with the observations made in the WHC, final G*, and NMR. The bigger pore sizes facilitate the water dynamics, which is perceived physically as reduced WHC. At the same time, the water molecules have more space to freely move in the CMs gels, which explains the higher water dynamics found in the NMR results. And the lower connection degree within the protein network is responsible for the reduced final G* compared to the pure pea gels.

Compering 80:20 and 100:0 gels, it is clear the inhomogeneity created in the gel microstructure is promoted by pea protein presence, increasing the pore sizes in relation to the pure CMs gels. Nevertheless, the same CMs gel organization can still be visualized, however, with the presence of denser aggregates probably composed of pea protein, which were responsible for the higher final G* compared to pure CMs system. Comparing the 20:80 ratio with the systems composed only of pea protein, it was observed a remarkable difference in the microstructure. The 20:80 sample microstructure is inhomogeneous with larger empty spaces. At the same time, the proteins are very closely packed and with the same nano-scale pore presented in the 0:100 sample. In the intermediated systems, 60:40 and 40:60, the microstructure seems to be a combination of the observed in pure CMs and pea gels. They present small protein aggregates uniformly distributed, more connected than CMs gels, and less connected than pure pea gels. Thus, the formation of two distinct networks is hypothesized. The application of thermal treatment increased the size of the protein aggregates as can be seen in Figure 5 A' – E', with no remarkable difference in pure pea proteins. The CMs gel microstructure also was affected by heat treatment, probably due to the small amount of whey proteins present in the casein powder used. The results of the microstructure agree with the found in the rheological analysis.

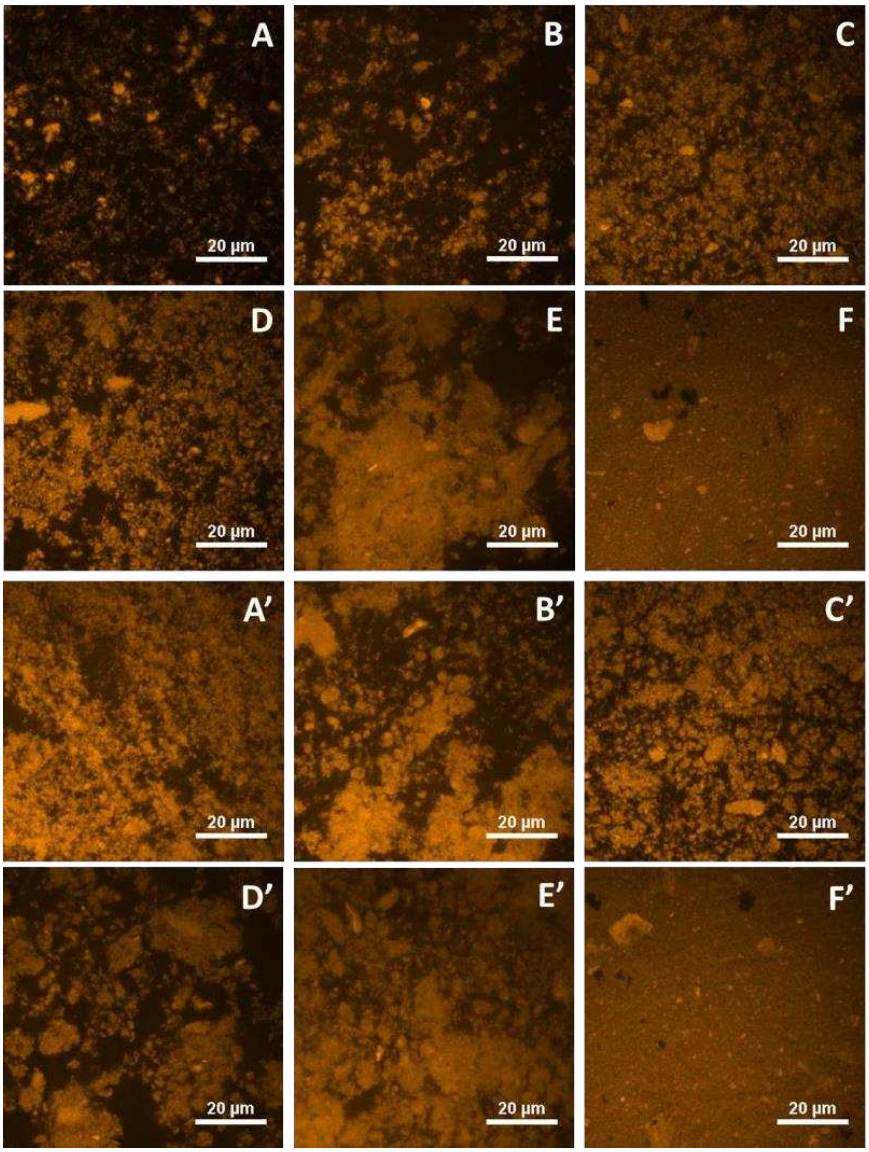

Figure 5. Microstructure of protein gels untreated (lower-case letters) and treated thermally (high-case letter) at 16% protein content. 100:0 (A, A'), 80:20 (B, B'), 60:40 (C, C'), 40:60 (D, D'), 20:80 (E, E'), 0:100 (F, F').

4. Conclusion

The applied techniques provided a comprehensive understanding of acidification systems and their impact on gel structures. CMs gels had larger pores and a less interconnected structure compared to pea protein gels. Thermal treatment affected CMs gels more significantly, while it reduced the strength of pea protein gels due to denaturation and prior aggregation. Gels with more pea protein had a stronger network and better water retention. Thermal treatment also reduced free phosphate content and increased gel elasticity, especially in gels with more pea proteins.

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Chapter IV: Molecular interactions involved in acid-gelation of mixed systems between pea protein and casein protein.

Molecular interactions involved in acid-gelation of mixed systems between pea protein and casein protein

The content of this chapter is being prepared for submission in: Food Hydrocolloids

Raiane Rodrigues da Silva, Guillaume Delaplace, Antônio Fernandes de Carvalho, Paulo Peres de Sá Peixoto Junior

Abstract

The development of new food products composed of mixed hydrogels, is dependent of the understanding of the structure, and the interactions are responsible for the maintenance of these. The aim of this work is to determine the interactions that are responsible for the structure of mixed hydrogels formed by casein micelles (CMs) and pea protein and the ratio impact on these interactions and structure. For that we conduct a heat approach utilizing rheology, NMR, and USAXS. On the rheology, the temperature impact was higher in the ratios that have more CMs, than the ones being formed by weaker interactions such as electrostatic interactions. Instead of this, the gels with high amounts of pea protein are formed by strong interactions, *e. g.* hydrophobic interactions. This result is also confirmed by NMR analysis since water mobility (T1) is higher on the gels formed majority by casein. On USAXS analyses, the effect of the ratio is more evident. This result suggests that the replacement of 20% of casein can approximate the pea protein chains forming a stronger gel than casein, which corroborates the results presented in other analyzes. Understanding the structure of mixed systems, the modification and improvement of interactions can be better studied opening the development of mixed gels with different characteristics.

Keywords: Mixed hydrogels; rheology; structure; USAXS;

1. Introduction

Nowadays food security is one of the bigger concerns in the world. The demand for food production rises as a result of the growing world population, rising calorie needs, and modern eating patterns that emphasize the consumption of meat and dairy products. However, the expansion of livestock becomes difficult and the need for a change in eating habits becomes increasingly evident (Depenbusch & Klasen, 2019).

