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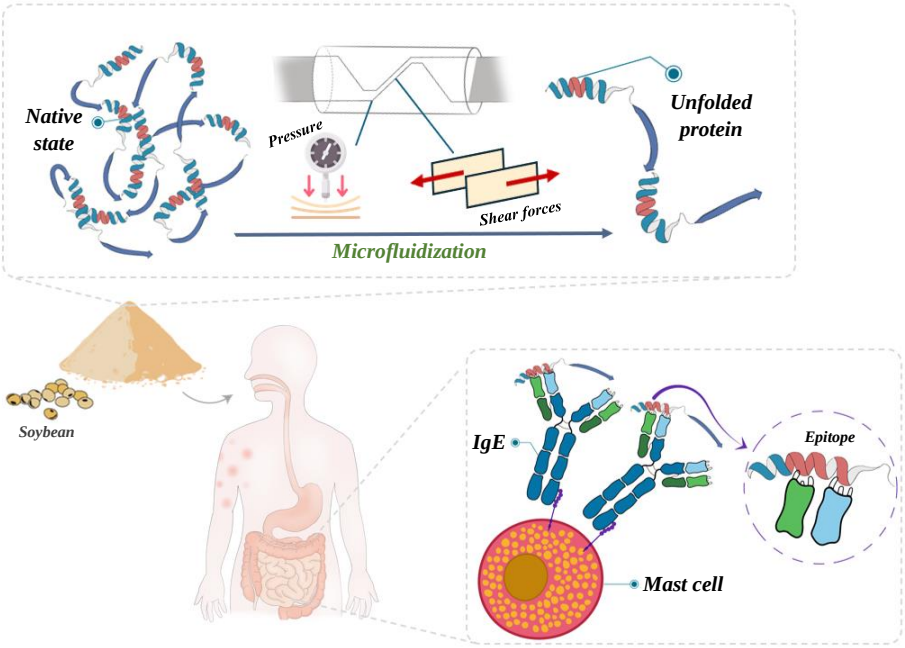
Effect of microfluidization on structural properties and allergenicity of soy proteins



Author: Andreea Diana Kerezsi

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COMMUNAUTÉ FRANÇAISE DE BELGIQUE
UNIVERSITÉ DE LIÈGE – GEMBLOUX AGRO-BIO TECH

EFFECT OF MICROFLUIDIZATION ON STRUCTURAL PROPERTIES AND ALLERGENICITY OF SOY PROTEINS

Andreea Diana KEREZSI

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Agronomic Sciences and Biological Engineering

Supervisors: Dr. Nicolas JACQUET and Prof. Dr. Christophe BLECKER

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Abstract

Andreea Diana Kerezsi (2024). "Effect of microfluidization on structural properties and allergenicity of soy proteins" (PhD dissertation in English).
Gembloux, Belgium, Gembloux Agro-Bio Tech, University of Liege. 183 pages, 34 figures, 12 tables

Food allergies represent significant challenges to human health, and soybean is a major allergen responsible for many cases. The increasing demand for plant-based protein-rich foods and the need for sustainable solutions necessitates the development of novel methods to reduce soybean allergenicity. This thesis explores the impact of microfluidization on soybean protein structure and a combination of microfluidization and enzymatic hydrolysis on allergenicity.

The first part of this research focuses on the relationship between protein structure and allergenicity, highlighting the importance of understanding protein modifications under different conditions. The study investigates the effects of microfluidization on soybean protein structure, including primary, secondary, and tertiary structures, under various conditions, such as temperature or not control and different cycle numbers. The results indicate that microfluidization can unfold protein, open hydrophobic regions, and increase surface hydrophobicity. Moreover, the behavior of soy proteins is different in powder and solution form.

The second part of this research examines the impact of microfluidization, enzymatic hydrolysis, and their combination on soybean allergenicity. The study combines microfluidization with enzymatic hydrolysis to assess the synergistic effects on allergenicity reduction. The results show that microfluidization alone was insufficient to decrease soybean allergens. Enzymatic hydrolysis effectively reduces soybean allergens, while combining microfluidization with enzymatic treatment should be confirmed because of the high variability of the enzymatic treatment alone.

Overall, this thesis contributes to understanding the effects of microfluidization and enzymatic hydrolysis on soybean protein structure and allergenicity, providing insights into developing novel methods for reducing soybean allergenicity and improving food safety.

Keywords: Physical processes, Shearing, Microfluidization, Allergenicity, Soybean proteins, Protein structure.

Résumé

Andreea Diana Kerezsi (2024). "Effet de la microfluidisation sur les propriétés structurelles et l'allergénicité des protéines de soja" (Thèse de doctorat en anglais)

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Les allergies alimentaires représentent un défi important pour la santé humaine, et le soja est un allergène majeur responsable de nombreux cas. La demande croissante d'aliments riches en protéines et le besoin de solutions durables nécessitent le développement de nouvelles méthodes pour réduire l'allergénicité du soja. Cette thèse explore l'impact de la microfluidisation, sur la structure des protéines de soja. l'impact de la microfluidisation et de l'hydrolyse enzymatique, appliquées seules ou en combinaison, à également été établi pour l'allergénicité.

La première partie de cette recherche se concentre sur la rélation entre la structure des protéines et l'allergénicité, soulignant l'importance de comprendre les modifications des protéines dans différentes conditions. L'étude examine les effets de la microfluidisation sur la structure des protéines de soja, y compris les structures primaires, secondaires et tertiaires, dans diverses conditions, Les résultats indiquent que la microfluidisation peut déplier les protéines, ouvrir des régions hydrophobes et augmenter l'hydrophobicité de surface. De plus, le comportement des protéines de soja est différent sous forme de poudre et de solution.

La deuxième partie de cette recherche examine l'impact de la microfluidisation, de l'hydrolyse enzymatique et de leur combinaison sur l'allergénicité du soja. L'étude combine la microfluidisation et l'hydrolyse enzymatique pour évaluer les effets synergiques sur la réduction de l'allergénicité. Les résultats montrent que la microfluidisation seule est insuffisante pour réduire les allergènes du soja. L'hydrolyse enzymatique réduit efficacement les allergènes du soja, tandis que la combinaison de la microfluidisation avec le traitement enzymatique doit être confirmée en raison de la grande variabilité du traitement enzymatique seul.

