Universidade do Minho
Escola de Engenharia

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Effects of ohmic heating technology on ultra-high-temperature processing of bovine milk proteins.

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Finally, a word of thanks to everyone I have met throughout my academic journey. The good or bad influence that you may have had on me made me more determined and helped me grow as a person.

Statement of Integrity

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

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Resumo

Efeitos da tecnologia de aquecimento óhmico no processamento a temperatura ultraelevada das proteínas do leito bovino.

As proteínas alimentares são macromoléculas com uma grande importância nutricional e um potencial notável em várias áreas da biotecnologia. Alguns subprodutos provenientes da indústria alimentar, como o soro de leite, podem ser valorizados através do desenvolvimento de uma variedade de produtos funcionais, promovendo também para a sustentabilidade ambiental. A β-lactoglobulina (β-lg) é a proteína em maior abundância na constituição do soro de leite e tem sido estudada extensivamente e destacada como uma proteína modelo no estudo da desnaturação proteica. No entanto, certas interações ainda precisam de ser documentadas, como os efeitos de campos elétricos moderados (MEF) na estrutura desta proteína. O aquecimento óhmico é um método térmico emergente que resulta da aplicação de MEF. De momento, poucos estudos exploraram os efeitos de temperaturas ultraelevadas nas proteínas alimentares através desta tecnologia. Assim sendo, o objetivo principal deste trabalho é caracterizar e mapear os diversos passos de desnaturação proteica de β-lg através de aquecimento óhmico, como também tentar esclarecer como as diferentes características químicas e físicas podem ser correlacionadas. Para este efeito, foram realizados vários aquecimentos de soluções de "Whey Protein Isolate" (WPI) contendo β-lg numa tentativa de caracterizar os efeitos promovidos por MEF e aquecimento óhmico. Os resultados obtidos destacam certos intervalos de temperatura entre 40 °C e 165 °C para processos como hidrólise, alterações na estrutura secundária e formação de fibrilas amiloides. Assim sendo, foi estabelecido que os intervalos de temperatura que aparentam mais zonas hidrofóbicas expostas na estrutura de β-lg é de 90 °C – 110 °C, 90 °C – 120 °C para o aumento da exposição de triptofano, 140 °C – 165 °C para processos de hidrólise e 130 °C – 165 °C para a formação de "coil". Estes resultados contribuem para a caracterização do processo de desnaturação de β-lg através de aquecimento óhmico, que demonstra ser uma ferramenta efetiva com um grande potencial de aplicação em várias áreas da biotecnologia alimentar.

Palavras-chave: aquecimento, β-lactoglobulina, caracterização, óhmico, UHT.

Abstract

Effects of ohmic heating technology on ultra-high temperature processing of bovine milk proteins.

Food proteins are macro molecules with great nutritional importance, as well as presenting remarkable potential in many fields of biotechnology. Some subproducts from the food industry, such as whey, can be valorized into many functional products whilst reducing environmental load. β-lactoglobulin (β-lg) is a protein that makes up a major part in whey's constitution and has been studied thoroughly and recognized as a model protein in the study of protein denaturation, though several interactions still need to be evaluated, such as the effects of moderate electric fields (MEF) on this protein through ohmic heating (OH), an emergent heating method in the last decades that originates from the application of MEF. There is still a notable scarcity of studies that explore the effects of ultra-high temperatures on food proteins with this technology. As such, the main objective of this work is to characterize and map the different denaturation steps of β-lg under ohmic heating, as well as to offer a better understanding about and how different physical and chemical properties of this protein can be correlated. To address these goals, several heating assays on Whey Protein Isolate (WPI) samples containing β-lg were performed to attempt to characterize the effects promoted by MEF and ohmic heating. The results obtained highlights several temperature intervals between 40 °C and 165 °C for processes such as hydrolysis, tryptophan exposure, changes in protein secondary structure and amyloid fibril formation. Therefore, it was established that the temperature ranges optimized for exposure of hydrophobic patches in the structure of β-lg was 90 °C-110 °C, 90 °C – 120 °C for the exposure of tryptophan, 140 °C – 165 °C for hydrolysis rate, and 130 °C – 165 °C for coil formation. These results contribute to the characterization of the denaturation process of β-lg through ohmic heating, which proves to be a reliable and effective tool with remarkable potential of aplication in multiple fields of food biotechnology.

Keywords: β-lactoglobulin, characterization, heating, ohmic, UHT.

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1. Introduction

1.1. Protein function and structure

Proteins are macromolecules found in all living organisms. These molecules are polypeptides that can have a distinct complexity due to the possible variety in structure and function (Damodaran & Parkin, 2017). Additionally, they make up a significant part of the structure of cells and carry out every reaction that occurs in biological systems (Whitford, 2013). Proteins can partake in a variety of biological roles, such as biocatalysts (enzymes), structural components of living systems (collagen, elastin, keratin, among others), hormones (insulin, human growth hormone among others), involved in the transport of other molecules (such is the case of hemoglobin), among others, as presented in Table 1 (Damodaran & Parkin, 2017). The abundance of biological roles that proteins exhibit further emphasizes their biological importance in biotechnology and biomedical fields.

Table 1: Protein roles in biological systems.

Protein role	Detailed function	Example	Reference
Enzymes	Regulate various biochemical reactions	α-amylase, lactase	(Inanan, 2022; Shukla et al., 2015)
Hormones	Regulation of bodily functions	Insulin, growth hormone	(Bidlingmaier & Strasburger, 2010; Wilcox, 2005)
Transport	Transporting other molecules by binding to them	Hemoglobin, transferrin	(Kawabata, 2019; Mairbäurl & Weber, 2012)
Structure	Reinforce the architecture of biological structures	Collagen, fibrillin	(Timpl et al., 2003; Tzaphlidou, 2005)

The large variety of proteins and their diverse spectrum of biological roles is mainly due to their complexity and arrangement in different systems. Proteins are

composed of combinations of 20 different amino acids, and the several possible combinations of these amino acids are a key factor in determining protein function, structure, and several additional characteristics (H.-D. Belitz , W. Grosch, 2004).

Protein structure is highly dependent on the three-dimensional arrangement of a protein in space, which can be described through four different structural bases. The primary structure of a protein is determined by the amino acid sequence, as previously mentioned (Branden & Tooze, 2012). The repeated dihedral angles that determine the spatial organization of the peptide backbone of the protein results in the formation of several micro-structures that can have a specific geometry dependent on the angle. The main structures exhibited are α-helices and β-sheets, which describe the protein's secondary structure (Pelton & McLean, 2000). The full three-dimensional spatial organization of the peptide chain is described as the tertiary structure of a protein and the formation of protein complexes consisting of several peptide chains make up the quaternary structure of a protein (Alberts et al., 2002). Protein studies throughout the years have tried to predict secondary and tertiary structures of proteins through analysis of the primary structure, however it is still a topic that requires more investment to increase the accuracy of these predictions (Branden & Tooze, 2012).

Protein structure is a crucial factor in determining protein function and possible interactions with other molecules (Lee et al., 2007). In 2017, only 0.8 % of the 60 million protein sequences deposited in the UniProt protein database had manually annotated functions in SwissProt (C. Zhang et al., 2017). Most of these documented structure-function data has been collected through sequence homologous transfer, utilizing tools such as BLAST (Altschul et al., 1997). In recent years, better tools are being developed to increase the accuracy of prediction of protein function through its structure (C. Zhang et al., 2017). Establishing the protein structure-function connection is an important consideration when evaluating proteins of interest that could show good potential in various fields of biotechnology.

1.2. Biotechnological advancements involving proteins.

The scope of the food industry is considerably large, focusing on several properties of food products to improve their overall quantity and quality through various processes and production lines. There are several challenges on this field that are pushing the

development of new technologies and techniques to enhance better health and well-being (Hui et al., 2007), as shown in Table 2.

Table 2: Incentives for the development of innovative processes in the food industry.

Aim	Description	Reference
Food Abundance	Further increases in world population demands a production of large quantities of food.	(Divi & Krishna, 2009; Fitton et al., 2019)
Food Quality	Pleasant aromas, textures and flavors are a demanding factor from consumers when it comes to purchasable food products.	(Pagnossa et al., 2020)
Food Safety	Food preservation and packaging are essential to increase shelf-time of products and combat food waste.	(McFadden & Huffman, 2017; Pagnossa et al., 2020)

Macronutrient Composition	High-protein foods are very commonly sought by consumers. Additionally, many strategies to reduce hunger in several civilizations involve the addition of high-protein foods in the population's diets.	(Kwak, 2019; Shewry, 2007)
Efficient Food Processing	The abundance of byproducts from food processing incentivizes the development of strategies to utilize these byproducts and reduce food waste.	(Ciriminna et al., 2017; Jovanović et al., 2005)
Reduced Environmental Impact	Environmentally sustainable processes are becoming a crucial detail of every industry due to the negative impacts of climate change.	(Rischer et al., 2020)

Allergens alternatives	Alternatives to several food products (most noticeably milk derivatives) are a necessity to increase food choices of allergic individuals.	(Fasolin et al., 2019; Pereira et al., 2020)

Proteins can serve multiple roles in the food industry and are thoroughly studied to achieve the goals listed in Table 2, either as catalysts in the production of food items or as an important macronutrient in food.

Many enzymes have been thoroughly studied and engineered to aid in food processing. There are many examples of these enzymes, such as the use of lacasses in food and beverage processing to catalyze the oxidation process of aromatic compounds (Atalah et al., 2019); glutamate dehydrogenase as a biosensor to quantify ammonia quantities in food products, contributing to food safety procedures (Atalah et al., 2019); rennet as an important agent in the coagulation of milk and the production of milk-based products like yogurt and cheese (Uribe-Alvarez et al., 2021); among many more that continue to be part of optimized food processing procedures.

Protein is a dietary macronutrient essential in human diets (Loveday, 2019) and is readily available in several types of food. The nitrogen contents in human bodies have a crucial role in determining daily intake of dietary protein and is the main reason for the consumption of foods with a high protein content (WHO, 2007). Additionally, an increase in protein intake in human diets are linked to weight loss and reduced risk of heart disease and type 2 diabetes (Clifton, 2012), proving to be a possible approach in combating these modern diet-related problems (Khonje et al., 2020).

Flavor is an essential quality of food that allows for the perception of food ingredients, nutritional value, and locale of production of a specific food item (Shahidi & Abad, 2018). Consequently, it is a determining factor in food production, as pleasantly flavored food items lead to more interest from individuals. Food proteins contribute to the flavoring profile of certain foods when considering phenomena such as Maillard reaction that can occur during cooking or other heating treatments and influence food flavor and color (Zhang et al., 2021). Protein does not contribute to the flavor profile of

food, however, there have been plenty of studies that explore the ability for proteins to absorb and/or bind to flavor compounds, which can result in a change in flavor of a certain product. This characteristic can also be detrimental since proteins can absorb or bind to off-flavor compounds during processing (Barros et al., 2019). Protein denaturation and aggregation during heat treatments also influence the flavor of food items during processing, as denaturation of proteins can release flavor compounds previously entrapped by the protein's structure. In the case of the formation of protein aggregates during heat treatments, functional aspects can be altered such as protein-flavor binding capacity (Zhang et al., 2021).

Protein also has a myriad of roles in food products, contributing to important functional and technological properties such as foaming, gelling and emulsifying (Kang et al., 2021). Evidently, proteins serve a multitude of functions in the food industry, highlighting their importance in food processing and the manufacturing of new and improved food products or ingredients. There is potential in exploring these interactions with innovative methods to optimize processes and work forward the goals listed on Table 2.

1.3. Whey protein isolate and β-lactoglobulin.

Food waste is a global concern that becomes more prominent yearly. As world population increases exponentially, there is a significant increase of waste produced, namely food waste (Ravindran & Jaiswal, 2016). The European Green Deal encompasses some measures to attenuate food waste, such as the "Farm-To-Fork Strategy" which projects changes to the production of food products to promote a sustainable food system (Spani, 2020). Furthermore, European countries are aiming to halve food waste generated per capita at the retail and consumer level by 2030 (Santagata et al., 2021).

Food waste and byproducts from the food industry are an environmental problem and require large expenses to discard safely. This is due to their high microbial load deriving from processes such as fermentation, that increase the risk of environmental damage or complications to public health (Zhu et al., 2020).