An alternative to maintain the levels of protein consumption is to utilized proteins of plant origin, such as soy protein, chickpea protein, and pea protein, that require a smaller production area and do not impact the greenhouse effect (Silva et al., 2019). However, these proteins have low acceptability for the population due to their flavor, and their applications are limited, due to their low solubility, for example. Despite that, the mixture along products with high acceptability, such as dairy products, is interesting to increase the consumption of vegetable proteins while reducing the consumption of animal protein (Oliveira et al., 2022; Silva et al., 2019).

However, to develop new products combining animal and vegetable protein is necessary to understand the interactions, properties, and stability between them (Beghdadi et al., 2022). Bovine milk protein is compoused by casein (80% wt) and milk serum proteins (20% wt), mainly beta-lactoglobulin, alfa-lactalbumin, and bovine serum albumin (BSA) (Walstra et al., 2006). Casein is a phosphoprotein composed of four different types, αs1-casein, αs2-casein, β-casein, and κ-casein (~ 23.6, 25.2, 23.98, and 19.55 kDa, respectively). In milk, caseins are present in the form of micelles composed of casein molecules, water, and salts, mainly calcium phosphate (binding agent between submicelles). These fractions are held together through hydrophobic and electrostatic interactions (Krishna et al., 2021; Silva et al., 2019; Silva et al., 2019; Walstra et al., 2006). Due to their constitution, casein micelles (CMs) can suffer several types of modifications depending on ambient conditions, e.g., pH, temperature, ionic strength, and enzyme presence, which is of paramount importance to their application in the dairy industry (Silva et al., 2019).

Vegetable proteins, like soy, pea, beans, and chickpea, have seen increased use in the food sector during the past few years. Among these, pea protein has had a lot of focus, mostly because of its low allergenicity, high nutritional value, availability, low cost, and production sustainably (Lam et al., 2018; Shanthakumar et al., 2022). Pea protein is composed of globulin and albumin, which make up 70–80% and 10–

20% of the protein content in pea seeds. Globulins are salt-soluble storage proteins and can already be divided into other three groups, legumin (300-400 kDa), vicilin (150-170 kDa), and in minor quantity convicilin (70 kDa) (Kornet, Penris, et al., 2021; Lam et al., 2018). On the other side, albumin is a small protein (4-26 kDa), a water-soluble protein, that is responsible for seedling growth. (Kornet, Penris, et al., 2021; Shanthakumar et al., 2022). Because they are different, the application of each pea protein fraction can enable different commercial applications.

The protein gelation has been going on for many years, such as in yogurt, cheese and gelatin. Hence, the application of different proteins and gelation techniques continues to be explored to the development of new food products (Felix et al., 2017). The thermal gelation occurs due to the unfolding of native protein exposing hydrophobic residues and followed by aggregation and rearrangement with several interactions, i.e., hydrophobic, Van der Waals, hydrogen bonding, and covalent interactions (Ben-Harb et al., 2018; Felix et al., 2017). Acid gelation or cold gelation is another technique utilized to produce protein gels. This process normally occurs utilizing glucono-delta-lactone (GDL) which is a weak acid that dissociates releasing protons in solutions. Those are responsible to reduce the electrostatic repulsion by protonation of a charged carboxyl group. The protein aggregates approaches to each other to form a three-dimensional macromolecular network (Chihi et al., 2018; Oliveira et al., 2022).

The gelation of pea protein and casein protein was approach by many researches but the focus of these studies was physical properties, mainly rheological (Ben-Harb et al., 2018; Chihi et al., 2018; Felix et al., 2017; Mession et al., 2017a, 2017b; Oliveira et al., 2022). So far there are no studies that explain the molecular interactions involved in the gel formation or stabilization of casein and pea protein mixed gels, neither the influence of the ratio on these interactions. Thus, this paper aim is to understand the molecular interactions responsible to form and maintain the casein and pea protein mixed gel formed by acid gelation with glucono-delta-lactone.

2. Material and methods

2.1. Materials

Micellar casein (CMs) powder protein (Promilk 852B, 85.4% of protein (w/w)) was kindly donated by Ingredia SA (Arras, France) and pea protein (PPs) powder (Nutralys F85F, 84% of protein (w/w)) was kindly provided by Roquette SA (Lestrem, France).

2.2. Suspension preparation

The casein and pea protein suspensions were prepared by solubilization in ultra-pure water at a concentration of 16% (w/w). The rehydration occurred overnight at 25 °C and 600 rpm. To avoid microbiological development was added to the suspension 0.003% of sodium azide.

After the rehydration, the suspensions were prepared by mixing casein suspension and pea protein suspension in the ratios 80:20 (CMs:pea protein) and 20:80 (CMs:pea protein) for 2 hours at 30 °C at 600 rpm. The ratios studied were defined based on previous studies (not yet published). The pure suspensions were also analyzed.

2.3. Gelation process

The gelation occurred by the addition of glucono-delta-lactone (GDL) (Sigma-Aldrich, EUA) to the suspensions, 1.2% (w/w) to suspensions with high PPs concentration, and 2% (w/w) to suspensions with high CMs concentration and then mixed for 1 minute at 400 rpm. After this process, the suspensions were placed in a water bath for 4.5 hours at 30 °C until pH 5.2.

2.4. Suspension characterization

2.4.1. Particle size analyses

The particle size distribution of the mixed systems was measured by diluting the suspensions 100 times in ultrapure deionized water and placed in a polystyrene

cuvette and then measure by Dynamic Light Scattering (DLS) equipment (DynaPro Nanostar, Wyatt, CA, USA) at a 90° scattering angle in 658 nm of wavelength at 25 °C.

2.4.2. Molecular weight distribution analysis

The molecular weight distribution was determined by the electrophoresis technique following the methodology described by (Beghdadi et al., 2022). The samples were prepared by a first dilution to 8 mg/mL in ultra-pure water and a second dilution on the buffer for reduced (0.055 M Tris HCl, 2% SDS, 5% β-mercaptoethanol, 7% glycerol, 0.0025% bromophenol blue) and non-reduced conduction (125 mM TRIS HCl, 4% SDS, 20% glycerol, 0.4% bromophenol blue). The mini gels were composed of 4 % stacking and 12 % separating gels. After gels solidification, 20 µL of the samples was placed in the well and the running of the gel was carried out using a running buffer (0.025 M Tris, 0.192 M glycine, 0.1 % SDS) at pH 8.3, applying 150 V during 1 hour. After the protein migration, the gel staining was done by immersion of them in a solution of 0.15% of Coomassie® Brilliant Blue R-250 dissolved in acetic acid, methanol, and water for 30 minutes under agitation followed by discoloration in acetic acid solution (10%). After this process, the gels were scanned and analyzed using ImageJ.

2.5. Gels characterizations

2.5.1. Rheology measures

The structuration and strain of the gels were evaluated in a strain-controlled rheometer (ARES, TA Instruments, USA) using a cone-plate geometry with an angle of (0.06 rad and 112 µm gap). The linear viscoelastic region was determined by a strain sweep test (0.01 to 100%) at 1Hz and 30 ℃, and through this analysis, it was determined the strain to apply during the rheology tests. The flow behavior was determined through three cycles varying to shear stress from 0.1 to 300 s^{-1} to verify the thixotropic behavior.

Following the addition of GDL to the suspensions, the rheometer was filled and it was performed the time sweep during 4.5 hours at 30 °C, 0,1% amplitude. After the gelation, to understand the interactions responsible to maintain the mixed gels were applied a temperature sweep according to the methodology described by Andlinger and Kulozik (2023), with minor modifications. The temperature was set to 20 °C and

maintained there for 10 minutes to allow the equilibration in order to standardize the starting temperature after gelation on the rheometer. This was followed by a temperature sweep from 20 °C to 95 °C, with an increase of 5 °C·min^{-1}.