Dans l'ensemble, cette thèse contribue à la compréhension des effets de la microfluidisation et de l'hydrolyse enzymatique sur la structure et l'allergénicité des protéines de soja, ce qui permet de développer de nouvelles méthodes pour réduire l'allergénicité du soja et améliorer la sécurité alimentaire

Mots-clés : Procédés physiques, Cisaillement, Microfluidisation, Allergénicité, Protéines de soja, Enzymes, Structure des protéines.

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15 October 2024, Andreea Diana Kerezsi

Table of contents

List of figures

Figure 6-16. Combined effects of microfluidization and enzymatic hydrolysis on soy protein structure and epitope recognition.

List of tables

List of abbreviations

1P, 3P, 5P: The number of passes used for microfluidization treatment
1PN, 3PN, 5PN: MF samples with temperature-controlled
1PW, 3PW, 5PW: MF samples without temperature-controlled
2-DE: Two-dimensional electrophoresis
Ab: Antibody
ANS: 1-anilinonaphthalene-8-sulfonic acid
AP: Alkaline Phosphatase
ATR: Attenuated total reflectance
BSA: Bovine Serum Albumin
C: Control (Non-microfluidized sample)
C: Control (SPI without enzyme addition, same hydrolyzed conditions)
CAPP: Cold atmospheric pressure plasma
CI: Confidence interval
Conf: Conformational epitope
DC Protein assay: Detergent-compatible protein assay
DH: degree of hydrolysis
DHPM: Dynamic high-pressure microfluidization
DIC: Controlled instantaneous pressure drop
DM: dry matter
E: Enzymatic hydrolysis
EAACI: European Academy of Allergy and Clinical Immunology
EAST: Enzyme allergosorbent test
ED_{05}: Eliciting dose
EFSA: European Food Safety Authority
ELISA: Enzyme-linked immunosorbent assay
EPIT: Epicutaneous immunotherapy
EU: European Union
FAO: Food and Agriculture Organization of the United Nations
FDA: U.S. Food and Drug Administration
FI: Fluorescence intensity
F_{max}: Maximum fluorescence
FTIR: Fourier Transform Infrared Spectroscopy
GMP: Good manufacturing practices
HHP: High hydrostatic pressure
HIU: High-intensity ultrasound
HPH: High-pressure homogenization
If/g × µM: Intensity fluorescence/gram × microMolar - measurement unit for PSH
IgE: Immunoglobulin E

ISM: Industrial and Scientific Medical
IU: International unit
IUIS: International Union of Immunological Societies
Izimab: Generated mouse monoclonal antibodies
kDa: Kilodalton
kGy: Kilogray
kU/l: Kilounits per liter
kvpp: Practical peak kilovoltage
M0: Non-microfluidized (native SPI)
M1, M3, M5: Microfluidized samples for 1, 3, and 5 times
M1E, M3E, M5E: Combination of MF and E for 1, 3, and 5 passes
ME: Combination of microfluidization and enzymatic hydrolysis
MF: Microfluidization/Microfluidized
MIR: Mid-infrared spectroscopy
MW: Molecular weight
NACMCF: National Advisory Committee on Microbiological Criteria for Foods
Native-PAGE: Native polyacrylamide gel electrophoresis
Nijs SPI: Soybean protein isolate (RGT Shouna variety – farmer Nijs, Belgium) obtained by laboratory-scale
nsLTP : Non-specific lipid transfer protein
OIT: Oral immunotherapy
P34: Gly m Bd 30K
PAL: Precautionary Allergen Labeling
PED: Pressure Equipment Directive
PL: Pulsed light
PSH: Protein surface hydrophobicity
PUV: Pulsed ultraviolet light
PVDF: Polyvinylidene fluoride
ROS: Reactive oxygen species
Rpm: Rotation per minute
RT: Room temperature
S: Svedberg sedimentation coefficient
SDBD: Surface dielectric barrier air discharge
SDS: Sodium dodecyl sulfate
SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SF: Defatted soybean flour
sIgE: Allergen-specific IgE
SLIT: Sublingual immunotherapy
SPI: Soybean protein isolate
SVM: Support vector machines
TBS: Tris-buffered saline

TBST: Tween Tris-buffered saline

Terryn SPI: Soybean protein isolate (RGT Shouna variety – farmer Terryn, Belgium) obtained by lab-scale

TI: Trypsin inhibitor

Tris-SDS: Tris-glycine-SDS buffer

UAP: Unintended Allergen Presence

USDA: United States Department of Agriculture

UV: Ultraviolet (light)

WAO: World Allergy Organization

WHO: World Health Organization

WW: Wet weight

γ-irradiation: Gamma-irradiation

γ-rays: Gamma-rays

λ_{max}: Maximum emission wavelength

1

General introduction

Chapter 1. General introduction

Context

Food allergies have emerged as a significant global public health issue, impacting approximately 220-250 million people (Meinlschmidt et al., 2016). When a person ingests a food allergen, the body mistakenly identifies proteins as harmful and produces IgE antibodies. These antibodies trigger the release of histamine, causing an allergic reaction (Huang et al., 2014). To contextualize the severity of food allergies, a comparison with diabetes can be addressed (537 million people worldwide affected (Ong et al., 2023)), to highlight the importance of food allergies as a public health issue. Food allergies not only impact individuals' quality of life but also represent a substantial financial burden. In the USA alone, food allergies generate $24.8 billion in expenses annually, with costs attributed to essential medications like epinephrine auto-injectors, clinician visits, hospitalizations, and caregiver job disruptions (Warren et al., 2020).