Recently, there have been many advances in the development of new technologies that facilitate the recovery of these byproducts, mainly due to the aggravation of the environmental crisis, unveiling new possibilities for the valorization of these substances (Zhu et al., 2020). Ideally, all industries would benefit from the development of new and

improved products containing materials with good functionality that are easily handled with minimal cost. However, the urgency of reducing the environmental impact of food waste by exploring the industrial potential of byproducts from the food industry is a general incentive to utilize cheap materials as a base for new processes and food products.

During the processing of dairy food items, there is a large amount of a byproduct that is recovered, "whey". Whey is a liquid substance that is collected from the cheese-making process and the coagulation of milk casein (Mollea et al., 2013). In 2020, 54.8 million tonnes of liquid whey were recovered from the production of milk derivatives in the European Union (Tsermoula et al., 2021). Whey is a difficult substance to dispose of, given that when it is present in wastewater, it complicates wastewater treatment due to its high organic load. Additionally, whey components are remarkably difficult to degrade (Yadav et al., 2015).

Whey is a very nutrient dense resource, being a reliable source of lactose, protein, lipids, and several vitamins, which underlines its potential for the development of enriched food products. Due to its nutrient dense nature, whey has recently been considered as a co-product, besides being a byproduct (Tsermoula et al., 2021). Liquid whey undergoes an extraction process to recover certain components, such as whey protein concentrate (WPC) and isolate (WPI). Other fractions are also recovered, such as whey permeate and lactose (Yadav et al., 2015).

WPI is a food ingredient utilized in the production of various food items, mainly to increase the protein content of certain products and its nutritional value (Minj & Anand, 2020). Two main proteins are present in WPI, α-lactalbumin and β-lactoglobulin (β-lg), the latter being the most prevalent protein in WPI, making up around 60% of total bovine milk proteins, dictating the functional and technological proprieties of WPI (Bu et al., 2009).

β-lg is arranged as a dimer, although it can form native monomers when at a pH value below 3.5 or above 7.5. While in a monomer state, it is a globular protein of 162 residues (Barbiroli et al., 2022) with a subunit molecular weight of approximately 18 kDa, presenting two sulfide bridges Cys^{66}-Cys^{160} and Cys^{106}-Cys^{119} (Bu et al., 2009). It also presents a free sulfhydryl group (-SH) Cys^{121}, which is involved in interactions with free -SH groups of other whey proteins or casein (Kleber & Hinrichs, 2007). Intriguingly, β-lg exhibits various structural-functional regions where several interactions occur, leading to deformities in protein structure in these regions (Kontopidis et al., 2004). Interactions

responsible for these possible irregularities in structure may be of ionic nature, covalent bonding, or even hydrophobic interactions (Barbiroli et al., 2022). Consequently, β-lg is noticeably vulnerable to chemical interactions, which makes this protein a prime target for protein studies to evaluate conformational changes of proteins.

A representation of the protein structure of β-lg is shown in Figure 1, as well as the Cys^{106}-Cys^{119} sulfhydryl group and Cys^{121} free sulfhydryl group mentioned previously (RCSB Protein Data Bank, 2023).

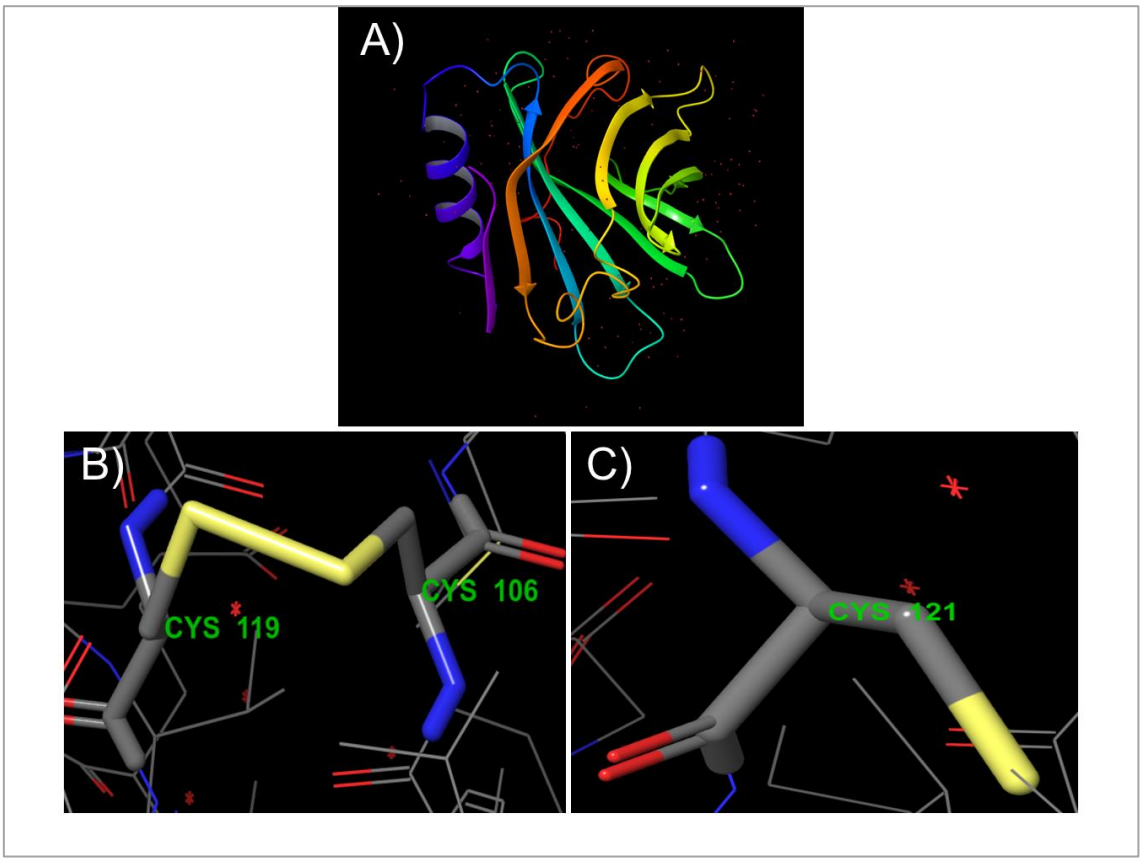

Figure 1: Representation of β-lactoglobulin: A) Complete protein structure (ribbon). B) Cys^{106}-Cys^{119} sulfhydryl group. C) Cys^{121} free sulfhydryl group. Protein model from RCSB Protein Data Base (PDB ID: 2Q2M).

1.4. Protein denaturation in protein studies.

Protein structure is a crucial characteristic to consider, when discussing protein function, especially in the case of enzymatic proteins (Stollar & Smith, 2021). This structure is determined by various intra-molecular interactions within the polypeptide chain and the interactions of the amino acids with the surrounding medium. At a physiological pH value, under specific temperature and ionic strength conditions, the protein structure adopts a folded conformation, where the free energy of the protein is at its lowest. This balanced structure arises from the optimization of several intra-molecular

interactions, including van der Waals interactions, steric strains, hydrogen bonds, electrostatic interactions, and hydrophobic interactions (Hui et al., 2007).

When the conditions of the protein´s surrounding environment are significantly altered (such as temperature, pH, ionic strength, or presence of chaotropic agents), it can lead to the disruption of its native conformation, a phenomenon known as protein denaturation. During this process, there is previous unfolding of the protein´s structure due to loss of native tertiary and/or secondary structure (Anema, 2020).

This loss of native structure can be reversible, partially reversible or irreversible, depending on the severity of the imposed changes (Nikolaidis & Moschakis, 2018). Regarding β-lg, thermal protein denaturation occurs in distinguishable steps. At first, an increase in temperature causes the monomer-dimeric equilibrium of the protein to be disrupted, resulting in a monomeric state (N). Secondly, there is a reversible rearrangement of the conformation of the monomers that leads to exposure of the Cys^{121} (a free thiol) and a hydrophobic patch (R). There is an irreversible second arrangement of monomers (S), and lastly there is a process of aggregation that originates from the formation of disulfide bonds and hydrophobic interactions in certain protein regions (Loveday, 2016). The thermal denaturation process of β-lg is demonstrated in Figure 2. An important observation regarding this denaturation process is the fact that these denaturation stages do not appear to happen simultaneously, but rather successively (Loveday, 2016). At around a temperature of 60 °C, there are changes in tertiary structure that result in exposure of a free cysteine and possible disulphide aggregation. Above 60 °C, β-lg is described as adapting a "molten globule" state (Qi et al., 1997), which is a critical transition point that exhibits a complete unravelling of the α-helix structure and the exposition of the Cys^{121} free thiol group, as well as part of the hydrophobic core (Tolkach & Kulozik, 2007). The middle point of β-lg thermal transition is measured to be at around 74°C in physiological conditions. Conformational changes can still occur at temperatures above or below this middle point due to the different thermal stability in various regions of β-lg, which result in different level of preservation in certain protein regions at different temperature values (Rodrigues, Avelar, Vicente, et al., 2020; Tolkach & Kulozik, 2007). When β-lg is partially unfolded during the denaturation process it exhibits thiol reactivity, an increase in tryptophan (Trp) exposure, as well as exposed hydrophobic patches. These events increase the potential for β-lg to interact with several other molecules during the

denaturation process. These potential interactions are the foundation for the development of new protein functionalities (Rodrigues, Avelar, Machado, et al., 2020).

Protein aggregates that formed during the denaturation process present certain characteristics depending on different medium conditions. In fact, pH value, ionic strength and other denaturing factors lead to different aggregate morphology -e.g., spherical or fibrillar – and structural organization – e.g., fine-stranded, or particulate gels –, which indicates it is possible to consistently replicate a protein aggregation pattern by inducing protein denaturation in a controlled medium (Pereira et al., 2017) . Considering this, β-lg denaturation can be effectively controlled to customize various biomaterials, including functional delivery systems for entrapped bioactive substances through nano and microencapsulation systems, protein hydrogels with specific rheological properties, scaffolds for tissue engineering, among others (Guo et al., 2017; Pereira et al., 2017; Simion et al., 2015).

1.5. Conventional heating vs. Ohmic heating

High temperature is a defining factor in protein denaturation. The bonds and interactions that are responsible for a protein's secondary and tertiary structures are disturbed during significant temperature changes (Hédoux et al., 2006). It is essential to study this phenomenon and its related mechanisms as to uncover potential applications through the denaturation process. This can incentivize experimentation with alternative methods and tools that might prove more cost-efficient than the more commonly followed methodology.

The standard protocol for thermal studies requires the use of a stirred reactor with a double-jacketed vessel containing the desired solution in indirect contact with hot water, using a controlled water bath (Pereira et al., 2010). This system leads to an increase in temperature of the protein solution through indirect heat transfer. When the temperature is high enough, proteins in the solution will undergo the denaturation process. This heating method is commonly referred in literature as traditional or " conventional heating" (Cov) (Frank Czerwinski, 2012; Pereira et al., 2010).

Conventional heating is based on the energy being generated externally and transferred to the desired material by heat transfer phenomena, such as conduction, convection, and radiation (Costa et al., 2015), underlining that these processes can

happen simultaneously during the heating process, mimicking the general pasteurization processes that apply plate-and-frame heat exchangers (Gut & Pinto, 2003). Numerous challenges can arise from the use of this technology, with one significant concern being the substantial energy transfer required to heat solutions containing large particles. This is because these heat transfer phenomena is not direct (Ferreira et al., 2021), resulting in costly procedure as well as potentially leading to the degradation of the outer portions of the solution due to overprocessing. The thermal load is higher in indirect heating systems due to the temperature gradients required to heat up the sample's cold regions (Varghese et al., 2014). In fact, heat transfer to a solution or a product is not uniform and is time costly, which incentivizes experimentation with alternative heating methods that prove more efficient in terms of both time and energy input.

Ohmic heating (OH) has surged in recent years as an alternative heating method for changing protein function. The concept of OH encompasses the energy transfer properties of Joule's law (Pereira et al., 2010). OH is also known as electro-heating method which results from electric current flow through a semi-conductive material that serves as an electrical resistance when an electric field of certain intensity is applied. This process generates heat due to internal energy transformation within the material (electrical to thermal), without the occurrence of heat transfer phenomena (Knirsch et al., 2010). This heating process is direct, allowing a uniform heating pattern throughout the material. Consequently, it results in significantly shorter heating times when compared to conventional heating (Cappato et al., 2017). The main disadvantages of the OH effect is that it relies on physical contact with electrodes (metal pieces), as well as it depending strongly on the electrical conductivity of the food product (Shao et al., 2021).