2.5.2. Nuclear magnetic resonance (NMR)

The gels were prepared as described in section 2.2.2, with the substitution of the water by deuterium oxide. After this, the gel was analyzed by solid-state nuclear magnetic resonance spectroscopy (NMR). It was measured the protons dynamic (^{1}H NMR) on a 9.4T AVIII Bruker spectrometer using a 4 mm probe operating at a spinning frequency of 700 Hz. To analyze the effect of the temperature, measurements of the water proton NMR relaxation times T1 were performed on the different hydrogels ratios and temperatures, 20°C, 30 °C, and 40 °C.

2.5.3. Ultra-small angle X-ray scattering (USAXS)

For USAXS analysis, the gels were prepared in the same way described in section 2.2.2, with a fixed capillary inside. The measurements were carried out according to Nogueira et al. (2023). The USAXS measurements were performed on synchrotron facility SOLEIL (Gif-sur-Yvette, France) on the SWING beamline operating at ~12 keV of photon energy. The scattered intensity was recorded on a detector placed at ~6.2 m from the sample. For each sample, data were first recorded at a short exposure time (typically ~0.2 s) to avoid any radiation damage (aggregation) that could result in artifacts at low q values. Subsequently, data were recorded at long exposure times (typically ~15 s) using a larger beam stop to obtain a good signal/noise ratio at high q values without damaging the detector. Intensities recorded at the two exposure times were then radially averaged and combined to get a scattering curve covering a q-range of 1.03 x 10^{-3} to 0,18 Å^{-1}. The corresponding intensities were discarded before the merging procedure was carried out. For each sample, the intensity scattered from the solvent (ultra-pure water) in the same capillary has been measured and subtracted from the gel sample pattern. The measurements were taken at 20 °C, 30 °C and 40 °C. The resulting corrected intensity is denoted by $q^2I(q)$ (a.u.).

2.6. Statistic analyze

The data were processed using variance analysis (ANOVA) with SAS University edition software to access the influence of the ratios and temperature. The Tukey HSD test was applied to compare the mean values. $P < 0.05$ was used to determine significance.

3. Results and discussion

3.1. Diluted suspension characterization

To characterize the hydrodynamic radius of the aggregates, present in pea proteins, in pure or mixed samples a characterization of the proteins has been done. The results of dynamic light scattering analysis are presented in Figure 1.

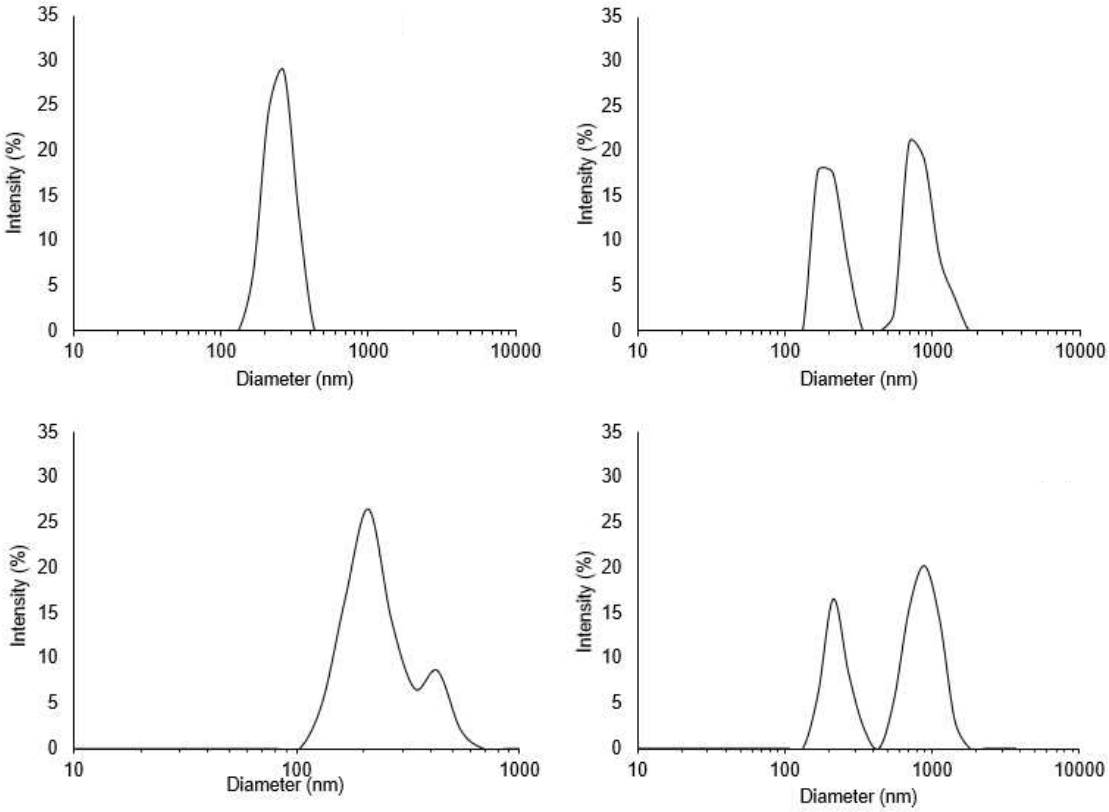

Figure 1. Particle size distribution of different ratios: 100:0 (A), 80:20 (B), 20:80 (C), 0:100 (D).

The apparent diameter in the ratio 100:0 (pure caseins) was 269 nm, which is in accordance with the results described by Beghdadi et al. (2022), which was 280 nm. In the ratio 0:100 (pure pea), the suspension presented two peaks. The first one is around 212 nm and the second one is around 880 nm, which is in accordance with the results presented by Kornet et al. (2021). For pea protein is expected a diameter of around 15 nm, hence, in the pea protein suspensions there is the presence of aggregates. Previous works argued that these aggregates are formed during the obtention of pea protein isolate, which is made by precipitation, purification, and drying (Barac et al., 2010; Beghdadi et al., 2022; Chihi et al., 2016). The presence of these aggregates is also confirmed by electrophoresis. In the mixed gels, 80:20 and 20:80, one can find sizes that correspond to sizes previously reported in pure proteins samples which indicates that no new type of aggregate is formed.

The electrophoresis technique is applied to determine the protein constitution of the powder. Figure 2 presents the molecular weight distribution of casein and pea protein suspensions. The molecular weight was calculated based on the R_F. The molecular weight of caseinfractions, α-, β-, and, κ-, varies from 39 to 32 kDa. The presence of serum proteins in the casein powder was also detected by the presence of BSA represented in the first peak (76 KDa) and the last peak referent to b-lactoglobulin (16 KDa). The presence of these proteins in casein powder may occur as a residue, from the production line.

Related to the pea protein suspension, in Figure 2B is possible to identify the fractions, convicilin, legumin, and vicilin. The first peak in the molecular distribution is related to convicilin, a storage protein with 83 KDa. Legumin is another common protein with a sedimentation coefficient of 11s. This proteins are divided into three subunits, acid (-α, 48 kDa), basic (-β, 19-27 KDa) and Leg-αβ, subunit covalently linked by a disulfide bond (73 kDa). The vicilin protein has also three subunits vicilin-α (55 kDa), vicilin-β (39 KDa), and vicilin-γ (16 KDa). Similar results were found by Beghdadi et al. (2022) and Shand et al. (2007) during studies about the impact of heat treatment on the formation of pea protein gels.