Symptoms of food allergies can be cutaneous (rash, angioedema, urticaria), related to the respiratory system (coughing, sneezing, nasal congestion, asthma) and the gastrointestinal tract (diarrhea, vomiting, abdominal pain) (Jain, 2023). These allergic reactions can range from mild to severe, with the most dangerous being anaphylaxis, a life-threatening condition. During anaphylaxis, the immune system releases a flood of chemicals that can lead to shock, causing a sudden drop in blood pressure and narrowing of the airways, blocking breathing. The primary treatment for anaphylaxis is epinephrine auto-injectors like EpiPen® (adrenaline) (FDA, 2024; White et al., 2015).

In addition, 9 allergenic foods are responsible for more than 90% of IgE-mediated allergies, such as milk, eggs, fish, shellfish, tree nuts, peanuts, wheat, soybean and sesame. Sesame was officially recognized as the 9th major food allergen in the United States, with the regulation becoming effective on January 1, 2023 (FDA, 2024).

Our project focuses on soybean, one of the leading sources of plant-based protein, known for its high-quality protein content, but also one of the most problematic allergen. Moreover, the recent introduction of soybean cultivation in Belgium and other northwestern European countries offers significant potential for sustainable agriculture (Pannecoucque et al., 2022). Currently, Europe relies on imported soybeans for food and feed, leading to negative socioeconomic and environmental impacts (Van Dingenen et al., 2022). This has generated interest in increasing local soybean production, which motivates my research project on this crop. In this way, our variety used in some experiments was RGT Shouna, from two different farmers from Flanders (Nijs and Terryn).

Regarding allergies, a diverse community of professionals, including allergists, immunologists, dietitians, nutritionists, researchers, advocacy groups, nonprofit organizations, food industry experts, educators, and school staff, are actively engaged in addressing this issue (Sansweet et al., 2024; Warren et al., 2021).

Various strategies exist for managing food allergies, including avoidance or partial curative solutions (Anagnostou et al., 2023). An example of a partial solution for managing food allergies is oral immunotherapy (OIT). Palforzia, approved by the U.S. Food and Drug Administration (FDA) for treating peanut allergies in children aged 4-17, is an OIT product. However, Palforzia is specific only to peanut allergies and may not be suitable for all patients. Other immunotherapy options include sublingual (SLIT), where food protein is dissolved under the tongue (Kim et al., 2019), and epicutaneous immunotherapy (EPIT), which involves applying allergen patches to the skin (Fleischer et al., 2019). Clinical trials show that EPIT can desensitize individuals, particularly to peanuts, with a better safety profile than OIT.

However, our focus is on finding ways to decrease soybean allergenicity and there is an interest in developing methods to reduce food allergens. While thermal treatments (roasting or boiling) are commonly used, it is important to explore other physical treatments (Cabanillas et al., 2018). Several physical techniques have been identified in the literature to reduce food allergens, including pressure-based treatments (Yin et al., 2019; H. Li et al., 2012; Cuadrado et al., 2011; Peñas et al., 2004) and wave-based treatments (Meinlschmidt, Ueberham, et al., 2016; Moriyama et al., 2013). In addition to physical treatments, non-physical such as fermentation (Song et al., 2008), and enzymatic methods (Pang et al., 2024; Peñas, Restani, et al., 2006), have also been shown to reduce allergen levels effectively.

Recent trends show a shift towards using new physical methods due to concerns about the potential negative effects of thermal processing on food quality and nutritional value. After conducting a comprehensive literature review, it was observed that various techniques have been employed to mitigate the allergenicity of soybeans. However, a gap was identified where microfluidization, as a technique for reducing soy allergenicity, has not been investigated.

Regarding new physical treatments, microfluidization is an emerging technology in the food industry that improves product stability and reduces particle size while protecting the organoleptic properties of the final product (Kavinila et al., 2023; Sethi et al., 2022). Since proteins are the primary triggers for allergic reactions, it is crucial to assess the impact of microfluidization on soybean protein structure before determining the optimal conditions for allergen reduction. This assessment will help establish the relationship between the structural changes induced in proteins and the presence of allergens. The existing literature has described the impact of microfluidization on the structure of various proteins, including soybean protein isolate, peanut protein isolate, ovalbumin, β-lactoglobulin, pea globulin, and potato protein isolate (Ozturk & Turasan, 2021; Shen & Tang, 2012).

However, there is limited information regarding the effect of microfluidization under different conditions, such as controlling or not controlling the temperature during the process and varying the number of passes, on the structure of soybean proteins and, consequently, their allergenicity.

2

Objectives, research roadmap and outline

Chapter 2. Objectives, research roadmap and outline

2.1. Objectives

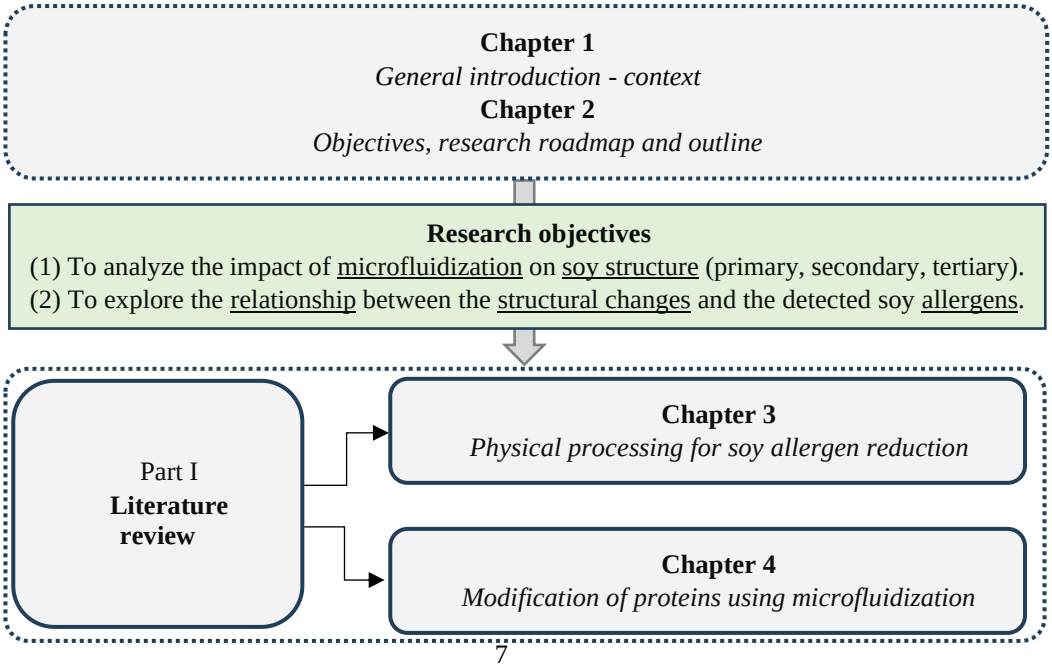
Provided the previous background, the main aim of this thesis is to investigate the possibility of reducing soybean allergens by initially employing a physical treatment (microfluidization) and subsequently combining it with a traditional method, enzymatic hydrolysis, to enhance allergen reduction. To accomplish this objective, a detailed understanding of soybean protein structures and selecting optimal conditions were crucial preliminary steps in applying the treatments to reduce soybean allergens. These preliminary steps were necessary for several reasons. Identifying allergens requires the use of the Western Blot method, which is time-consuming and costly due to the use of human antibodies. Therefore, having a comprehensive understanding of the protein structure was essential for optimizing the experimental design. Consequently, the objectives include two significant aspects:

(1) To analyze the impact of microfluidization on soybean structure (primary, secondary, tertiary).

(2) To explore the relationship between the structural changes and the detected soybean allergens.

2.2. Research roadmap

The manuscript is organized into eight chapters, and the thesis outline is presented in Fig. 2-1.



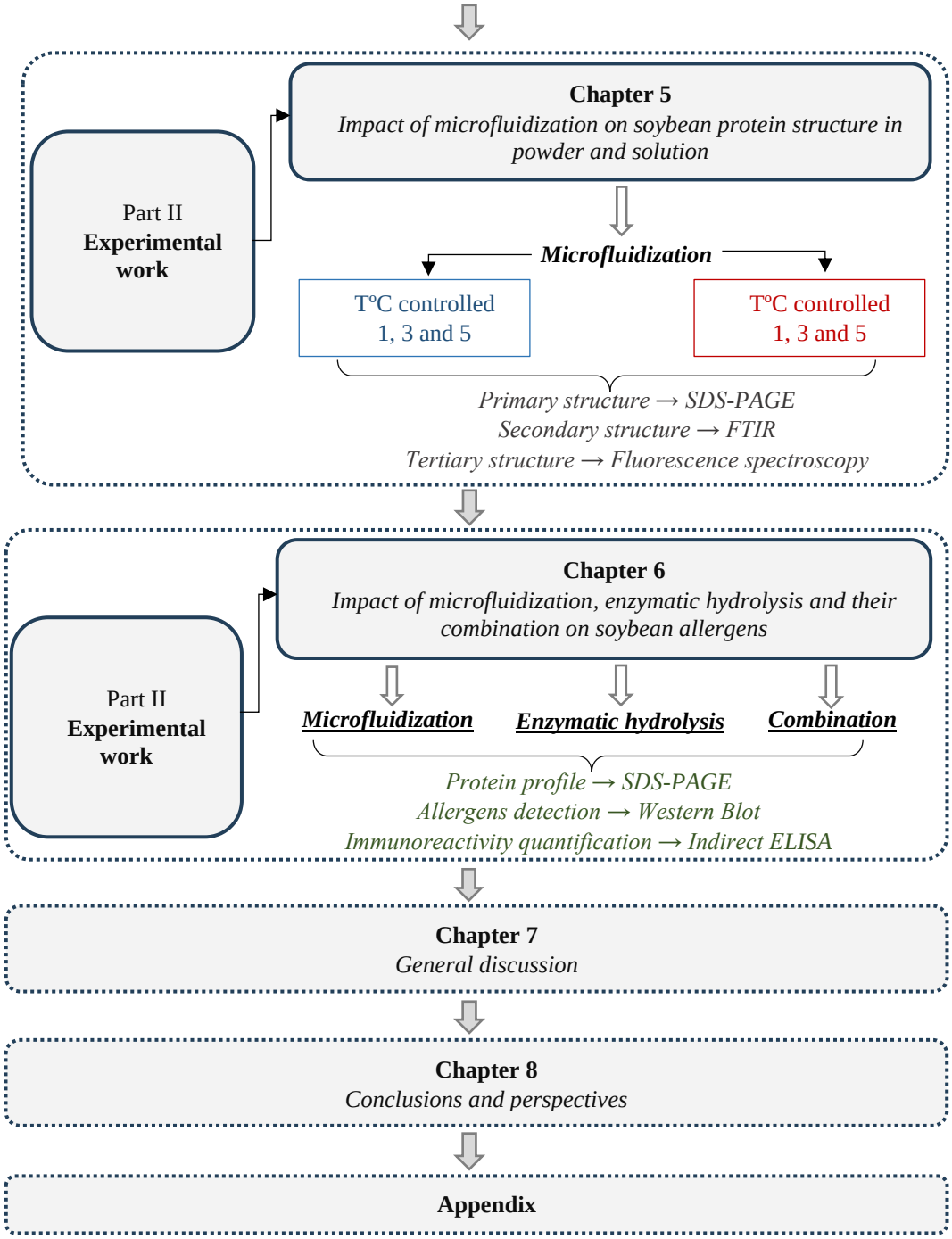


Figure 2-1. Summary of the thesis structure.

2.3. Outline

Chapter 1 introduces the thesis with a general overview, including the context.

Chapter 2 describes the objectives, thesis roadmap and outline, with a description of each chapter.

Chapter 3 is part of the literature review, describing all the physical treatments to reduce soybean allergens. Several types of physical treatments are outlined in two groups. One is related to the pressure and the second group is presented in the form of waves. All the techniques described the impact on protein structure and the subsequent correlation with allergen reduction.

Chapter 4 is also part of the literature review and addresses the gap in research concerning the effects of microfluidization on soy allergens. This chapter will detail the protein modifications resulting from high-shear treatment (microfluidization) and provide insights into other allergens studied.