Heating rate through this methodology is dependent on several parameters, namely the intensity of the electric field applied to the system and the electrical conductivity of the material being heated (Cappato et al., 2017). Electrical conductivity indicates the ability for the material to conduct electricity through a unit of area (Adeline Goullieux, 2014). This parameter can be calculated in an ohmic heating system through Equation(1):

$$\sigma = \frac{L}{A} \bullet \frac{I}{V} \qquad \textit{Equation(1)}$$

where A indicates the area perpendicular to the passage of electric current (m^2), L is the distance between the electrodes of the system (m), I is the alternating electric current (A), and V indicates the voltage applied to the system (V). Electrical conductivity (σ) is represented as Siemens per meter (S/m) (Shao et al., 2021). This equation indicates that, theoretically, it's possible to replicate ohmic heating thermal profiles with consistent heating rates if the conductivity of the material is maintained, as well as all the electric field parameters. This is important not only in a laboratory setting for studying this methodology but also in an industrial setting for calculating monetary and time costs. This is particularly crucial in industrial production lines.

OH still requires optimizations to increase range of applications. Additionally, more studies need to be conducted to further explore and document the effects of moderate electric fields (MEF) on WPI network systems. It has demonstrated that MEF interfere with the aggregation and gelation process of whey proteins, as well as their immunoreactive properties towards their specific antibodies (Pereira et al., 2020; Rodrigues, Avelar, Machado, et al., 2020). Furthermore, a characterization of β-lg denaturation during heat assays involving OH methodology is crucial in determining optimal treatment conditions for different types of protein studies. For example, several medium conditions and parameters such electric field intensity or frequency can strongly influence different properties of β-lg that need to be addressed (Rodrigues et al., 2019).

Overall, the emergence of OH effect in protein studies has proved to be beneficial, not only as a novel processing technology through the distinctive effects of electric fields in the denaturation and aggregation process but also as a highly energy efficient method – i.e., at about >95 % (Rodrigues et al., 2019).

1.6. Current state-of-art of ohmic heating in food protein studies.

The substantial potential of the OH method in protein studies, particularly in WPI systems and, more specifically, in β-lg protein solutions, has spurred numerous research endeavors. These studies aim to delve deeper into the effects of this technology, gaining a better understanding of the possible interactions of β-lg with various molecules and factors related to the medium (such as pH value, ionic strength, and temperature shifts). Furthermore, these investigations seek to enhance our comprehension of the denaturation and aggregation processes of this protein, contributing to the theoretical

basis for novel products in various fields of biotechnology. They also open new avenues for applications of OH technology.

There are several interactions that can occur between proteins and the medium surrounding them, or even protein-protein interactions that lead to the formation of biostructures or complex matrixes (Ramos et al., 2017). This body of knowledge can be implemented in various applications such as the formation of films or coatings, as well as the encapsulation of active compounds for pharmaceutical use (Pereira et al., 2017). An example of these functional structures is the formation of gels. Heat-set or thermotropic gels originate through the heat denaturation of proteins in a certain solution followed by an aggregation process while retaining specific degree of order in the resulting protein matrix. Many factors can contribute to the rearrangement of this matrix, such as pressure, acidification of the medium as well as an increase in its ionic strength, protein concentration and temperature (Totosaus et al., 2002). Alternatively, cold-set gels can also be produced having 3 distinct and separated steps associated with the process, namely denaturation, aggregation, and gelation. Cold-gelation is distinguished process from thermotropic gelation where denaturation, aggregation and gelation occur in the same heat-induced treatment (Alting et al., 2003). In cold-set gel formation, heat denaturation of proteins in a solution occurs initially, resulting in the formation of repulsing aggregates. Secondly, after a certain cooling period, the pH of the solution is lowered to the isoelectric point of the proteins, or alternatively electrostatic interactions with counter ions are promoted, leading to a specific aggregation process. Moreover, there is a certain relation between the aggregation and gelation processes, allowing studies to inquire how the aggregation process influences the characteristics of the resulting gel (Alting et al., 2003). Due to the physicochemical characteristics and its ability to maintain most of its structure during heating assays, β-lg aggregates have been extensively studied in gel formation processes (Kuhn et al., 2010; Nicolai et al., 2011). Corroborating these studies, OH has been tested in the formation of cold-set gels to evaluate the effects of MEF in the denaturation process. Changes in the electrostatic interactions of proteins alter the denaturation patterns in tertiary and secondary structures, which could result in different characteristics of the resulting gel (Pereira et al., 2017). This process is still speculative since the addition of salts in cold gelation also has a considerable effect in gel formation.

OH has also been experimented in the production of probiotic dairy foods. There are reports that indicate that exposure of these products to MEF lead to an increase of enzymatic activity in probiotics at non-lethal temperature heat treatments, which potentially enhances fermentation. This OH application still requires optimization of the electric field parameters to maximize the beneficial effects of this heat treatment. However, it holds promise as an application of OH in dairy-related products. (Pereira et al., 2018).

Cow's milk allergy is a prevalent food allergy developed in early childhood and has the possibility of continuing present into adulthood. Affected individuals have no choice but to completely exclude dairy products from their diets. In fact, cow's milk allergy is among the most common causes of food-induced anaphylaxis, along with peanuts and tree nuts (Flom & Sicherer, 2019). β-lg constitutes close to 10 % of total protein fraction in cow's milk (Pereira et al., 2020) and it presents as one of its major allergens, namely Bos d 5 (Villa et al., 2018). There is a focus in exploring alternatives to dairy products, but several studies still aim to understand the allergenic proprieties of β-lg. Immunoreactivity trials are assays that allow to test the ability of a potential allergen to provoke an immune reaction. The most common assay that is conducted to this purpose is ELISA - Enzyme-linked immunosorbent assay (de Luis et al., 2009). ELISA detects an antigen in a solution by immobilizing the intended molecule in a microplate with specific antibodies, creating an antigen-antibody complex. This complex indicates the presence of the targeted antigen in a solution and can possibly allow for the quantification of the antigen afterwards (Shah & Maghsoudlou, 2016). Theoretically, conformational changes to the structure of β-lg could result in the lack of formation of an antigen-antibody complex when evaluated through ELISA. This would indicate that there would be no allergic reaction resulting from the presence of altered β-lg in milk ingested by an allergic individual. OH has unveiled interesting results, since some intensities of MEF compromises β-lg structure integrity and antibody interactions. Consequently, previous studies indicate a potential reduction in immunoreactivity of β-lg in OH treated samples (Pereira et al., 2020). There are still many optimizations to be made in this field, as well as some considerations regarding possible effects of MEF in the nutritional value of milk-based products. MEF and its attendant OH effect could be used to develop hypoallergenic foods, but it is still necessary to understand the nutritional impact of this treatment.

Edible films are a recent technology that has gained more significance in recent years due to the aggravated symptoms of climate change. Packaging materials have increased by about 8 % annually, and since only less than 5 % of all plastics are being recycled, there is an overwhelming accumulation of plastic in the environment (Tavassoli-Kafrani et al., 2016). There is an urgent incentive to develop new technologies that increase shelf-life of products, as well as attenuating the environmental crisis. An alternative to regular packaging has been the production of edible films or coatings constituted by biopolymers, which provide biodegradability to these food packages. Additionally, it prevents moisture from seeping into the food product, not allowing for the development of pathogens, thus prolonging shelf-life and assuring the safety of the food product (Pereira et al., 2010). WPI and β-lg aggregates have been tested as materials for the manufacturing of edible films (Pereira et al., 2010; Seydim & Sarikus, 2006). Development of β-lg edible films undergo several steps, namely the formation of the aforementioned β-lg aggregates with the sequential addition of a plasticizer agent such as glycerol to increase the spacing between biopolymers This results in the creation of a flexible matrix that is dried afterwards and used as a film (Pereira et al., 2010). Furthermore, there are significant differences when WPI aggregates are formed with conventional heating or OH. Protein aggregates resulting from OH produced edible films with less thickness than the edible films produced with conventional heating. OH films presented less permeability to water vapor, an important factor in food safety to reduce pathogen development, whilst maintaining similar tensile and moisture barrier properties (Pereira et al., 2010). These findings exhibit a potential novel method to produce edible films with interesting proprieties and good functionality.

Evidently, OH studies show good potential in several fields of biotechnology, most notably when related to the food industry. Further research is required to better understand the effect that MEF and the OH effects associated with them can have in the structure of proteins and its influence in the protein aggregation process. An increase in knowledge regarding the effects of OH could lead to the development of new processes in the food industry as well as the possible creation of novel food products and applications, especially when utilizing WPI as a substrate to make the most out of an abundant byproduct with interesting functional and nutritional properties.

1.7. Aim of this work - Characterization of β-lactoglobulin in ohmic heating related heat treatments.

Despite a lot of recent research on OH usage, studies that explore temperatures above 100 °C and above are quite rare. In fact, although achieving ultra-high temperatures through OH is much more efficient than with techniques such as conventional heating (as mentioned above), treatments at such severe conditions can lead to electrode corrosion, as well as still requiring a great amount of energy, which could prove a financial challenge (Indiarto & Rezaharsamto, 2020). However, experimenting with the effects of high temperature through ohmic heating is still a necessary step in understanding the capabilities of this technology and its effect on biomolecules.

Although there are studies related to the properties of β-lg and the effects OH treatments in this protein, reports characterizing this process are scarce. This leads to the development of work protocols that explore these interactions in unoptimized medium conditions for the specific β-lg functionalities that are trying to be better understood, since different temperature thresholds during OH heat treatments result in different denaturation stages of β-lg (Pereira et al., 2020).

There are attempts to characterize β-lg in other experimental conditions. Literature identifies 6 different pH related structural states of β-lg, which is already an advancement in characterizing the effects of pH on the conformation of β-lg (Taulier & Chalikian, 2001). Additionally, protein aggregation of β-lg at different pH values during conventional heating has also been characterized (Zúñiga et al., 2010), however the characterization of β-lg denaturation and the formation of protein aggregates with the influence of MEF has yet to be documented and discussed.

The recent developments of protein studies with OH would benefit from this specific characterization, which would facilitate the optimization of methodology parameters. As a result, this work explores various conformational changes of β-lg to further survey the effects of OH on this protein. The objective of this work is to characterize effects of different heating temperatures - i.e., from pasteurization to ultra-high-temperature (UHT) – under generation of OH effect, thus providing important insight on the interactions present in this temperature range and further emphasizing the possible applications of this technology on food proteins. As far as it is known, this is the

first attempt at the characterization process of β-lg through OH/MEF at ultra-high temperatures.

2. Material and methods.

2.1. WPI Solution.

Protein solutions were prepared by diluting WPI in a 0.250 mol/L phosphate buffer ($N_2H_2PO_4$). The resulting solution's protein concentration is about 2 %. The mixture's pH was adjusted until a value of 4.6 was reached. Subsequently, the mixture was centrifuged at 2817 g of force for 10 minutes. The resulting supernatant was transferred to an alternate container and further diluted until the protein concentration of the solution represented 1 % of the total liquid. The resulting solution's pH value was adjusted to 2 and it was preserved right after at 5 °C until heat treatments were applied.

2.2. Ohmic heating system.

2.2.1. Ohmic heating reactor.

Heat treatments were conducted in an ohmic heating tubular reactor with 2 stainless steel electrodes connected to each edge. A sinusoidal electric wave was generated in a digital function generator (1 Hz – 25 MHz and 1-10 V from Agilent 33220A, Penang, Malaysia) and amplified further (Peavey CS3000, Meridian, MS, USA). Temperature was controlled by adjusting amplified voltage from 0 to 180 V.

2.2.2. Heat assays.

Protein solutions were heated inside the reactor vessel described previously until various established target temperatures were reached (from 40 °C to 165 °C). The heating process followed a linear profile amongst all the treated samples. Heating assays were conducted independently for each target temperature and replicated at least three times. After the heating procedure, the protein samples were recovered and immediately refrigerated in an ice bath and afterwards stored at 5 °C for further analysis. Various protein samples that did not undergo heat treatment, serving as control, were also stored for analysis and for comparison with the treated samples.

2.3. Fluorescence analysis.

2.3.1. Intrinsic fluorescence analysis

Intrinsic fluorescence analysis was conducted through Aqualog fluorometer (HOBIBA-Jobin Yvon, Inc. Japan). WPI solutions were previously diluted in distilled water and phosphate buffer (300 μL of WPI solution in 2.7 mL buffer + water). β-lg inherent fluorescence was obtained through excitation of tryptophan (Trp) in the samples at 280 nm, followed by emitted light in a range of 295 nm to 450 nm. In this range, fluorescent signal was captured and recorded for further analysis.