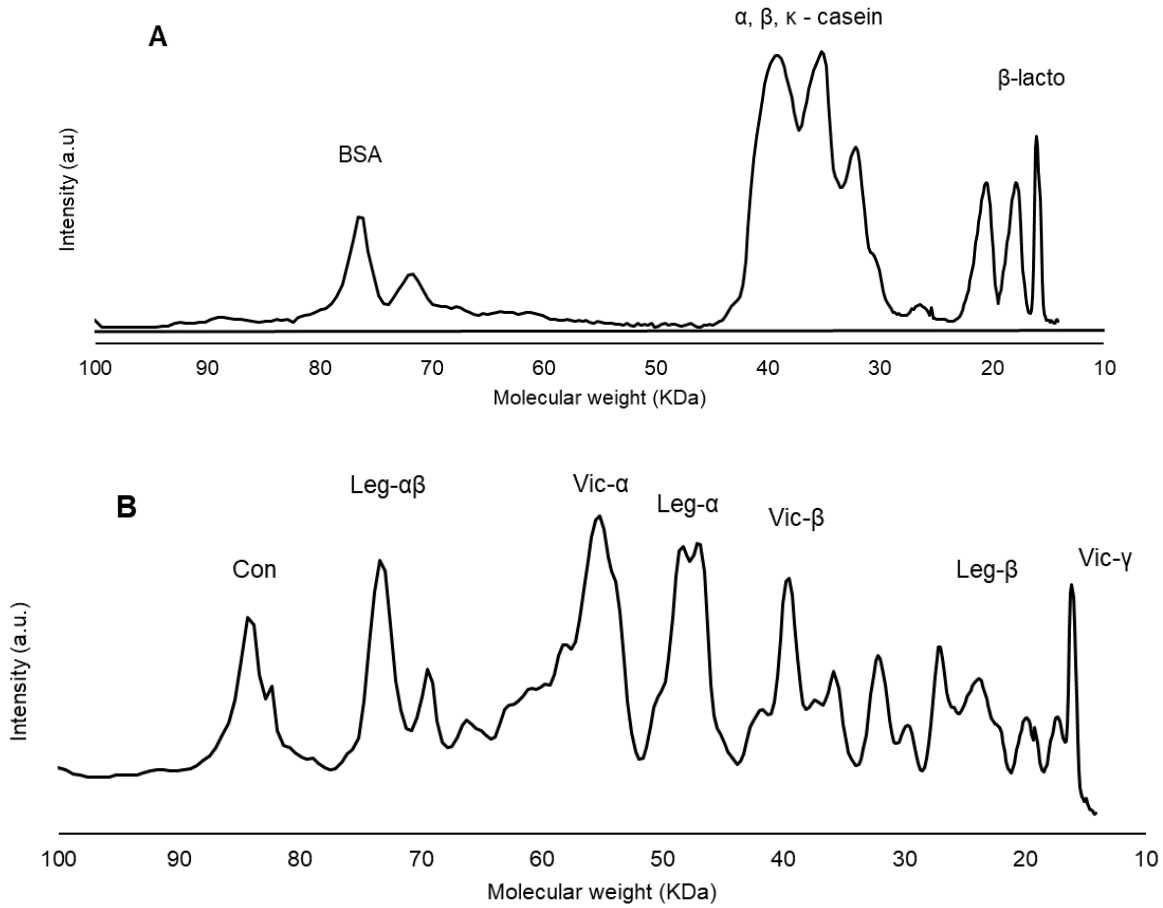

Figure 2. Molecular weight distribution of casein suspension (A) and pea protein suspension (B) at 16% (w/w).

At the top of the electrophoresis gel (Figure 3) it can be seen that not all of the sample added to the well was able to penetrate the gel, which agrees with the hypothesis that in the pea suspension, there are large aggregates. These aggregates are known as native aggregates, and they often develop as a result of the peas proteins isolation process. These aggregates also appeared in studies developed by other researchers (Beghdadi et al., 2022; Kornet, Shek, et al., 2021). Even with the treatment with SDS and β-mercaptoethanol, the aggregates cannot enter the gel. This result shows that besides disulfide and non-covalent interactions, other networks are responsible for the formation and maintenance of these protein aggregates (Sun & Arntfield, 2012).

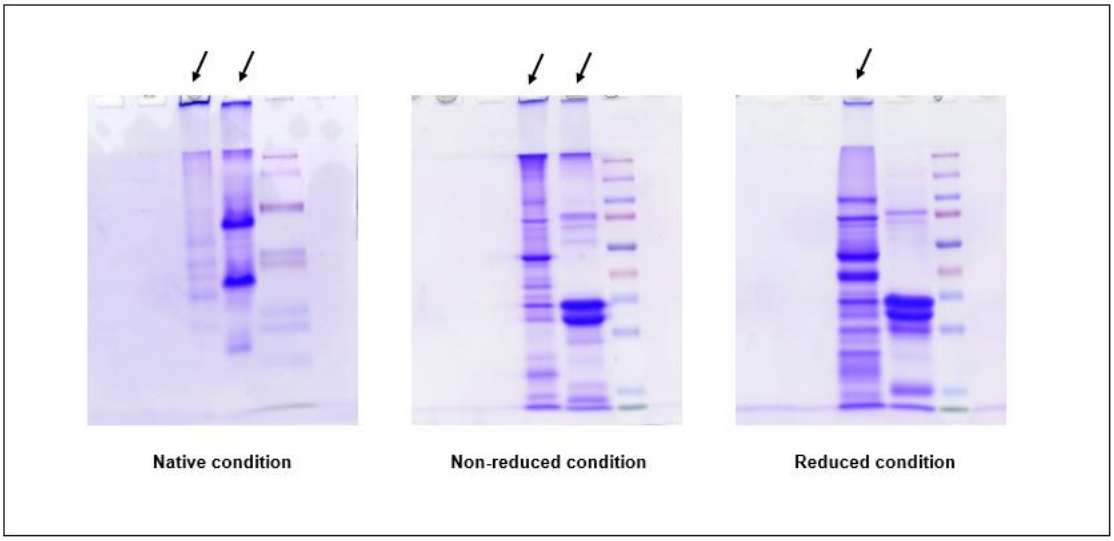

Figure 3. Electrophoresis gel in native condition, non-reduced condition, and reduced condition.

3.2. Gels characterization

3.2.1. Rheology measures

The development of new products originated from a mix of milk and pea protein is still difficult since only a few studies have investigated the interaction of these two components and the interactions through which they are associated.

In the rheology test, the presence of strong bonds that contribute to the elastics characteristics such as electrostatic interactions and hydrogen bonds modify the G′ values whereas G″ values are due to weaker bonds such as hydrophobic and Van der Waals bonds. Besides that, tan δ is related to the type of bond present, regardless of its quantity (Gastaldi et al., 2003).

In Figure 4, CMs gel (100:0), can be seen that the G' has a strong dependence on the temperature when this is raised to 55 °C. Analyzing the tan δ, until 60 °C occurs an increase of the magnitude. This reduction in G' and increase of tan δ, can be indicative that electrostatic interactions, such as hydrogen bonds and van der Waals are involved in the CMs gel (Andlinger & Kulozik, 2023). The same results were demonstrated by Schorsch et al. (2000) in an acid casein gel produced at 20 °C.