Chapter 5, part of the preliminary findings, aims to assess the impact of microfluidization on soybean structure in various states, such as powder and solution. This chapter will enhance our comprehension of protein structure and help us in selecting optimal conditions for allergen reduction. Additionally, this study fills another research gap by exploring different parameters, such as the number of passes and temperature control during treatment. SDS-PAGE (under reducing and denaturing conditions) was used for primary structure analysis, FTIR for secondary structure evaluation, and fluorescence spectroscopy for tertiary structure assessment. This comprehensive analysis of soybean behavior in various states was concluded and published as a research article.

After determining the optimal conditions, we conducted temperature-controlled microfluidization on soybean allergens. Recognizing the insufficiency of a single treatment in allergen reduction, we introduced supplementary enzymatic hydrolysis. The individual and combined effects of these treatments were investigated in Chapter 6. We employed three methods to assess the impact on soybean allergenicity: SDS-PAGE for protein separation based on molecular weight, Western blot for allergen detection through antibody-antigen interaction, and indirect ELISA to quantify allergen response (immunoreactivity).

Chapter 7 provides a comprehensive overview of the entire thesis within the general discussion.

Chapter 8 presents the conclusions and future perspectives of this thesis. This section highlights the significance of the findings, the novelty of the research and provides suggestions for future investigation in this research area.

Additional information has been included in the appendices covering optimization of the Western Blot method, the SDS-PAGE profile, the Western Blot analysis of local Belgian soybean and its chemical composition, as well as the particle size distribution of the microfluidized samples.

PART ONE

Literature Review

Chapter 3

Physical processing for soy allergen reduction

Chapter 4

Modification of proteins using microfluidization

3

Physical processing for soy allergen reduction

Description of Chapter 3

This chapter describes the physical treatments applied in the food industry to reduce soybean allergens. It also provides insight into the structure of proteins and their relation to food allergies. Understanding how proteins behave is essential because these molecules trigger allergic reactions in humans.

This work is an original contribution adapted from Kerezsi, A. D., Jacquet, N., & Blecker, C. (2022), "Advances on physical treatments for soy allergens reduction - A review." This paper was published in *Trends in Food Science & Technology*, *122*, *24–39*, on 8 February 2022 (https://doi.org/10.1016/J.TIFS.2022.02.007).

Chapter 3. Physical processing for soy allergen reduction

3.1. Introduction

The increasing prevalence of food allergies has prompted the exploration of various technologies to mitigate allergenicity, such as thermal (dry heat -roasting and moist heat -autoclaving and boiling), enzymatic, and fermentation treatments (Vanga et al., 2017). These treatments require necessary amounts of energy and water, which can lead to high costs, limit the process's sustainability, and decrease food quality (Dong et al., 2020). In this way, physical processes could be a more durable solution in our current society's context.

Different physical processing methods can be applied to proteins, particularly for reducing soybean allergens. This chapter will discuss these approaches, but first, we will describe some general considerations regarding food allergens.

3.2 General overview of food allergens

3.2.1. Food allergies: mechanisms and epitope classification

Food allergies affect adults and children and have risen in the past two to three decades (Seth et al., 2020). A food allergy is an adverse immune reaction that occurs mainly against dietary proteins, usually considered harmless and caused by the immune system's inability to develop or maintain tolerance. The immune response is triggered when specific regions of the allergenic protein, known as epitopes, are recognized by immunoglobulin E (IgE) antibodies. These epitopes are key to initiating the allergic reaction, as they bind to IgE on immune cells, releasing inflammatory mediators. In the first case, with IgE (type 1 or immediate hypersensitivity), reactions quickly develop (in a few minutes up to 2 hours). In the second one, the symptoms appear in hours or days (type 4 or delayed hypersensitivity) (De Angelis et al., 2019).

In addition, the epitopes are categorized into two groups: linear and conformational. Linear epitopes come from short, continuous amino acid sequences (8-10 amino acids), while conformational epitopes depend on a specific three-dimensional structure formed by protein folding, involving discontinuous amino acid segments (X. Li et al., 2015). Figure 3-1 illustrates the structural differences between the two types of epitopes in their interaction with IgE antibodies.

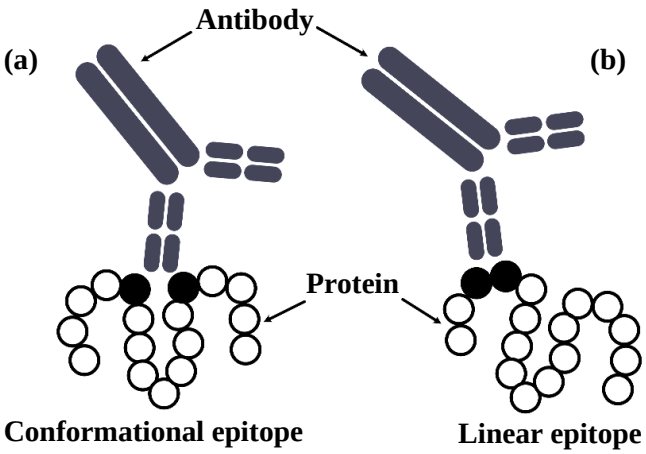


Figure 3-1. Conformational (a) and linear (b) epitopes.
Adapted from (Bogahawaththa et al., 2017).

The following Table (3-1) presents examples of linear and conformational epitopes derived from Gly m 6 (glycinin G1) and Gly m 4 obtained from PDB (Protein Data Bank) and IEDB (Immune Epitope Database) (PDB, 2024; IEDB, 2024). These examples illustrate the distinct characteristics of each epitope, highlighting their structural differences and positions within the protein sequences. Each letter (S, G, C and so on) corresponds to an amino acid. Moreover, the table does not include all the soybean allergens with their epitopes.

Table 3-1. Examples of linear and conformational soybean epitopes.