2.3.2. Extrinsic fluorescence analysis (8-anilino-1-naphthalenesulfonic acid)

8-anilino-1-naphthalenesulfonic (ANS) was used as a hydrophobic fluorescent probe (sourced from Sigma-Aldrich). This probe can aid in identifying proteins' hydrophobic regions. The ANS solution was prepared at a concentration of 1.5 mmol/L in methanol and subsequently stored at 4 °C whilst shielded from light. Afterwards, 9 μL of ANS solution was added to a microplate containing 270 μL of WPI solution. Fluorescence analysis of the formed ANS-protein complex occurred by excitation of samples through wavelengths of 370 nm and measured emission at 480 nm.

2.4. Circular dichroism spectroscopy.

Circular dichroism (CD) was conducted on a Jasco J-1500 CD spectrometer (Jasco Inc., Tokyo Japan). Spectra readings were recorded in a wavelength interval between 190 nm and 250 nm in a 1 mm quartz cuvette. Additionally, other parameters were followed: scanning speed of 50 nm/min, 3 accumulations, protein concentration of 0.2 mg/ml and path length of 0.1 cm.

Results obtained were further analyzed using the DICHROWEB software to observe changes in secondary structure of proteins present in heat-treated samples and control samples (Miles et al., 2022). Readings occurred at room temperature.

2.5. Particle size analysis.

Size of protein aggregates were evaluated through dynamic light scattering (DLS) using a Zetasizer Nano (ZEN 3600 Malvern In-struments Ltd., Malvern, U.K.). Protein samples were centrifuged prior to examination. Measurements occurred at (25 ± 0.5) °C. The poly-dispersity index (PDI) obtained for each sample during DLS was also measured,

representing the width and overall variance in particle size distribution within each sample. All measurements were done in triplicate.

2.6. Zeta potential measurement.

Zeta potential of protein samples was measured using Zetasizer Nano (ZEN 3600 Malvern In-struments Ltd., Malvern, U.K.). Protein samples were centrifuged prior to examination, in similarity with the process undergone in particle size analysis. Measurements occurred at (25 ± 0.5) °C. Zeta potential values for each sample were obtained, as well as conductivity of each protein sample. All measurements were done in triplicate.

2.7. Conductivity profile measurement.

Very brief heat assays were conducted for each protein sample to calculate the corresponding conductivity. A voltage value in the range of 32.2 V – 50 V was used throughout the heating process. Samples were heated from around 20 °C to 30 °C, with temperature values following a linear profile. Temperature changes during this interval were recorded during the heating process. Changes in alternating current intensity throughout the heating process were recorded and for calculation of conductivity profile through Equation Equation(1).

2.8. Hydrolysis analysis.

Through the use of 2,4,6-trinitrobenzene sulfonic acid (TNBSA), available amino groups were measured in WPI samples. DL-Serine was diluted with sodium bicarbonate buffer (0.1 M pH, 8.5) at a maximum concentration range of 0.01 % (v/v). WPI samples were diluted to 0.01% (v/v) with sodium bicarbonate buffer (0.1 M, pH 8.5) prior to analysis. Afterwards, TNBSA solution was added [0.01 % (v/v)] and incubated for 2 hours at 37 °C with absence of light.

After the incubation period, SDS was added to the solution at 10 % (v/v) and HCl at 1M to stop the reaction. The concentration of available amino groups in all WPI samples were obtained through a microplate reader (Cytation3) with use of Biotek's 5 Software, measuring the absorbance values at a wavelength of 335 nm. These reading occurred in triplicate.

2.9. Fibril quantification using Thioflavin T (ThT).

A stock solution composed of 10 mM phosphate and 150 mM NaCl buffer, and 3.0 mM of Thioflavin T (ThT) was passed through a 0.2 mm syringe filter (Millex-GS, Millpore). This stock solution was subsequently preserved by an amber glass container at 4 °C covered in aluminum foil as to prevent light exposure.

The stock solution was diluted 50 times through the previously mentioned phosphate-NaCl buffer, with the final ThT solution having a concentration of 60 mM.

200 µL of sample was mixed with 3 mL of the previously mentioned solution. This mixture was rapidly vortexed and kept at room temperature, shielded from light. Afterwards, the mixture's fluorescence was analyzed through Biotek's Gen5 Software microplate reader, Cytation3. Excitation and emission wavelengths applied occurred at 440 nm and 482 nm, respectively. The obtained results of heat-treated WPI samples were then adjusted by subtracting the obtained fluorescence value obtained in non-heat-treated WPI samples.

2.10. Statistical analysis.

All statistical analysis was conducted in Origin Pro 2018 SR1 software (version b9.5.1.195). This software allows for a better understanding of the data obtained through all the procedures of sections 2.3 – 2.9. Additionally, principal component analysis (PCA) was carried out through this software, representing all the data previously obtained in an organized manner (May & Stevenson, 2009).

3. Results and Discussion

For an improved visualization and interpretation of the obtained results, WPI solutions were labelled as follows: « WPI "number corresponding to maximum temperature achieved" "assigned letter to differentiate between triplicate solutions (A, B

or C)" ». For example, the second element of the triplicate of the WPI solution heat-treated at 50 °C was assigned as "WPI 50B".

3.1. Conductivity profile.

Electrical conductivity profile was assessed to understand the effects of denaturation and aggregation processes, as well as effects of MEF on WPI samples. Since the conductivity profile of WPI samples were tested several days after the initial heating assays, measuring this parameter can help theorize the reversible/irreversible conformational changes in β-lg at different thermal treatments through potential changes in calculated conductivity values of WPI samples (Llave et al., 2018). Conductivity is calculated through Equation(1), where the electric current and the voltage applied are key factors in determining conductivity (Shao et al., 2021). Additionally, temperature is also responsible for conductivity, with an increase in temperature leading to an increase in conductivity in a linear pattern (Sakr & Liu, 2014). Since the geometric characteristics of the reactor, electrical current and applied voltage are the same throughout the brief heating assays conducted, potential conductivity changes can be attributed to ionic mobility through conformation changes in β-lg (Llave et al., 2018).

Figure 2 exhibits the calculated conductivity values of WPI samples at 20 °C and 30 °C. At a temperature of 20 °C, there are no significant differences between the non-treated WPI samples and the treated samples. The only exception are the results obtained at 150 °C, which presents a significant drop in conductivity when compared to non-treated samples, heat-treated samples at a maximum of 90 °C and the samples heat-treated at 140 °C (p-value < 0.05). This statistical analysis is valid at 20 °C and at 30 °C, solidifying that there is indeed a considerable drop in conductivity value recorded in the WPI samples heat-treated at 150 °C. Overall, the control sample presents a higher conductivity value compared to the majority of the heat-treated WPI samples, with samples heated at 70 °C and 80°C being the exception at a temperature of 30 °C.

Literature describes that the electric conductivity of certain pre-heated samples through ohmic heating might present a lower value than non-treated samples (Castro et al., 2004), although these were results obtained in starchy solutions, not WPI solutions. There might still be some relevant interactions that lead to the decrease of electrical conductivity in most samples, mainly attributed to conformational changes in β-lg (Llave

et al., 2018). Literature is still relatively scarce in documentation of effects of pre-heating treatments through ohmic heating on electric conductivity. It is a difficult parameter to evaluate the direct effects of MEF in this parameter, however it is a fact that electric field intensity and voltage are key factors to consider (Shao et al., 2021).

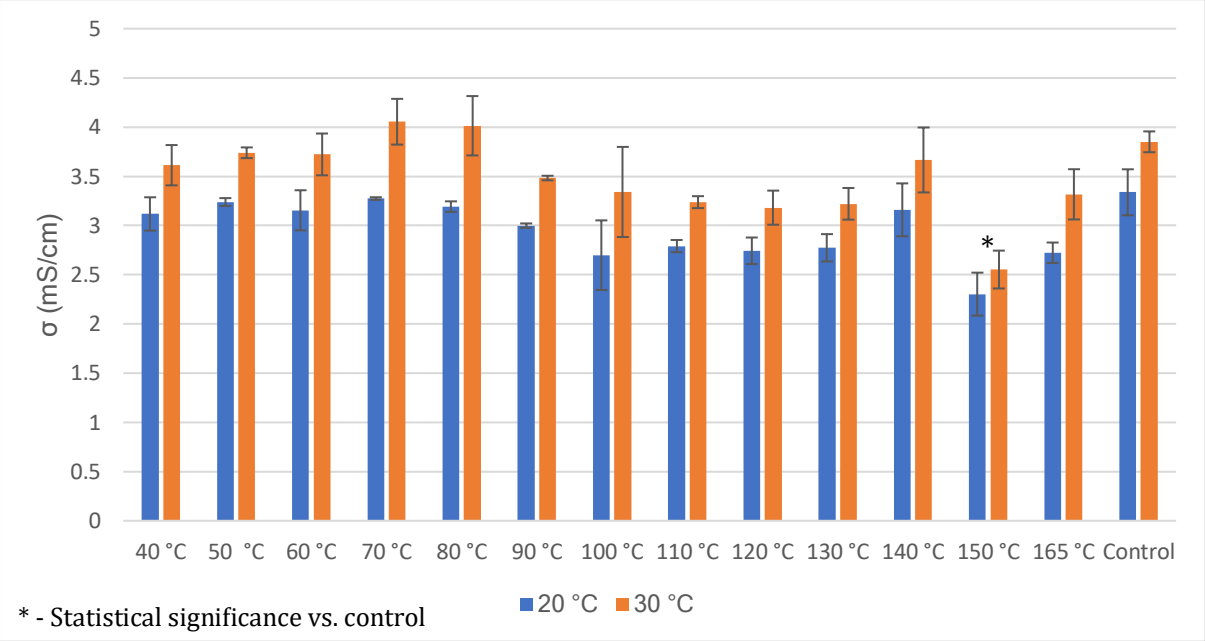

Figure 2: Calculated conductivity profile of WPI samples.

3.2. Fluorescence.

3.2.1. Intrinsic fluorescence.

Intrinsic fluorescence can aid in determining conformational changes of proteins with aromatic amino acids by analyzing a specific wavelength. Tryptophan (Trp) is an amino acid present in the hydrophobic barrel of β-lg and near disulfide bridge Cys^{66}-Cys^{160}. (Simion et al., 2015) Therefore, studying the intrinsic fluorescence of the heat-treated WPI samples in a wavelength range of 295 nm - 450 nm allows for the detection of Trp exposure, which helps determine conformational changes in β-lg proteins. When an increase in intrinsic fluorescence at this wavelength is detected, it may be a result of exposure of Trp that was previously buried by the native protein's structure (Pereira et al., 2020).

Results regarding intrinsic fluorescence difference between heat-treated and untreated (control) protein samples are displayed in Figure 3. From temperature values of 40 °C – 80 °C this intrinsic fluorescence different is slightly negative, which gives

indication of conformational rearrangemetns of protein structure. Upon heating there is a noticeable transition in exhibited intrinsic fluorescence different at temperatures betweem 70 °C and 90 °C. Conformational changes to β-lg structure at this temperature interval is supported by the results shown in literature (Pereira et al., 2020; Rodrigues et al., 2019). Conformational changes in the hydrophobic barrel of the structure β-lg due to unfolding and denaturation results in Trp exposure and, therefore, a higher intrinsic fluorescence intensity at this wavelenght. Fluorescence intensity gradually decreases in temperatures of 130 °C and above, which could be a result of the formation of protein aggregates and quenching of Trp, and thus decrease of the fluorescence signal.

Intrinsic fluroescence values of all treated WPI samples present statistically significant differences to eachother (p-value < 0.05), except the cases of 50 °C – 60 °C (p-value = 0.64) and 100 °C – 150 °C (p-value = 0.19).

These results indicate that conformational changes to β-lg during OH start to occur at temperatures below 50 °C and that a singificant structural transition occurs above 80 °C and 120 °C , highlighting these temperatures as a key parameters to monitor in studies regarding conformational changes in β-lg.

Due to the scarcity of studies that conduct heat treatments at temperatures above 100 °C, confirming possible deductions in relation with the decrease in intrinsic fluorescence in temperatures above 130 °C is difficult. However, it can be theorized that amino acid degradation may be a possible reason for this decrease (Körner, 2021). However, in-depth analysis of amino acid integrity in treated WPI samples at this temperature would have to be conducted to affirm this possibility.