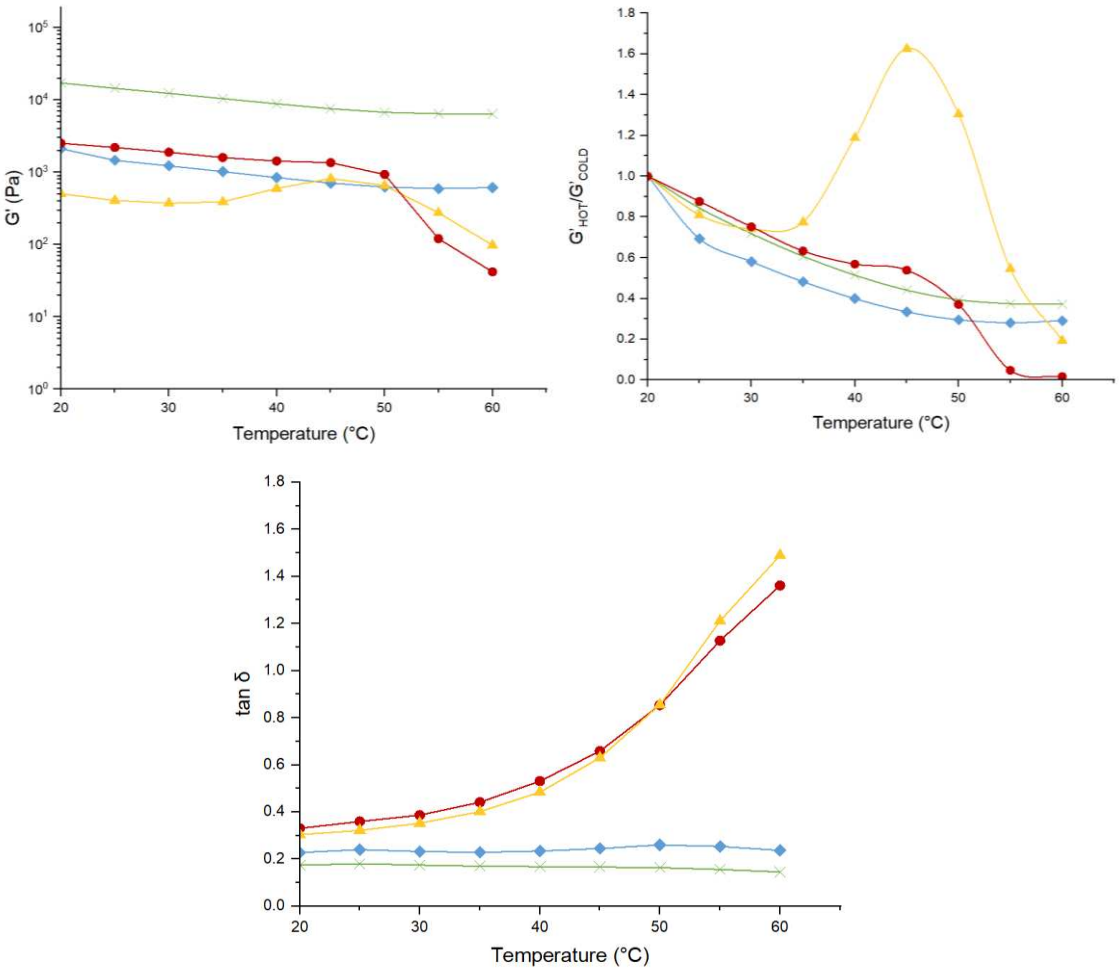

Figure 4. Temperature-dependence of G' (A), G'hot/G'cold (B), and tan δ (C) of mixed gels of CMs:pea protein ratios at 100:0 (red), 80:20 (yellow), 20:80 (blue), and 0:100 (green).

On the ratio 80:20, there is a reduction of G' until 35 °C. After this temperature, occuredan increase until 45 °C followed by a decrease when the temperature increases until 60 °C. In their study, Schorsch et al. (2000), identified the same behavior in casein gels. They established that at 50 °C, the gel expels water from its structure, a process known as syneresis. Upon the tan δ, the ratio 80:20 present the same behavior of the gel 100:0 in a smaller strength.

The ratio 20:80 present until 60 °C a decrease in their strength. Compared with the ratio 80:20 this value can be associated with a syneresis of the gel, which can occur later because of the reduction in the casein amount. Analyzing the tan δ, this ratio is almost unaffected by the increase of the temperature being reduced from 0.227 to 0.124. This low-temperature dependence of this ratio can be associated with the presence of hydrophobic bonds in these gels (Andlinger & Kulozik, 2023).

The ratio 0:100 presented a smaller temperature dependence for both G' and tan δ, which is associated with hydrophobic interactions, similarly to the ratio 20:80. Sun and Arntfield (2012) and Tanger et al. (2022) worked with pea protein gel and, reported that the majority of the pea protein interaction was non-covalent. Many forms of interactions, including hydrogen bonds, hydrophobic interactions, and electrostatic interactions, are included under non-covalent interactions. Sun and Arntfield (2012), treated the suspension with different blocking agents and observed that these agents interfered with the gel-forming process. They concluded that among the non-covalent interactions, the most representative was hydrogen bonds, hydrophobic interactions, and electrostatic interactions, been these last two responsible for the initial structure development during gel formation.

Given the changes that occur in the gel structure as it undergoes temperature changes, NMR analysis was performed to bring a better understanding of the effect of temperature on gels and insights into the effect of the ratio on the gel structure.

3.2.2. Nuclear magnetic resonance (NMR)

Solid-state 1H NMR was used to analyze the impact of the temperature on the water dynamics and in the proteins on the hydrogels. The water in hydrogels can be divided into three groups: bound water, intermediate water, and free water (Baumgartner et al., 2002; Mariette et al., 2002). The temperature effect on the water mobility is presented in Figure 5.

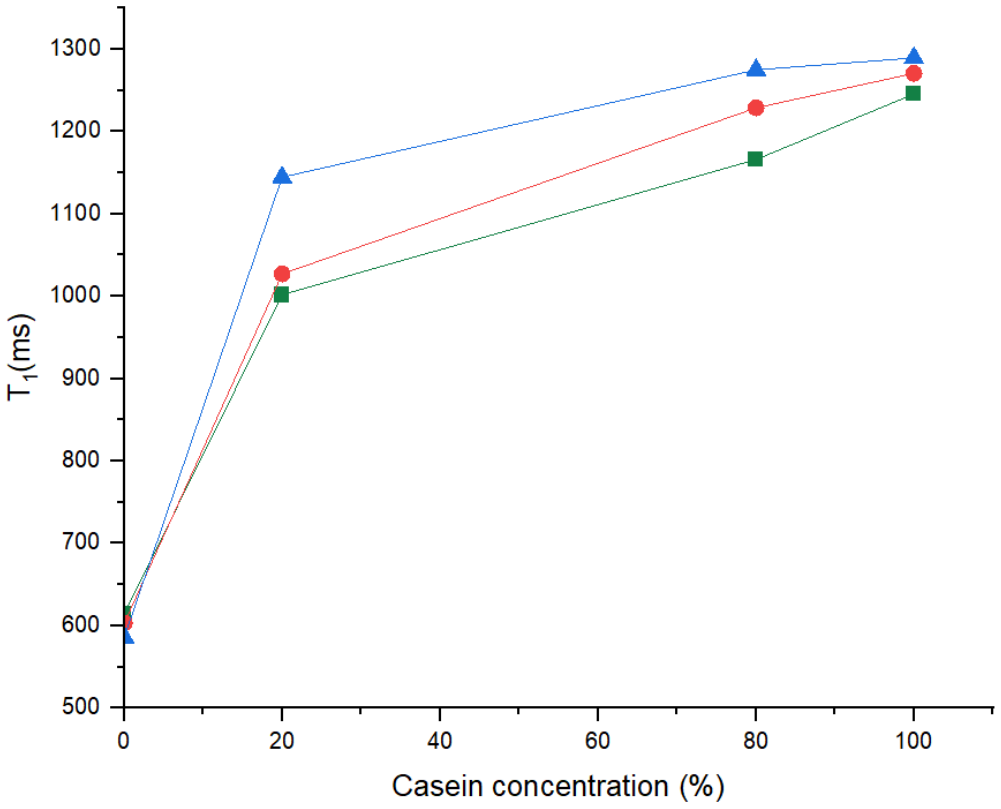

Figure 5. Spin-lattice relaxation times (T1) of water as a function of the casein concentration on the gels. Green line: 20 °C; red line: 30 °C; blue line: 40 °C.