Allergen	PDB	IEDB ID	Epitope Type	Start Position	End Position	Epitope Amino Acids
Gly m 6	1FXZ	914076	Linear	14	20	SGCCFAF
		913981	Linear	25	34	QPQQNECQIQ
		2265259	Linear	176	189	DQMPRRFYLAGNQE
		913210	Linear	192	198	FLKYQQE
Gly m 4	2K7H	581517	Conf.			V41, V44, A77, N78

3.2.2. Allergenicity prediction using in silico tools

Preventing allergenicity driven by food-related proteins is a major relevant nutritional, health, and industrial problem. The potentially multifactorial origin of this property turns its prediction into a highly complex challenge. Fortunately, the fast integration of statistical learning and data science methods to protein analysis also pushes the field of protein allergenicity prediction.

Due to the high risk of allergen proteins, the criteria to classify a novel protein as a potential allergen is very conservative. The Codex Alimentarius establishes that any protein with 35% identity (or higher) on a window of 80 amino acids, when compared with known allergens, must be considered a potential allergen (Hayes et al., 2015). Following this idea, the database AllergenOnline compares new proteins against large and updated reported proteins (Goodman et al., 2016). Proteins with an identity larger than 50% suggest a high risk for cross-reactivity.

Developing tools for predicting such properties has considerable research potential. This was proven when a large set of allergen protein structures was used to generate surface maps of discontinuous peptides (Bragin et al., 2013). Tools like AlphaFold are used to extend the information on protein structure prediction (Negi et al., 2023; Ivanciuc et al., 2003). Table 3-2 presents some examples of tools for the allergenicity prediction in food proteins.

Other models incorporate physicochemical and biological properties. For example, a research done on 29 total variables for training random forest allergenicity models (previously tested with insect proteins), reaching an accuracy of >85% (Westerhout et al., 2019). Most of the tools are proposing sophisticated methods for understanding the cross-reactivity or allergenicity potential of new proteins compared with those reported in databases. (L. Wang et al., 2021.)

In addition, AllergenOnline was already mentioned, but other databases exist for the same purposes. The Allergome, provides information on IgE-mediated allergens and associated clinical data (Mari et al., 2006). BIOPEP database contains biologically active peptide names, sequences, experimentally or predicted epitopes, and the AllFam allergen database for classifying allergens into protein families (Iwaniak et al., 2024; Radauer et al., 2008).

Using computational models for predicting protein allergenicity is a common task, depending critically on curated experimental information. Moreover, predicting potential allergenicity does not confirm an actual allergic reaction. Further biochemical tests, such as IgE blotting, and biological tests—including basophil activation test, skin prick tests, or *in vivo* challenge tests with allergic individuals—are required to validate protein allergenicity predictions (Hayes et al., 2015).

Table 3-2. *In silico* tools for allergenicity prediction in food proteins (Adapted from Hayes et al., 2015).

***In silico* tools**	**Description**	**References**
AllerHunter	-The predictor is based on pairwise similarity combined with SVM (support vector machines) models.	(Muh et al., 2009)
AlgPred	-Predicts protein allergenicity by detecting IgE epitopes based on similarity.	(Saha & Raghava, 2006)
PREAL	-SVM model that integrates subcellular locations and protein properties (molecular weight, polarizability, solvent accessibility, secondary structure propensity, hydrophobicity, normalized van der Waals volume, polarity, and length).	(J. Wang et al., 2013)
AllerTop 1.0	-This predictor uses protein representations (based on physicochemical properties) as input for five integrated machine learning models.	(Dimitrov et al., 2013; Dimitrov, Bangov, et al., 2014)
AllergenFP	-Model that uses Tanimoto similarities among protein properties (hydrophobicity, size, relative abundance, helix, and β-strand abundances).	(Dimitrov, Naneva, et al., 2014)
Databases	**Description**	
AllergenOnline	-Peer-review sequence database for cross-reactivity search. Used for research and industry	(Goodman et al., 2016)
SDAP 1.0-2.0	-Searches for cross-reactivity by detecting IgE potential binding 3D structures of the proteins and known allergens. It is now assisted by AlhaFold predictions.	(Ivanciuc et al., 2003; Negi et al., 2023)
Allergome	-Provides information on IgE-mediated allergens and associated clinical data.	(Mari et al., 2006)
BIOPEP	-Contains biologically active peptide names, sequences, experimentally or predicted epitopes	(Iwaniak et al., 2024)
ALLfam	-Database for classifying allergens into protein families. It is based on allergen data from the WHO/IUIS Allergen Nomenclature Database supplemented by FARRP allergen database, AllergenOnline, and protein family data from the Pfam database.	(Radauer et al., 2008)

3.2.3. Food allergens: overview of characteristics and the role of soybean

The most general definition of an allergen is any molecule that binds to IgE antibodies (Molecular Allergology User's Guide 2.0, 2022). Allergens are typically water-soluble proteins, often glycosylated, with a globular conformation, stable during processing and digestion, with a molecular weight of 10-70 kDa (Costa et al., 2020; Shriver & Yang, 2011; Sicherer & Sampson, 2010; Ebo & Stevens, 2001). However, proteins or peptides having 3.5 kDa can still trigger allergic reactions (Ballegaard & Bøgh, 2023; Bøgh & Madsen, 2016).

In addition, around 200 food allergens were identified, and nine allergenic foods account for more than 90% of the IgE-mediated allergic reactions (FDA, 2024). These include milk, eggs, peanuts, tree nuts, shellfish, fish, wheat, sesame, and soy (Messina & Venter, 2020; Sicherer & Sampson, 2010). Nowadays, the best treatment for individuals with food allergies is to avoid the consumption of these products (Ballegaard & Bøgh, 2023; Messina & Venter, 2020; Burks et al., 2012). However, this could be complex due to the difficulty of determining which products contain the allergenic ingredient or mislabeling the allergens (Bertheau & Davison, 2011; Wilson et al., 2005).

Soybean (*Glycine max*) is an attractive variety of legumes due to the high quality of protein and fat. Moreover, it is an essential bean in the world for producing different products. It is one of the few vegetal resources that present a better profile in amino acids required for the human body, compared with other plant-based foods. On a dry-weight basis, the chemical composition of soybeans consists of about 40% protein, 35% carbohydrate, 20% fat, and 5% ash (Snyder, 2003).