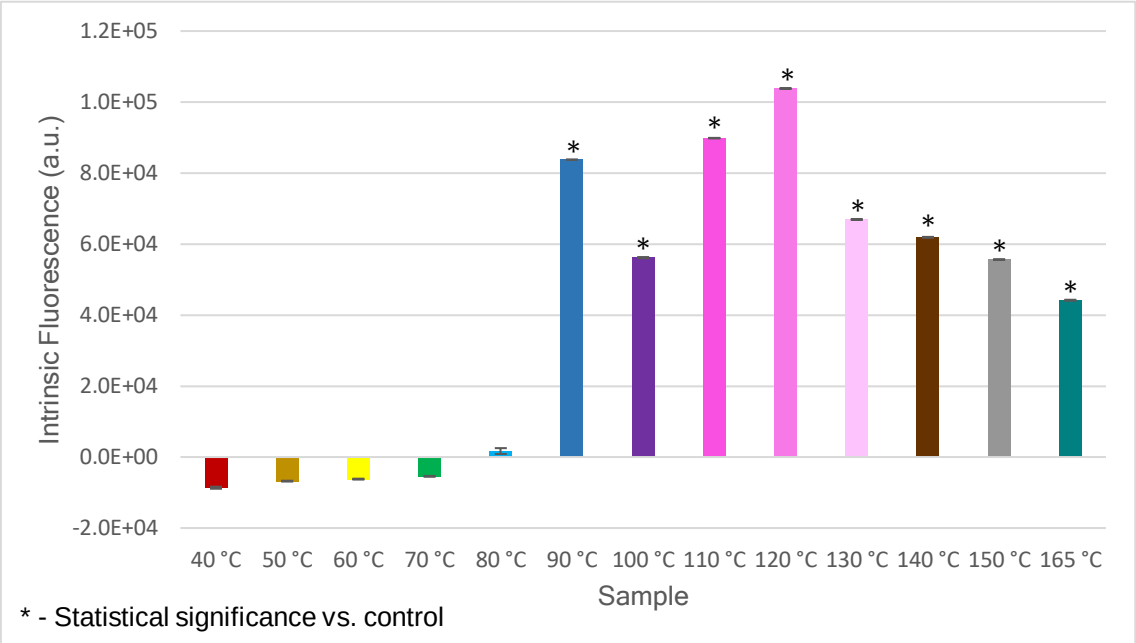

Figure 3: Intrinsic fluorescence of ohmic heating treated WPI solutions.

3.2.2. Extrinsic fluorescence.

Similarly to intrinsic fluorescence, measuring extrinsic fluorescence can help evaluate conformational changes in proteins. ANS identifies accessible hydrophobic regions of β-lg in this study (de Figueiredo Furtado et al., 2018).

Results obtained through extrinsic fluorescence analysis on all heat-treated WPI samples is shown in Figure 4. There is a noticeably drop in fluorescence intensity throughout higher temperature thresholds reached, specially from WPI samples treated at temperatures of 110 °C and above. These results indicate changes in the hydrophobic core of treated WPI samples that further support the results from intrinsic fluorescence analysis. As shown in Figure 4, the exposure of hydrophobic regions is much more prevalent in temperature ranges of 40 °C – 100 °C, with extrinsic fluorescence intensity values of the various WPI samples showing significant differences ($p < 0.05$) between them, except between samples heat-treated at 80 °C and 90 °C ($p = 0.73$). Literature also recognizes this temperature range as ideal for exposure of hydrophobic regions of β-lg (Ferreira et al., 2021; Rodrigues, Avelar, Vicente, et al., 2020).

Heat-treated WPI samples at 110 °C and above show less exposure of hydrophobic patches, probably due to the formation of protein aggregates at higher temperatures,

stabilizing the hydrophobic regions of the proteins in the inner parts of the aggregates, which would lead to less extrinsic fluorescence intensity in these temperature ranges.

The common pattern is that conformational changes in protein structure are more severe and noticeable throughout a steady increase in temperature, which is to be expected in β-lg due to the gradual heat denaturation, and eventually protein hydrolysis, resulting in the significant loss of hydrophobic patches at temperatures above 110 °C. High-temperature under OH can contribute to break hydrophobic domains, at β-lg pocket reducing binding affinity with ANS (Sponton et al., 2014). These results are further supported by the increase in intrinsic fluorescence observed within the temperature range of 90 to 120 °C. This can be attributed to the position of Trp19 within the central calyx. As the temperature rises and protein unfolding occurs, Trp19 becomes more exposed to the aqueous medium, making it available for fluorescence emission.

The obtained data through ANS fluorescence demonstrates the temperature range in which the hydrophobic barrel of β-lg (Loveday, 2016) is exposed, further elaborating on the effects of MEF and ohmic heating in the denaturation of this protein.

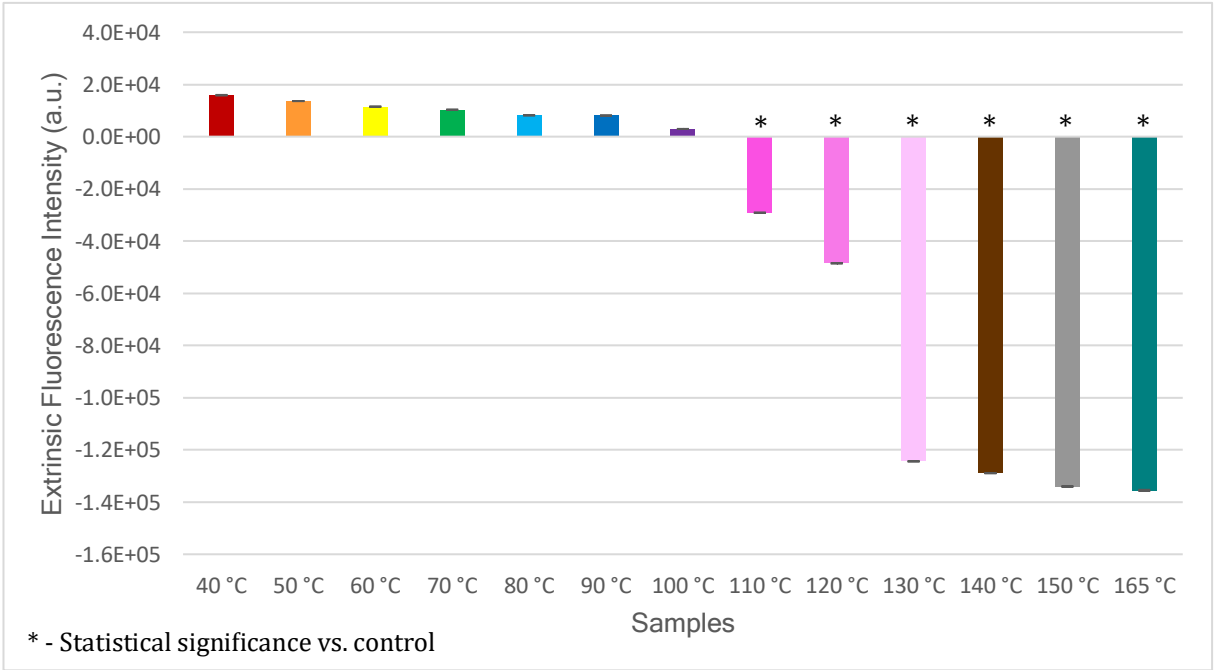

Figure 4: Extrinsic fluorescence of ohmic heating treated WPI solutions.

3.2. Circular dichroism spectroscopy.

As previously discussed, loss of native structural components of proteins is a defining factor of protein denaturation. Measuring the severity of these structural changes allows for insight on the effects of temperature in the conformation of proteins. Circular dichroism (CD) spectroscopy is a useful tool in assessing the effects of temperatures in the tertiary and secondary structure of β-lg (Miles et al., 2022). Figure 5 exhibits CD spectroscopy results of ohmic heating treated WPI samples as well as the average of the control protein samples.

Various observations can be made from Figure 5. Firstly, the control samples and all the treated samples seem to follow a similar spectroscopic pattern, even if at slightly different wavelengths and ellipticity (expressed as millidegrees – mdeg) values. Secondly, the control samples and heat-treated present a very similar ellipticity values up until the 80 °C temperature threshold. Onwards from these values, there is a slight decrease in ellipticity values from temperature thresholds of 90°C up until 110 °C. Afterwards, there is a very significant decrease in ellipticity values in the temperature interval between 120 °C and 165 °C. Furthermore, it is very noticeable that there is slight shift in wavelength where the minimum ellipticity value is recorded for each sample (for example, the average minimum ellipticity value of heat-treated samples at 80 °C was observed at a wavelength of 206 nm, whilst the minimum ellipticity value of the average heat-treated samples at 165 °C was observed at a wavelength of 198 nm). This blue shift in wavelength as temperature increases is concurrent with results obtained in the available literature and is mainly associated with the loss of secondary structure of proteins, specifically β-sheets and an increase in random coil (Rodrigues, Avelar, Vicente, et al., 2020). These transitions that are observed at CD measurements support previous results and are consistent with the fact that events such as unfolding, denaturation, and hydrolysis are being tracked.

CD results can be discussed further with the use of the DICHROWEB Software, allowing for an analysis of the percentage of secondary structures in each sample. As such, Figure 6 shows the ratio of secondary structures of proteins in average in each sample.

The percentage of α-helix structures in proteins is consistent, with a decline in heat-treated samples at 130 °C. This decline is significant for all WPI samples up to a maximum of 120 °C (p-value> 0.05), with an exception for WPI samples heat-treated at

80 °C, which presented a p-value of 0.07. The heat-treated WPI samples at 140 °C and 150 °C do not apparent a significant difference in α-helix percentage with each other, however, there are significant differences of WPI samples heat-treated at these temperatures with samples treated below 140 °C (p-value < 0.05). Furthermore, WPI samples heat-treated at 165 °C present significantly fewer α-helix structures than all other WPI samples (p-value < 0.05), except samples treated at 150 °C (p-value = 0.97).

The loss of β-sheet structures is more noticeable, with a slight decrease in percentage of these structures in heat-treated samples in the interval of 90 °C - 110 °C, which are results that correlate with the decrease of ellipticity values in Figure 5 previously discussed. The percentage of β-sheet structures in WPI samples treated at 110 °C present a significant difference when compared to samples treated at a maximum temperature of 80°C (p-value < 0.05). On heat-treated samples of 120 °C and above, there is a significant decrease in the percentage of these structures (p-value < 0.05), which could already have been predicted by analyzing Figure 5.

The percentage of turns in the secondary structure of proteins in all samples is relatively consistent, apart from a slight decrease that occurs from samples heat-treated at 120 °C and above. This decrease in these structures is still statistically significant compared to WPI samples treated at temperature of 110 °C and below (p-value < 0.05).

Coil/unordered structures demonstrate an inverted behavior when in comparison to percentages of β-sheet; there is a slight increase in coil/unordered structures starting at heat-treated samples between temperature values of 90 °C – 110 °C, with a noticeable increase at heat-treated samples of 120°C and above, which present a significant increase in percentage of these structures when compared to samples treated at a maximum temperature of 110 °C (p-value < 0.05).

It is apparent that conformational changes to β-lg start to be noticeable at temperatures of around 90 °C due to the unfolding process of the protein's structure. At temperatures of 120 °C and above there are severe losses in secondary structure of β-lg, with the loss of a significant percentage of both α-helix and β-sheet structures, with a great increase in random coil originating from this loss of secondary structure. These two temperature thresholds can be highly valuable for various types of studies, depending on the extent of thermal unfolding of β-lg that the study aims to elucidate. A partial unfolding might be beneficial in pharmaceutical studies, such as encapsulating certain compounds for delivery to a system. A more thorough unfolding process might be necessary in protein

studies to further enquire on the reversible/irreversible proprieties of the denaturation process of β-lg, as well as further understanding the effects of high temperatures on the conformational characteristics of β-lg. This information provides useful insights for development of protein functionalization strategies (interaction with other compounds, immunological aspects, among others).