The water mobility in the hydrogels is dependent on the ratio and the temperature. At 20 °C, the T1 of the gels increases with the rise of the casein concentration in the system, being the ratio 100:0 the ratio with higher T1.

High water dynamics inside the gel structure are implied by the higher water relaxation times T1, as seen in the casein gel (ratio 100:0; 100% of casein). Instead, pea protein gel have a low T1(ratio 0:100; 0% of casein), associated with less water dynamic. The high-water dynamic can also be associated with pore size. The water molecules in small pores, like those present in pea protein hydrogels, decay faster than those within large pores (casein hydrogels). A rapid decay fraction is generally related to a short relaxation time constant, often considered to be strongly linked to the matrix structure and with low mobility. Meanwhile a slow decay fraction is characterized by a long relaxation time constant, been considered to be weakly bound associated with matrix structure and more mobile (Ruan et al., 1997).

Comparing these results with those presented in the characterization session, the particles that have a higher density as pea protein form porous smaller than the

proteins that have low density (casein protein). The amount of matter in both systems is similar but the way proteins are organized changes substantially with the shape. This organization in CMs gels (huge porous) can leave a bigger space between the proteins, giving the water a higher mobility. On the contrary, pea protein gels which have a higher density, forming smaller pores and thus making water less mobile.

The temperature dependence of casein gels presented a linear increase with the raise of temperature. This behavior is similar to the water temperature dependence (Krynicki, 1966), showing that the presence of casein does not change the water behavior. This phenomenon does not happen, for example, in gels formed mainly by pea protein. When the gels are heated to 30 °C and 40 °C the same behavior as 20 °C is observed, although, the increase in T1 is not linear, showing that in the systems different networks are formed, as seen in section 3.2.1.

Comparing the pure gels and the mixed gels, there is a higher difference between 20 °C and 40 °C, being the ratio 20:80, the ratio with more difference. In the ratio 0:100, at 20 °C, the gel presented a T1 of 614.7 ms decreasing to 586.34 ms at 40 °C. The biggest difference was in the ratio 20:80, changing from 1002 ms at 20 °C to 1144.5 ms at 40 °C. Even with this difference presented by the ratio 20:80, the T1 on gels with more proportion of casein (ratio 80:20 and 100:0) is the biggest, showing that in these systems, water is less trapped and/or less associated with the gel. This result is a consequence of the weakest bonds formed between the caseins and their greatest dependence on temperature variation, as seen in section 3.2.1.

Comparing the behavior of some pea protein fractions that are present in the NMR spectrum on 2.79 ppm and 1.61 ppm (Figure 6 and 7), with the increase of casein concentration on the gels, the dynamics also increase, in Figure 6. On casein gels, the proteins are considered more dynamic due to the formation of a network that is more flexible. When there is an increase in pea proteins, this network becomes “harder”. However, this increase is not linear, been more intense on the gel with 20% of casein and less intense on the gel with 80% of casein. As seen in the other analyzes and more evident with the result of the USAXS, which will be presented later, the presence of caseins in pea gels has a great impact on the structure because the way the networks form are very different (Ben-Harb et al., 2018).

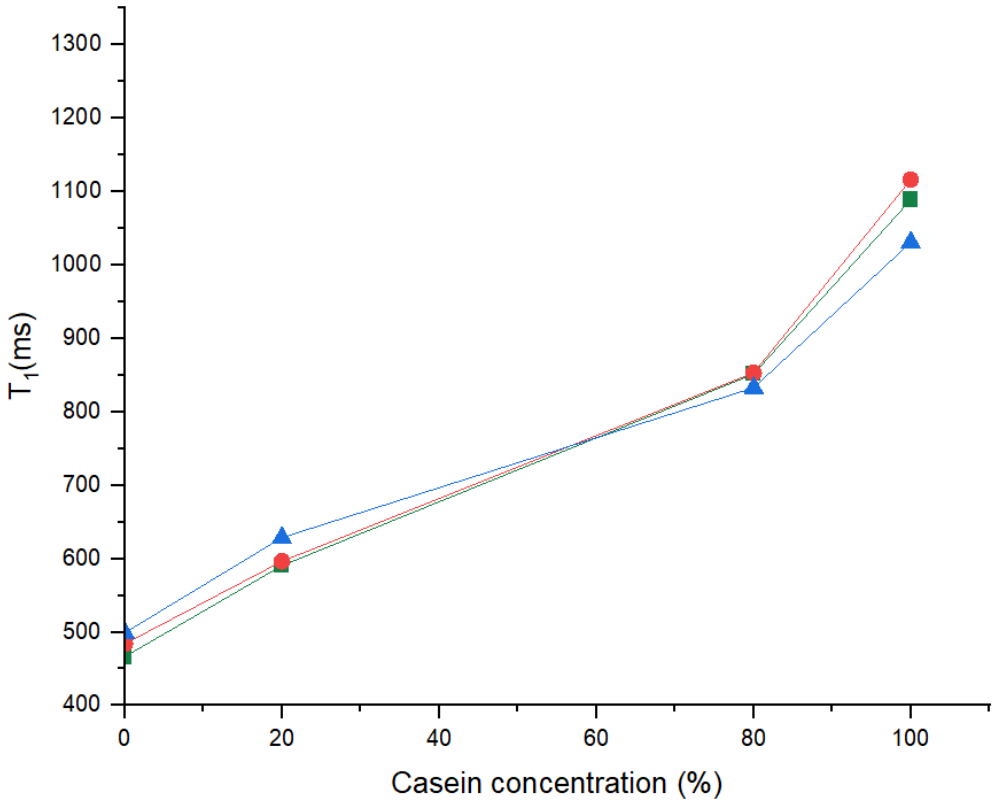

Figure 6. Spin-lattice relaxation times (T1) of pea protein (2.79 ppm) in function of casein concentration on the gel. Green line: 20 °C; red line: 30 °C; blue line: 40 °C.