About 90% of soybean proteins are represented by globulins, the primary storage proteins soluble in salt solutions, with the remaining 10% are water-soluble albumins. By acidification at pH 4.5-4.8, the defatted soybean can be extracted into storage globulin and whey fractions by obtaining the soy protein isolate (SPI), which contains a mixture of proteins, which are 2S, 7S, 11S, and 15S, "S" being the Svedberg sedimentation coefficient, where a higher number suggests a higher molecular weight (Sui et al., 2021; Nishinari et al., 2014).

The 2S fraction (~20% of total protein) includes 2S globulin, Kunitz trypsin inhibitor (~20 kDa), Bowman-Birk trypsin inhibitor (~8 kDa) and cytochrome C (Sui et al., 2021).

The 7S fraction (~40%) consists of the trimer glycoprotein β-conglycinin (7S globulin, ~180 kDa), composed of the α' subunit (~71 kDa), α subunit (~67 kDa), and β subunit (50 kDa). It also includes lipoxygenase, hemagglutinins, and α-amylase (Sui et al., 2021). An interesting observation was described by Murphy (2008) which noted that most citations after 1985 reported higher molecular weights for these subunits (68, 72, and 52 kDa for α', α, and β). However, her research group demonstrated that a deglycosylation step results in lower molecular weights (65.8, 68, and 53 kDa for

α', α, and β). Nevertheless, the literature presents varying molecular weights for these protein subunits.

The 11S fraction (~30%) primarily contains the hexamer glycinin (11 S globulin, ~360 kDa), which includes 12 unique polypeptides composed by acidic subunits (~35-40 kDa) and basic subunits (~20 kDa) (Sui et al., 2021; Murphy, 2008).

Finally, the 15S fraction accounts for ~10% and is probably a dimer of glycinin (Sui et al., 2021).

However, the two primary components of soybean storage proteins are the 7S globulin (β-conglycinin) and the 11S globulin (glycinin), shown in Figure 3-2.

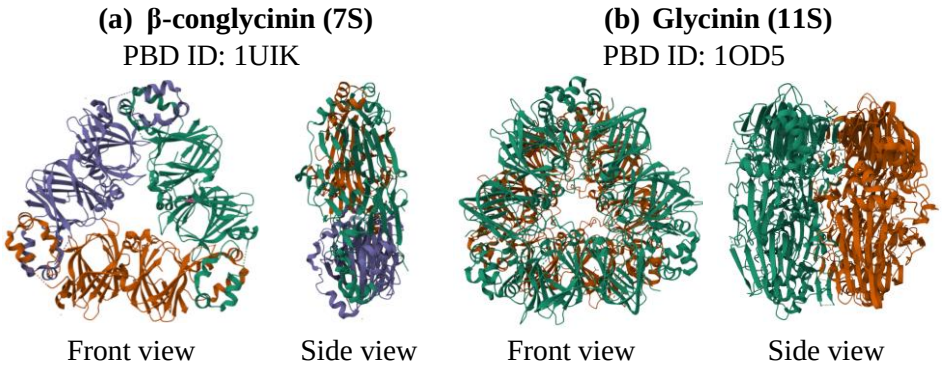


Figure 3-2 (a). The trimer β-conglycinin consists of three subunits α', α, and β and **(b)** the hexamer glycinin, both shown in a ribbon diagram (PDB, 2024).

Although soybean is frequently classified as one of the "Big 8" allergens in the literature, very recently the Expert Committee on Risk Assessment of Food Allergens concluded that soybean does not meet the criteria for inclusion as a global priority allergen due to its low prevalence, low potency, and the rarity of global reports of anaphylaxis (FAO & WHO, 2022). Moreover, in 2023, it seems it was recommended to update the list of eight priority allergenic foods or food groups, which includes milk, egg, fish, crustacean shellfish, peanut, tree nuts, sesame seeds, and gluten, adding sesame seeds and removing soybean (FAO & WHO, 2023).

The eliciting dose predicted to provoke allergic reactions in 5% of the population (ED_{05}) was estimated at 10.0 mg protein (CI 95%: 2.2, 54.6) for the discrete dosing scheme and 14.1 mg protein (CI 95%: 3.1, 76.2) for the cumulative dosing scheme, based on Bayesian modeling (Remington et al., 2020).

Furthermore, soybean was categorized in the lowest severity group (Group C [I]), as it had the lowest rate of anaphylaxis among allergens across all regions. Data confirms that soybean is an uncommon cause of anaphylaxis globally, with no cases of anaphylaxis observed at exposure levels below 200 mg of protein (Turner et al., 2022), aligning with other findings (Baseggio Conrado et al., 2021).

According to the International Union of Immunological Societies (IUIS) Allergen Nomenclature Sub-Committee (www.allergen.org), eight verified allergenic proteins have been registered in soybean seeds: Gly m 1 to Gly m 8 (Verhoeckx et al., 2015) presented in Table 3-3. As an illustration, the two major storage proteins, Gly m 5 and Gly m 6, are correlated with the critical effects in subjects from Europe (Verhoeckx et al., 2015).

Table 3-3. Soybean proteins responsible for allergenicity.