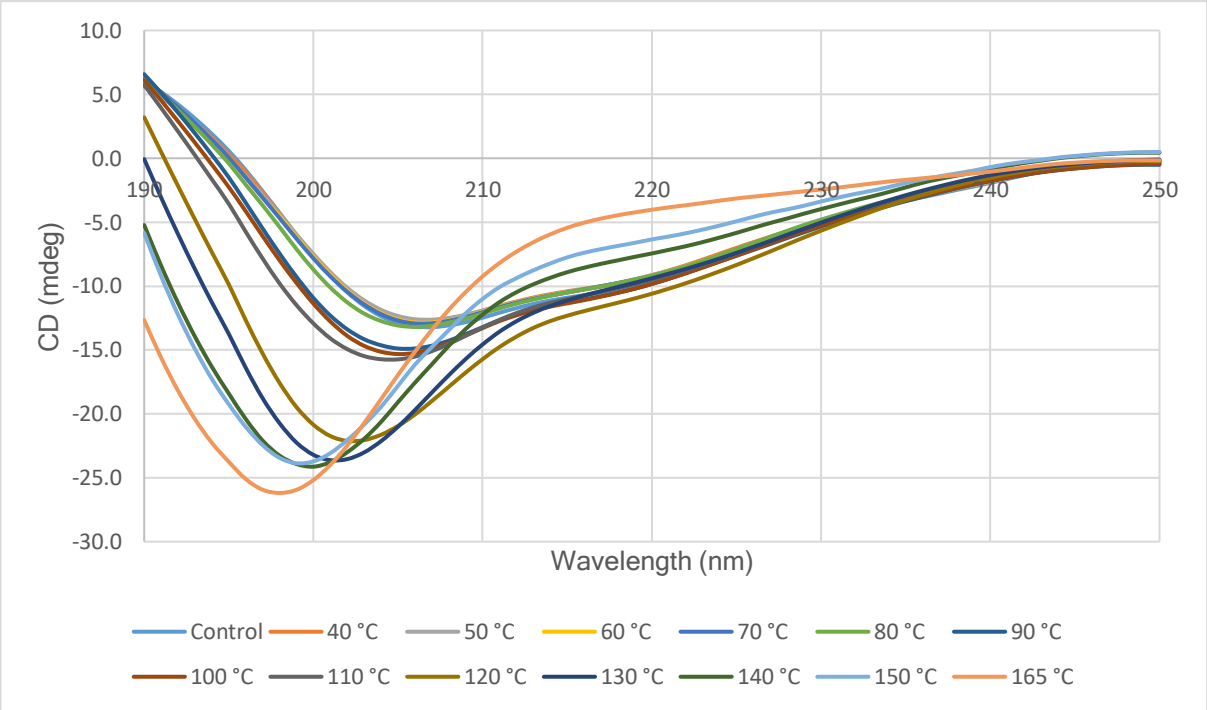

Figure 5: Circular dichroism spectroscopy results in ohmic heating treated WPI samples in comparison with a control sample.

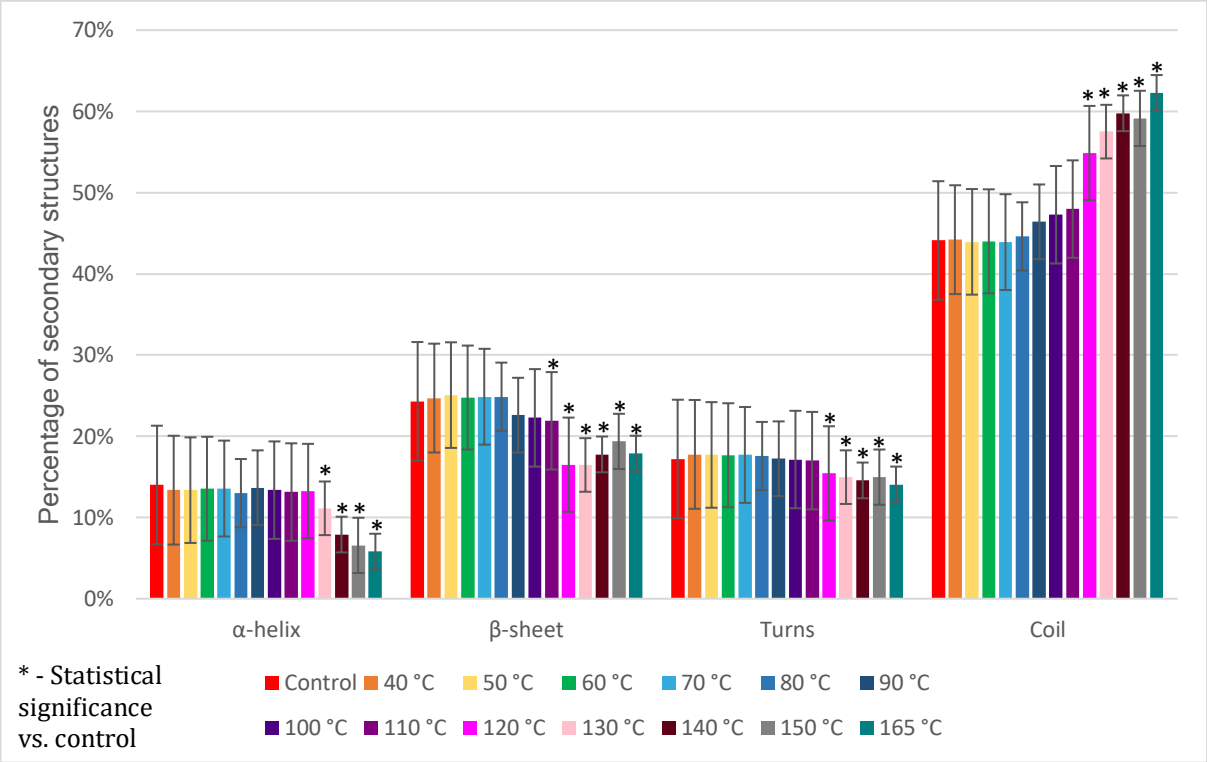

Figure 6: Distribution of secondary structure of proteins in all ohmic heating treated samples and the average of the control samples.

3.3. Aggregate particle size.

The advanced stages of the denaturation process of β-lg are characterized by the formation of protein aggregates. With the use of Dynamic Light Scattering (DLS), it's possible to evaluate the distribution of particle size in heat-treated sample, including average hydrodynamic particle size (Bhattacharjee, 2016). This data is beneficial for pharmaceutical applications to access the surface area of the protein aggregates, which directly correlate to bioavailability (Danaei et al., 2018).

Figure 7 represents the average PDI of all samples treated with ohmic heating as well as the control samples. PDI values are measured between values of 0 and 1, with a value of 1 indicating a very polydisperse protein sample with a wide array of particle sizes and a value towards 0 indicating a sample with particles following a uniform and consistent size. The results shown in Figure 7 are not conclusive, as there seems to be no apparent pattern of PDI values with an increase in temperature. Zeta average measures

the hydrodynamic particle size of a certain sample allowing to establish a potential relation with protein aggregation. Figure 8 presents the Zeta average of the WPI treated solutions and corresponding controls (untreated samples). Once more, it is difficult to trace a pattern of particle size within the WPI samples as observed for PDI results. This procedure would need to be tested with a pure β-lg solution to avoid interference of other proteins in PDI and zeta average reading. Results obtained from PDI and zeta average could indicate that DLS technology is not appropriate for measurements of these kind of protein aggregates. Working at an acidic pH often leads to the formation of fibrillar aggregates, which may not be suitable for analysis using the DLS technique. DLS is most accurate and effective when applied to particles that exhibit Brownian motion and have spherical symmetry (Hassan et al., 2015).

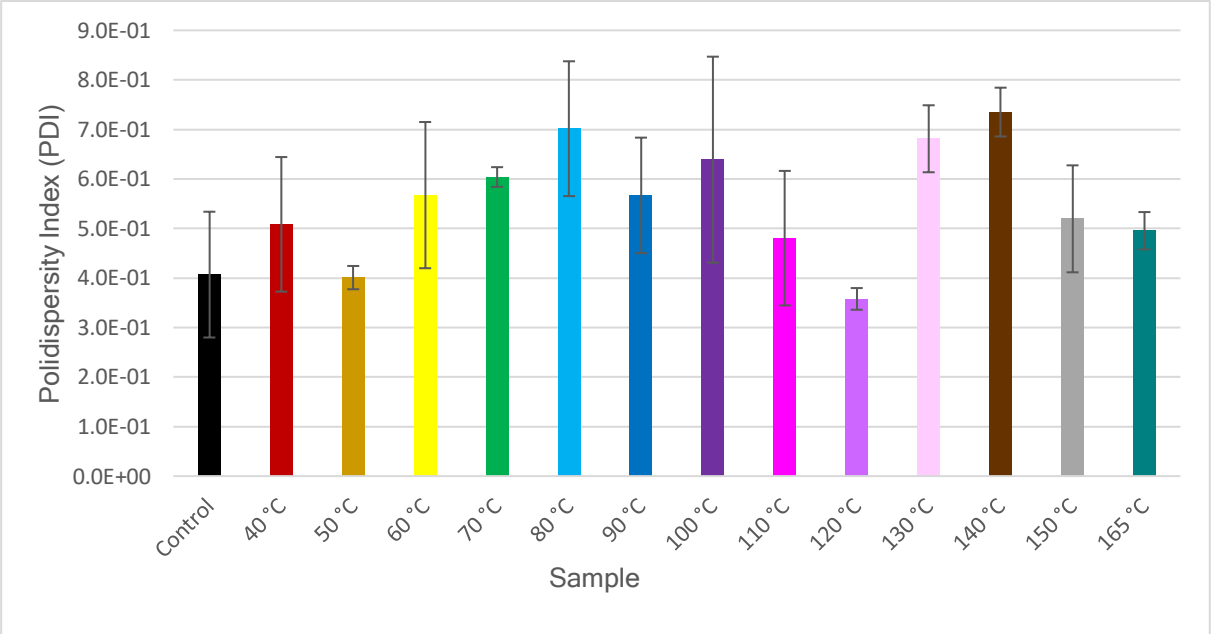

Figure 7: Average polydispersity index of ohmic heating treated samples and all control samples.

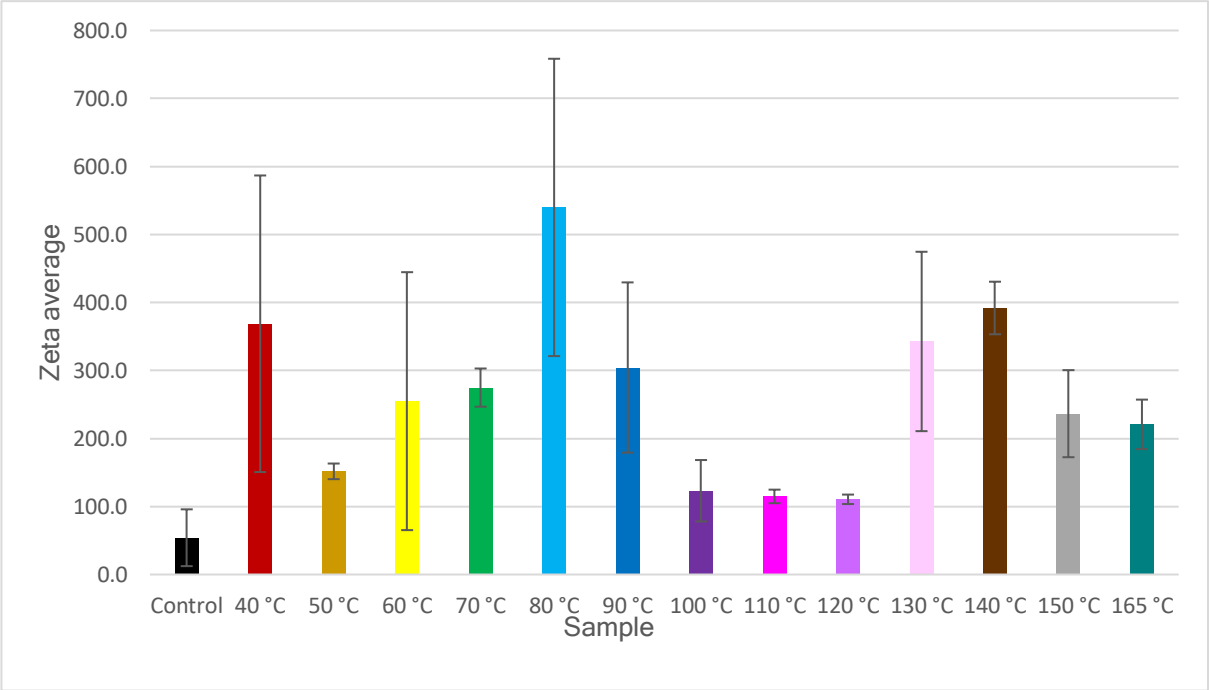

Figure 8: Zeta average values of ohmic heating treated WPI samples and the average of the control samples.