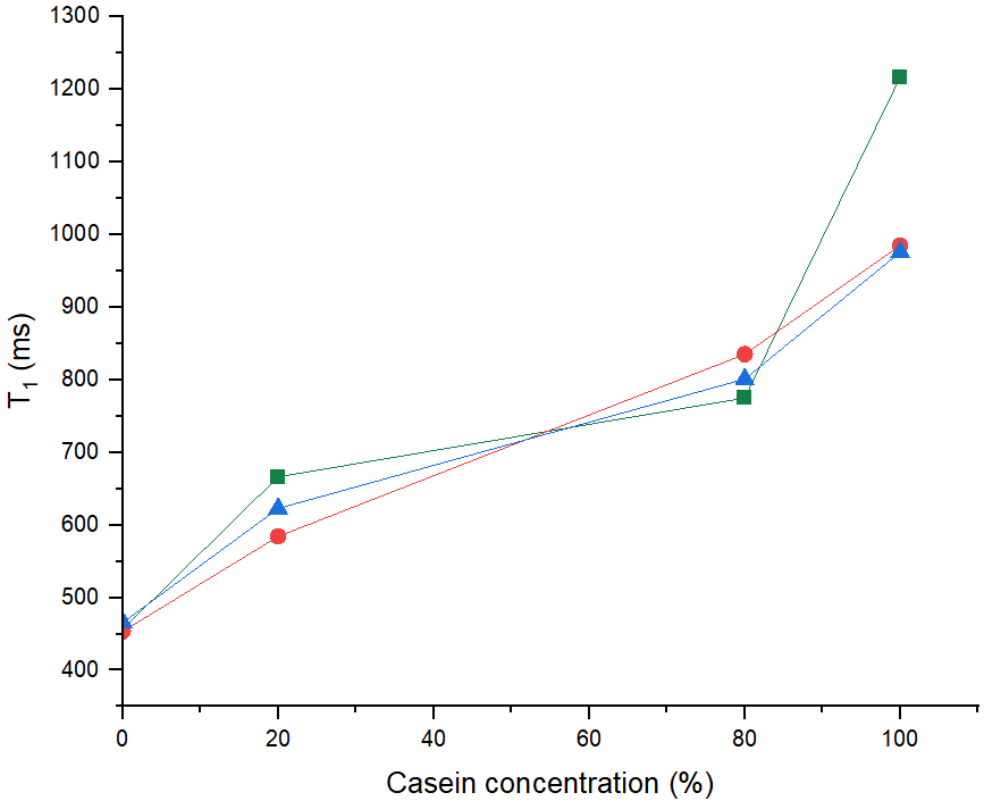

Figure 7. Spin-lattice relaxation times (T1) of pea protein (1.61 ppm) in function of casein concentration on the gel. Green line: 20 °C; red line: 30 °C; blue line: 40 °C.

3.2.3. USAXS

Ultra-small angle scattering is a technique thought is possible to determine the size, shape, form, and total surface area due to some mathematic models, and on the gels can supply information about protein aggregation (Chen et al., 2022). The USAXS spectra of casein present three levels that are related to three structures responsible to form the casein micelles. According to Bouchoux et al., (2010), the levels are a low q range (level 0), corresponding to a q range inferior to 6 x 10^{-3} $Å^{-1}$ correlates to the micellar envelope, which has a diameter of around 100 nm, and the distance between CMs in the current concentration. Intermediate q range (level 1), in the range of 6 x 10^{-3} $Å^{-1}$ to 2 x 10^{-2} $Å^{-1}$ correlates to smaller micelle structures (20 – 40 nm in diameter) that are referred to as "hard" because they are connected to incompressible structures when exposed to osmotic pressure. And, high range (level 2), with a range that represents two structures (higher than 7-8 x 10^{-2} $Å^{-1}$). The first one is the inter distance between calcium phosphate nanoclusters (CCP), assigned to a clear “shoulder” at a

“q” range between 0.042 and 0.27 nm^{-1}, that is called “hard” structures (~20 nm) as filled regions containing several CCP nanoclusters (~5 nm).

On the Kratky plot of Figure 8, it can be observed the temperature effect in all the ratios. At 20 °C, on the low q, all ratios, except the ratio 0:100 (black circle), present the same intensity, while at ratio 0:100 the intensity is reduced, indicating that the distance between the proteins is smaller than on the other ratios. On the high q, the intensity decreases as the proportion of pea protein in the system increases, similarly to what was presented above, probably due to the decrease of CCP amount.

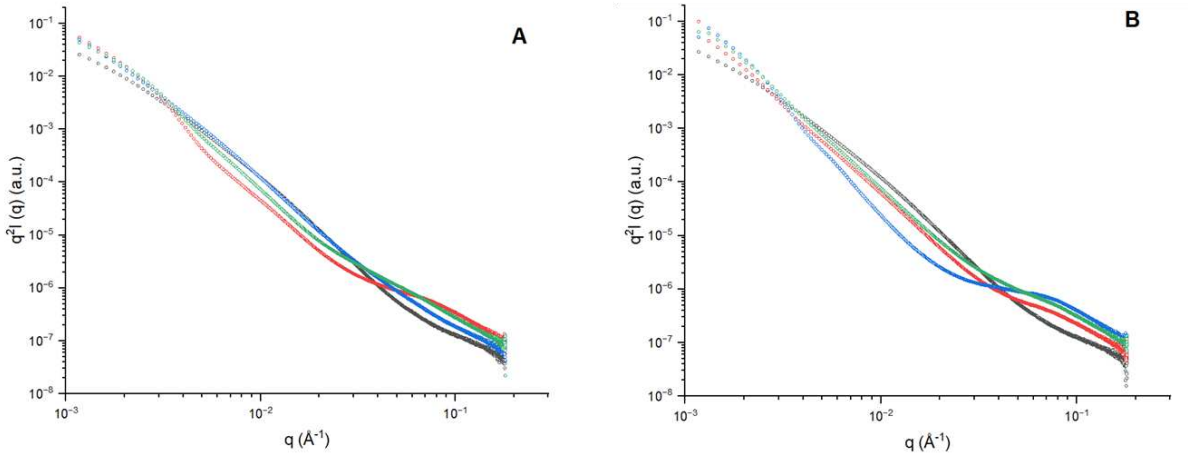

Figure 8. Kratky plots in a log-log scale of CMs:pea protein gels in function of temperature: A) 20 °C B) 40 °C. Red circle: 100:0 30 °C; green circle: 80:20; blue circle: 20:80; black circle: 0:100

In Figure 8B, on the low q, the intensity of the ratios 80:20 and 20:80 is higher than in the pure gels, which may indicate that most likely, in mixed media, there is the formation of larger aggregates (Chen et al., 2021). On the intermediate q region, which gives an idea about small particles with ~40-20 nm (Bouchoux et al., 2010), the ratio 20:80 present less intensity, probably because of the presence of casein. In this region, the ratio 0:100 presents the higher intensity, showing that this gel, at 40 °C has more small aggregates. On the high q, which has small units, *e.g* CCP nanoclusters and pea protein polypeptides, the pure gels presented low intensity than the mixed gels, which indicates that the inter-distance is less frequent in these samples (Nogueira et al., 2023b).

In all three levels, the substitution of 20% of casein, causes a big impact on the gel’s structure. Probably, the presence of casein in the gels, reduces the distance

between the proteins, being capable to form a stronger gel than other systems. On the NMR analysis, the ratio 20:80 has a T1 smaller than the ratio 80:20, showing that the presence of casein in the system, in a small amount can approximate the pea protein. This approximation favoring pea-pea links, being capable to form stronger gels than when the casein is in a higher amount, result that also corroborate with the results presented in rheology analyses.

Figure 9A presents the X-ray scattering caused by casein gel. No interferences of temperature happen in levels 0 and 1, while, on level 2, the q^2I is reduced, when the temperature reached 40 °C. This reduction of intensity is due to the demineralization of the system that occurs when the casein micelles gel is heated. However, the demineralization occurs just in a small part of the CMs, this can be concluded because if it occurred in most micelles there would be a change in the peak, which, as observed, does not occur (Nogueira et al., 2023b).

Comparing the Figure 9A and the Kratky plots presented by Bouchoux et al., (2010) and Nogueira et al., (2023b), observing level 2, it can be noticed that the shoulder is in the same position however in a smaller intensity than in the native condition, showing that in the gel structure, most casein micelles are lost. Nevertheless, there is in the low region the presence signal of the distance between CCPs, suggesting that at some points there are regions that have yet the casein micelle structure at a small scale.