3.4. Zeta potential.

Zeta potential determines the electrostatic potential of a certain particle. This parameter can help determine the reactivity of a particle, especially in the case of nanoparticles, where zeta potential can affect the particle's ability to establish electrostatic interactions with other molecules or permeate membranes (Clogston & Patri, 2011). In the case of protein aggregates, zeta potential is a factor to consider in determining the possible interactions between protein aggregates and other charged molecules, which can give valuable information in protein characterization studies (Tatkiewicz et al., 2015).

Measurements of the average zeta potential of all ohmic heating treated and control samples are shown in Figure 9. Zeta potential values in the interval of 40 °C - 80°C are very comparable to the ones from control samples. In fact, the measured zeta potential values of WPI samples heat-treated at this ranges are not significantly different with the control sample (p-value > 0.05). Samples heat-treated at 90 °C, 110 °C, 130 °C and 150 °C do present statistically significant values when compared with the control sample (p-value < 0.05). WPI samples heat-treated at 90 °C present the highest zeta potential of all samples, which, in theory, indicates that the proteins within this sample are the most conformationally stable out of all samples (Tatkiewicz et al., 2015). Since 90 °C is an

important temperature threshold in the denaturation process of β-lg, it can be theorized that this increase in zeta potential and protein stability could occur through the changes in protein conformation, leading to a better stabilization of β-lg conformation. However, this observation is merely hypothetical, since literature is scarce in the discussion of zeta potential values of β-lg that has undergone the denaturation process.

Moreover, studies that evaluate changes in zeta potential of β-lg are conducted at pH values above 6, where the changes in zeta potential are more noticeable and can be documented more efficiently (Świątek et al., 2019), which is a higher pH value than the one this work focuses on (pH = 2). Still, Figure 9 offers some information on the effects of MEF on the zeta potential of β-lg. However, it would be beneficial to conduct some more work at a higher pH value to further elaborate on changes in zeta potential of β-lg throughout the denaturation process through OH.

Overall, the results obtained in Figure 7 and Figure 8 appear to be very inconsistent, considering the results obtained with fluorescence and CD spectroscopy data. It would be necessary to repeat these analyses with better techniques and different sample concentrations to obtain more accurate results that allowed for better discussion and could provide a better understanding of these parameters in β-lg denaturation, as well as permitting a more complete characterization of this protein.

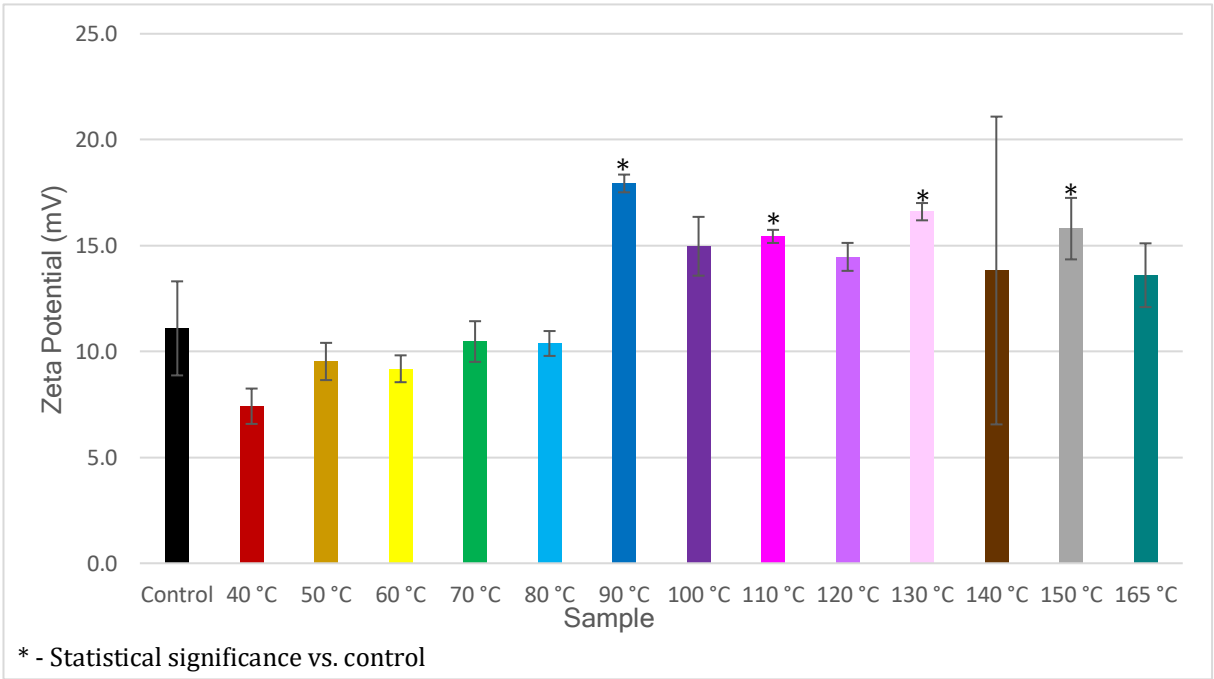

Figure 9: Average zeta potential of ohmic heating treated samples and control samples.

3.5. Hydrolysis.

β-lg hydrolysis can occur due enzymatic or chemical cleavage of its peptide bonds. Thermal treatments, such as heating at high temperatures, can also lead to β-lg hydrolysis (Tavano, 2013; Wang et al., 2020). Protein hydrolysis has several potential advantages, mainly in the context of food and dairy industries such as enhance the digestibility, reduce allergenicity, improved functional properties, development of bioactive peptides or superstructures (Ávila-Fernández et al., 2011; Chen et al., 2015; Kasera et al., 2015). For example, development of peptide nanofibrils, widely regarded as amyloid fibrils is recently attracting attention due to the study of many fibril-based diseases such as Alzheimer's and Parkinson's (Ghosh et al., 2021). These studies are unravelling the potential of amyloid fibrils as novel functional materials with a very abundant raw material source such as proteins. In this regard, β-lg has emerged as a model protein, since it follows many fundamental mechanisms of fibril formation (Ye et al., 2018). β-lg has a remarkable ability to form fibrils in various conditions, such as low pH, presence of alcohol, high temperatures, among others (Pan & Zhong, 2015). Protein hydrolysis is a key aspect in the development of β-lg fibrils (Pereira et al., 2016; Ye et al., 2018), and it is crucial to gather data for characterization studies.

Figure 10 demonstrates the average hydrolysis rate of all heat-treated samples and control samples through TNBSA results. It is evident that hydrolysis onset starts at temperatures above 110 °C. Treatments performed between 110 °C and 120 °C can provide a better environment to produce amyloid fibrils. However, heating at ultra-high-temperature (UHT) range (> 130 °C) can be very challenging due to interactions between proteins and polysaccharides through Maillard reaction. Previously, it was mentioned that this phenomenon affects organoleptic proprieties in proteins that can positively alter sensory quality. However, Maillard reactions may also alter detrimental proprieties in proteins, such as hypertensive, mutagenic, or even carcinogenic proprieties, which could have detrimental health impacts on consumers (Rodrigues, Avelar, Machado, et al., 2020). This temperature interval can also be associated with loss of protein nutritional quality (Perez-Locas & Yaylayan, 2010). Despite this fact, OH treatments seem to have a great advantage in comparison to conventional heating in this regard, since some studies report that UHT treatments utilizing ohmic heating allowed preservation of protein quality despite the high temperature values (Mesías et al., 2016; Roux et al., 2016). This is not observed in conventional heating methods which involve higher thermal loads

(Rodrigues, Avelar, Machado, et al., 2020). Recently, relevant results that indicate that MEF can increase enzymatic hydrolysis rate, as well as altering the physical proprieties of certain proteins (Li et al., 2021). Despite this, more research needs to be conducted on the definitive effects of MEF and OH in hydrolysis procedures, especially when evaluating protein quality and interactions with other molecules, since these properties are of high importance for application in several fields (Jin et al., 2014). Still, these results seem promising in that regard. Nonetheless, the direct heating properties of OH offers a great advantage over conventional heating by reducing heat kinetics and over processing (Rodrigues, Avelar, Machado, et al., 2020). To the best of our knowledge this work describes, for the first time , anassessment of OH effects at wide range of temperatures (up to 165 ºC) on hydrolysis degree of β-lg.

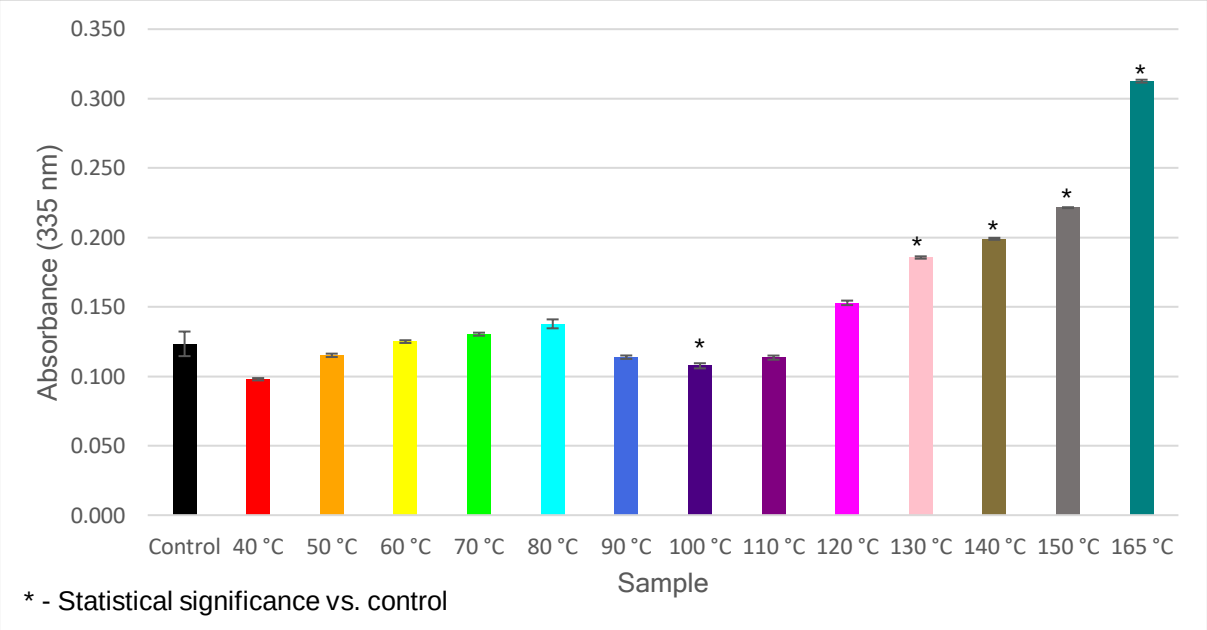

Figure 10: TNBSA results of the ohmic heating treated samples and control samples.

3.6. Fibril formation analysis.

Thioflavin T (ThT) is one of the most common amyloid fibril dyes used in several studies (Biancalana & Koide, 2010). Figure 11 shows the amyloid fibril formation data gathered through the ThT method in all ohmic heating treated samples, as well as the control samples.

The treatment at 100 °C appears to stand out as the base temperature for amyloid fibril formation. Analyzing the first set of triplicate WPI samples resulted in a very noticeable increase in absorbance at 482 nm. This result was further validated with the

preparation of a second set of triplicate WPI samples that were heat-treated at 100 °C. Although the absorbance value of the second set of triplicates heat-treated at 100 °C was slightly lower than the one observed in Figure 11, it was still the highest average value out of all other WPI samples with a significant difference over WPI samples treated at a maximum temperature of 80 °C and the samples heat-treated at 165 °C ($p < 0.05$), thus maintaining the ThT pattern observed in the data. It is possible to conclude that 100 °C is a desired temperature for the formation of β-lg fibrils. Discussing the data on Figure 11 and Figure 10, it seems that, despite hydrolysis occurring at a great rate at temperatures > 120 °C, (as shown in Figure 10), it does not represent the highest amount of fibril formation. These results are somewhat consistent with literature, where there are cases that report UHT treatments resulting in fewer formation of β-lg fibrils (Loveday et al., 2012). Before the formation of β-lg fibrils, several monomers converge into small oligomers during heat denaturation. Several tertiary structure changes in the protein occur before and after the formation of these oligomers, which in turn provide structural integrity to β-lg fibrils (Giurleo et al., 2008). It is possible that an increased thermal motion provoked by higher temperatures in samples above 100 °C might speed up the rearrangement patterns of fibril formation and lead to less stable (or straight up dysfunctional) structures (Loveday et al., 2012). These unstable structures would also be more susceptible to hydrolysis, leading to less detection of fibrils in WPI samples heat-treated at higher temperatures.