Observing Figure 9B, related to pea protein gel, the temperature does not change the intensity of the scattering. On the high q, the scattering curve is presented upturned, indicating the presence of aggregates with a high dimension (Chen et al., 2022). Unlike casein gel, pea gels are not mainly structured by weak physical forces too susceptible pH or to temperature, therefore, the temperature does not impact the Kratky plot. A different effect is seen in the ratio 20:80 (Figure 9D). In this ratio, the increase of the temperature to 40 °C promoted an increase in intensity, both in low “q” and in high “q”. Unlike the temperatures of 20 °C and 30 °C and the ratio 0:100, when heated to 40 °C, the high q curve is turned downwards, indicating the presence of several small aggregates. In the study developed by Chen et al., (2022), during the heating of pea protein solution, the R_g decreased from 2.76 at 20°C to 2.20 °C at 59°C, due to the changes in the protein structure or the polypeptides released from the hexamers. They also suggest that the structure of pea protein became less compact and with flatter structure. In our study, may the same behavior happens in the gel, since

the temperature promotes a greater unfolding, which can also be observed in the G' on the rheology.

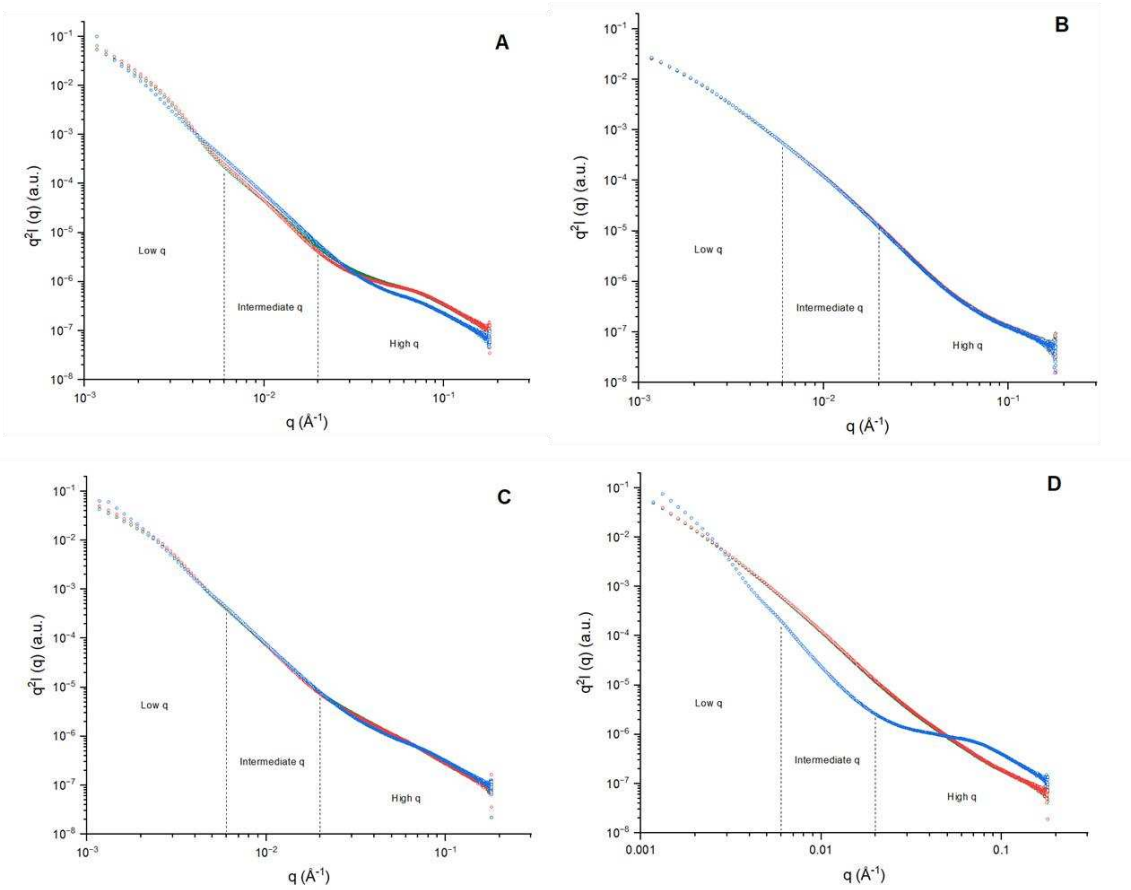

Figure 9. Kratky plots in a log-log scale of CMs:pea protein gels in function of concentrations: A) 100:0 B) 0:100 C) 80:20 D) 20:80. Green circle: 20 °C; red circle: 30 °C; blue circle: 40 °C.

4. Conclusion

Through a heat method, the molecular interactions existing in mixed pea and casein gels were determined. The gels that are formed mostly by casein protein are more sensitive to temperature changes and therefore have weaker interactions in their structure. Instead of this, gels formed mostly by pea protein present strong interactions on the gel, being less sensitive to temperature changes. It can also be seen that the replacement of 20% of casein can cause several changes in the gel, such as the approximation of the proteins, giving new characteristics to the mixed gels, however, in mixed systems, the different proteins do not interact, forming independent systems. In future studies, the optimization of the interactions between casein and pea for the

formation of gels with characteristics more similar to those already known should be studied.

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General conclusions and perspectives

1. Conclusions

In this dissertation, the mix between pea protein and casein micelles was evaluated.

In high protein suspensions, casein micelles and pea proteins seem to prefer interacting with themselves. The thermal treatment increased the interaction between the pea proteins, which increased the elasticity of the systems. This effect is even more pronounced in less concentrated systems. The formation of disulfide bonds occurs, but only between milk-milk and pea-pea proteins. Despite the absence of disulfide bonds between pea and casein micelles, a synergism in the complex modulus and the apparent viscosity of protein mixtures starting at 16% ($w.w^{-1}$) before thermal treatment and at 12% ($w.w^{-1}$) after thermal treatment was observed for 20:80 protein ratio. Thus, this study showed that the presence of a small amount of CMs interferes with the organization of the proteins and can induce more interactions in the overall system if a specific balance of forces is achieved.

After analyzing the impact of thermal treatment on suspensions, the formation of mixed hydrogels by acidification applying the same treatment was also evaluated. In a structure view, CMs gels had larger pores and a less interconnected structure compared to pea protein gels. Thermal treatment affected CMs gels more significantly, while it reduced the strength of pea protein gels due to denaturation and prior aggregation. Gels with more pea protein had a stronger network and better water retention. Thermal treatment also reduced free phosphate content and increased gel elasticity, especially in gels with more pea proteins.

The existing interactions on acid gels were evaluated through a heat method. The gels that are formed mostly by casein protein are more sensitive to temperature changes and therefore have weaker interactions in their structure. Instead of this, gels formed mostly by pea protein present strong interactions on the gel, being less sensitive to temperature changes. It can also be seen that the replacement of 20% of casein can cause several changes in the gel, such as the approximation of the proteins, giving new characteristics to the mixed gels, however, in mixed systems, the different proteins do not interact, forming independent systems. In future studies, the optimization of the interactions between casein and pea for the formation of gels with characteristics more similar to those already known should be studied.

2. Perspective

2.1 The effect of pea protein fractions on hydrogels structure

Evaluate the impact of the different pea protein fractions on the rheological and structural properties of the mixed hydrogels and select between than which one contributes more to the techno-functional properties.

2.2 Mixed hydrogels formed by the acidification by lactic acid bacteria.

In the development of fermented dairy products, the use of lactic acid bacteriaa is widespread. Thus, formulating mixed hydrogels using these bacteria can bring new insights to formulate new food products.

2.3 Strategies to increase CMs: pea proteins interactions.

The employment of methods with low environmental impact is preferable, with emerging technologies such as ultrasound and high hydrostatic pressure and pH shift being promising.