An intriguing observation is that WPI samples heat-treated at 165 °C recorded one of the lowest fibril formation rate out of all samples, considering that this temperature is linked to an increased in Maillard Reaction phenomenon (previously discussed to be linked to carcinogenic/mutagenic characteristics, which can be detrimental in certain pharmaceutical applications). Maillard Reaction can potentially increase fibril stability (Wei & Huang, 2019; Zou et al., 2016). There is a possibility that ultra-high temperatures can be beneficial for β-lg fibril formation with WPI samples with different conditions or heating assays with a slower heating process that could allow β-lg fibrils to form in a more stable condition at such a high temperature.

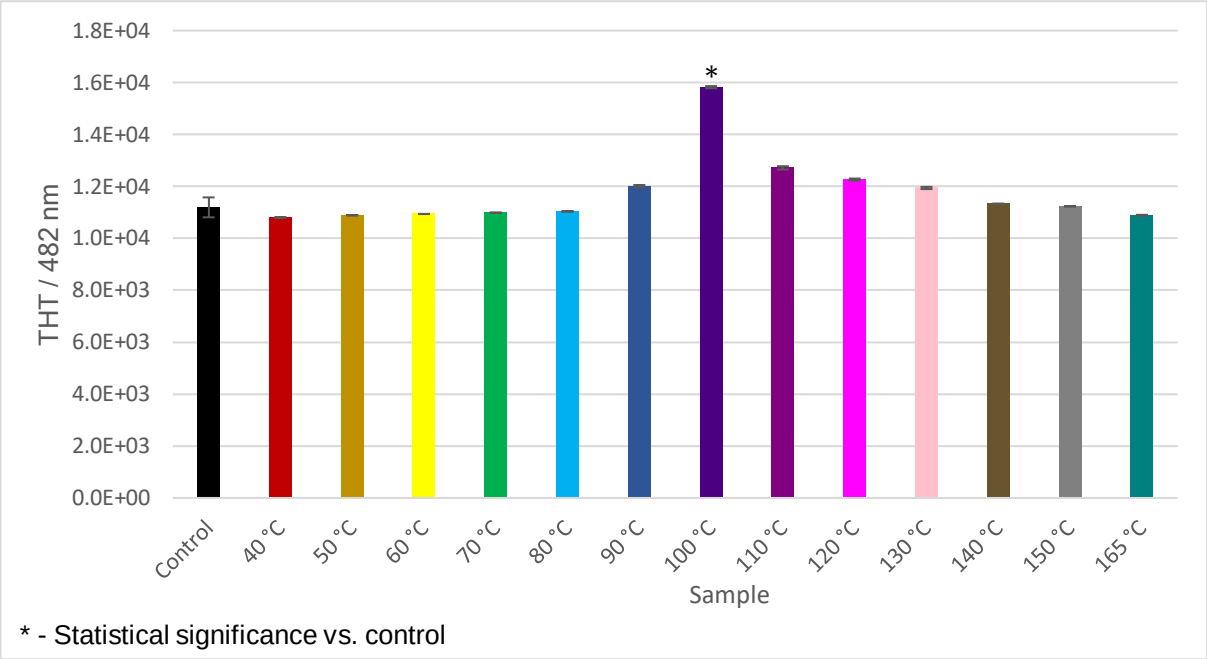

Figure 11: Average of the spectroscopy results at 482 nm (Thioflavin T sensitive wavelength) of ohmic heating treated samples and control samples.

3.7. Principal Component Analysis (PCA)

PCA was employed to understand the impact of the different treatment temperatures on β-lg structure and to find correlation between the different measured variables. PCA is a multivariate statistical tool that allows to reduce the dimensionality of data to two components (PC1 and PC2) while retaining as much relevant information as possible interpret complex data and reveal patterns or relationships. Throughout this work, several parameters of WPI samples were measured after heat treatment through ohmic heating to characterize β-lg during heating with this method. A considerable amount of data was collected on many samples, which can make difficult interpretation of results obtained through the sheer amount of data collected. In these cases, running a PCA can help to organize the data by assigning linear combinations of the original variables measured and presenting these combinations in quadrants. This analysis method significantly decreases visual clutter, resulting in an organized line graph that allows a better visualization of the results obtained (Ringnér, 2008). It is important to note that, since there is little documentation of effects of ohmic heating in WPI samples at a minimum temperature of 90 °C, and the most intriguing results were obtained past this temperature threshold in WPI samples during this work, this PCA only included the results obtained in the temperature range of 90 °C – 165 °C. The PCA of the

characterization of β-lg is presented on Figure 12. It is noteworthy that although zeta-potential, zeta average and particle size were evaluated parameters, the results obtained did not follow any evident trend. As such, this data was discarded from PCA to better visualize influence of other characteristics of β-lg. Figure 12 shows that there are evident specific characteristics that are prevalent in certain quadrants shared between certain treated WPI samples.

PC1 (horizontal axis) and PC2 (vertical axis) account for more than 85 % of the variability. This means that PCA can be used effectively for dimensionality reduction or data simplification. Where PC1 and PC2 collectively explain than 85% of the variance in the original dataset. PC1 represents the horizontal axis and its greater variance (75 %) is explained by the loss of native structure of β-lg imposed by OH. PC2 represents 12 % of variance and evidences the possible formation of β-lg fibrils. The first quadrant mainly encompasses the WPI samples with the highest THT value recorded – i.e., WPI samples treated in the temperature range of 90 °C – 110 °C had noticeably higher values than all other WPI samples. Additionally, WPI samples heat-treated in this temperature range also exhibit the most exposure of hydrophobic regions in β-lg, data obtained through extrinsic fluorescence analysis using ANS (Figure 6).

In the second quadrant, intrinsic fluorescence is represented close to the Y axis, with WPI samples treated at 120°C presenting the highest values. The presence of Coil / Unordered secondary structures is present in the second quadrant, this time close to the X axis, with emphasis in WPI samples heat-treated at higher temperatures, in the range of 120 °C – 165 °C.

Intrinsic fluorescence wavelength shift and TNBS results show similar results in the third quadrant, with WPI samples heat-treated in the range of 140 °C – 165 °C showing the highest values in both intrinsic fluorescence wavelength shift and hydrolysis rate, which indicates that ultra-high temperature is certainly a defining factor with a noticeable effect on these characteristics of β-lg.

Lastly, the fourth quadrant mainly encompasses non-treated WPI samples, which logically maintain the largest quantities of native β-lg, since no denaturation of these structures occurred through heat treatment. These samples also presented a significant amount of Turn secondary structures, since in WPI samples treated at higher temperatures, these structures likely hydrolyzed into Coil / Unordered structures.

Through this discussion and analysis of the data presented through PCA, Table 3 attempts to select several temperature ranges of WPI samples for the study of certain characteristics.

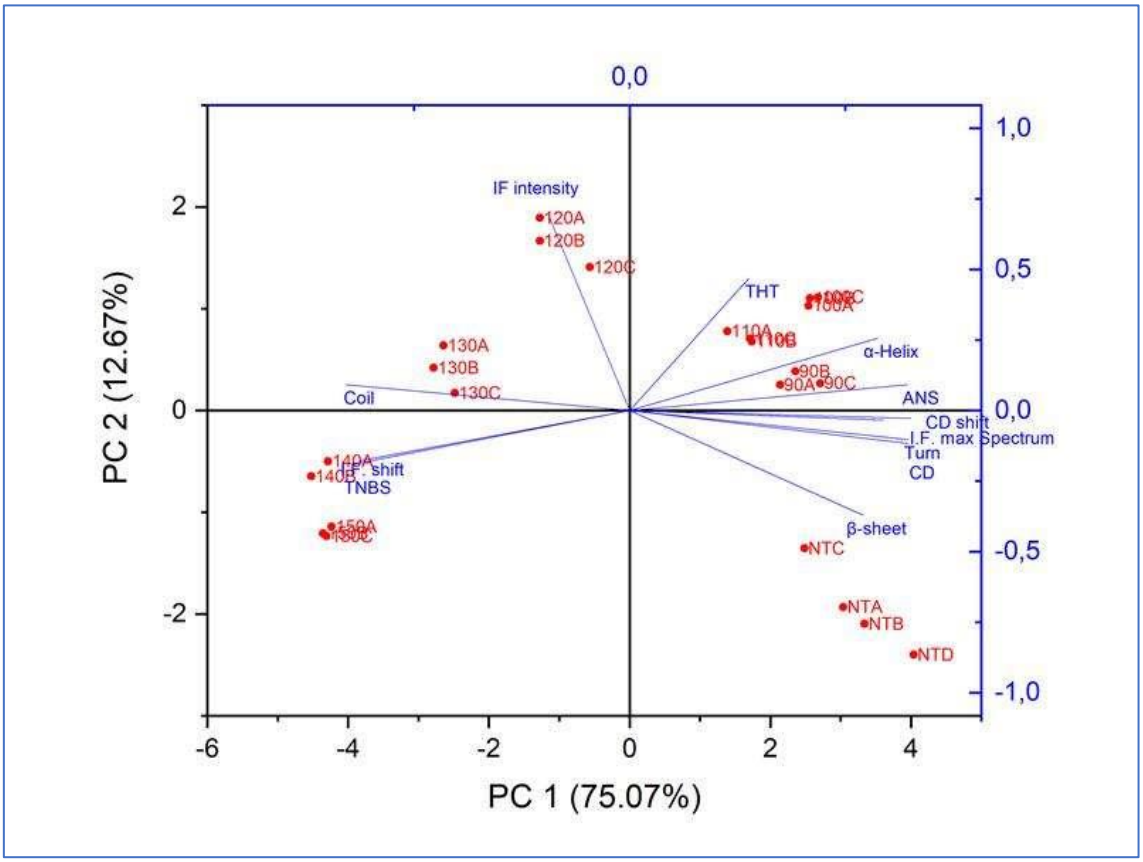

Figure 12: Principal Component Analysis regarding β-lg secondary structure, intrinsic fluorescence, extrinsic fluorescence, hydrolysis, and fibril formation.

Table 3: Optimal ohmic heating treatment temperature ranges for different study parameters.

Study parameter	Temperature range	Quadrants
Tryptophan exposure	90 °C - 120 °C	1,2
Exposure of hydrophobic regions	90 °C - 110 °C	1
Fibril formation		
Hydrolysis	140 °C - 165 °C	3
Coil formation	130 °C - 165 °C	2,3

4.0. Conclusion and future prospects.

Although the influence of MEF in protein denaturation and aggregation still requires further studying, conformational changes in β-lg through OH has shown intriguing results.

Clearly, the unfolding, denaturation, and aggregation processes of proteins of WPI were identified under OH effect. Furthermore, the direct heating capabilities of this method through Joule effect have also proven to be fast and cost-efficient.

Furthermore, it was possible to document several key temperature thresholds that highlight changes in conformation of β-lg, as well as processes such as hydrolysis and aggregation. These results aid in characterizing optimal study conditions for future projects.

Future studies could be developed to further understand the correlation between conformational changes in β-lg and MEF exposure, perhaps during longer periods of heating time maintaining a certain temperature and comparing structural properties of WPI samples at different time intervals during the OH process. There can also be attempts to change parameters such as protein concentration, pH value of WPI samples, electric field strength, conductivity through the addition of salts. The results obtained mark the first-ever denaturation mapping of β-lg by establishing correlations between multiple characteristics analyzed throughout this investigation. This achievement holds significance for future studies, as it provides a foundational understanding of β-lg denaturation and its relationships with various characteristics under OH. By establishing denaturation mapping and correlation in this manner, it creates a valuable framework for exploring and delving deeper into the behavior of β-lg under different conditions and settings. Researchers can use this knowledge as a reference point to investigate how changes in parameters or environments affect β-lg, leading to more informed and targeted research in fields such as protein science, biotechnology, and food chemistry.

Regardless, OH appears to be a promising method for the characterization of β-lg and the formation of protein aggregates, as well as a great tool in expanding the biotechnological and biomedical applications of these biostructures. Future studies should research how β-lg denaturation and its correlations with other characteristics influence interactions with other molecules, including ligands, other proteins, and small molecules. Knowledge of the denaturation process can inform strategies to reduce the allergenicity of β-lg and understanding the influence of having a denatured or partially

denatured forms of the protein. This insight can be important for food processing and product development. Additionally, intriguing challenges are intertwined with enhancing the precision and efficiency of data collection and analysis, which, in turn, will drive technological advancements. Lastly, delving into the biological and functional ramifications of β-lg denaturation assumes paramount importance within the context of its involvement in diverse biological processes.

5.0. References

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