Allergen	Protein family/Name of protein	Molecular weight (kDa)	References
Gly m 1.0101	nsLTP	7	WHO/IUIS (2019)
Gly m 1.0102	nsLTP	7.5	WHO/IUIS (2019)
Gly m 2	Defensin	8	Wilson et al. (2005)
Gly m 3	Profilin	14	Ogawa et al. (2000)
Gly m 4	Pathogenesis-related proteins (PR-10)	17	WHO/IUIS (2019)
Gly m 5	β-Conglycinin/7S globulin, vicilin, Cupin family	180	Sui et al. (2021)
	α	63-67	Wilson et al. (2005), EFSA. (2014)
	α’	71	
	β	42-50	Wilson et al. (2005), EFSA. (2014)
Gly m 6	Glycinin /11S globulin, legumin, Cupin family	360	Sui et al. (2021)
	Gy1	40-53.6	Verma et al. (2013)
	Gy2	22-52.4	Verma et al. (2013)
	Gy3	52.2	EFSA. (2014)
	Gy4	61.2	Verma et al. (2013)
	Gy5	55.4	EFSA. (2014)
Gly m 7	Seed biotinylated protein	76.2	WHO/IUIS (2019)
Gly m 8	2S albumin	20-28	Verma et al. (2013) WHO/IUIS (2019)
Gly m Bd30K	Serine protease	30-34	Wilson et al. (2005), Gonzalez et al. (1992)
Gly m Lectin	Lectin (agglutinin)	14.5	Mittag et al. (2006)
Gly m TI	Kunitz trypsin inhibitor	20	Burks et al. (1994)

3.2.4. Databases for protein and allergen information

Access to reliable and comprehensive databases is essential in studying allergens and their associated proteins. These databases provide valuable information regarding protein structures, sequences, and allergenic properties, facilitating research and

improving our understanding of food allergies. Below is a list (Table 3-4) of significant databases that cover protein and allergen-related data, along with their respective functions and resources (PDB, 2024; Molecular Allergology User's Guide 2.0, 2022).

Table 3-4. Most important databases covering proteins and allergen-related data.

Web tool	Website	Information
RCSB PDB (Research Collaboratory for Structural Bioinformatics - Protein Data Bank)	https://www.rcsb.org/	3D structure data for proteins, DNA, and RNA
UniProt (Universal Protein Resource)	https://www.uniprot.org/	Protein sequence and functional information
wwPDB (Worldwide Protein Data Bank)	https://www.wwpdb.org/	Archive of macromolecular structure
ALLERGEN NOMENCLATURE WHO/IUIS	https://www.allergen.org/	Allergen nomenclature database
IEDB (Immune Epitope Database)	https://www.iedb.org/	Experimentally determined B cell and T cell epitopes

3.2.5. Allergens labeling

Food labeling is crucial in protecting all consumers, especially those with food allergies. For instance, in the European Union (EU), allergens must be highlighted in ingredient lists, allowing consumers to easily identify them. According to Regulation (EU) No. 1169/2011, if an ingredient consists of multiple words, only the allergen itself needs to be emphasized (Chang et al., 2023).

Moreover, Precautionary Allergen Labeling (PAL) has emerged as an additional regulatory approach to address the risks posed by Unintended Allergen Presence (UAP) in food products. Introduced in the 1980s, PAL serves to inform consumers, particularly those with food allergies, about potential cross-contact during food production, mainly when allergenic ingredients are processed in shared facilities or with shared equipment (FAO & WHO, 2023). Common PAL statements, such as "May contain X, " "Produced in a facility that uses X, " and "Not suitable for someone with X allergy," help consumers make informed decisions about their food choices (Barnett et al., 2011).

However, unlike mandatory allergen labeling, PAL is not regulated by law in most countries, leading to inconsistencies in both its application and wording. In the EU, Regulation (EU) No. 1169/2011 specifies in Article 36 that any voluntary information provided to consumers, including PAL, must be clear, not misleading, and grounded in relevant scientific data when applicable (FAO & WHO, 2023).

3.3. Allergenicity and food processing

Thermal treatment, such as pasteurization and sterilization, is one of the most used methods for preserving food worldwide (Dewan, 2020). In addition to preservation, these methods can impact the protein structure, and they are also considered to influence food allergenicity. Studies have explored food allergies as a global health concern by examining how food processing can impact immunoreactivity (Vanga et al., 2017). Changes in allergenicity are often a result of modifications to the protein structures of known allergens.

When people started to cook food from the raw state, they did not think that cooking could impact the allergenicity of the food. In this way, roasting and boiling are believed to be efficient methods to reduce the allergenicity in some foods (Dong et al., 2020). Cabanillas et al. (2018) described that after 30 min of boiling soybean, the IgE reactivity was not affected by this treatment, but it was partially reduced after 60 min. Also, the authors described that a combination of heat and pressure had the highest impact on soybean allergenicity by a potential fragmentation of the proteins, even if some allergens (β-conglycinin) persisted after boiling. Interestingly, despite the effectiveness of thermal treatments in some cases, allergens in tree nuts, wheat, celery, and soybean often exhibit heat stability, remaining unaffected by high temperatures (Dong et al., 2020).

While traditional thermal treatments are beneficial in reducing food allergens, they have also some limitations, impacting negatively the sensory qualities of food, such as nutritional value, color, flavor, and texture (Hogan et al., 2005).

The mechanism for reducing soybean allergenicity is the denaturation of proteins, affecting their structure. The result is a change of conformation into a random coil or unfolded proteins. Also, the epitopes can be destroyed, or new ones can appear through thermal treatment. For example, between 55°C-70°C the secondary structure of the protein is lost, at 70°C-80°C the disulphide bonds are cleaved, new interactions are formed at 80°C-90°C and at 90°C-100°C, aggregates are created (Wal, 2003).

Compared to thermal treatment, non-thermal procedures presented more advantages, not only in terms of organoleptic properties (by having a better profile on flavor, color, texture, smell) and improving antioxidants and phenolics components but also in decreasing the allergenicity of shrimp, peanuts, and soybean (Dong et al., 2020).

Several types of physical treatments are outlined in this chapter by describing two groups. One is related to the pressure, such as extrusion, high hydrostatic pressure (HHP), high-pressure homogenization (HPH), and controlled instantaneous pressure drop (DIC). The second group is presented in the form of waves: gamma irradiation (γ-irradiation), pulsed ultraviolet light (PUV), cold plasma, microwave, and ultrasonication. Although all of these are considered physical treatments, some, such as extrusion, HHP, DIC, or microwave, also involve the application of temperature. This overlap can lead to confusion in the literature, where certain methods may be classified as either thermal or non-thermal depending on the context. Moreover, cold

plasma, is often seen in the literature as either physical treatment or physico-chemical treatment, due to its ability to induce both physical and chemical changes.

Some of these treatments can be used alone or in combination with thermal treatment. However, all these techniques have the same common goal: to reduce soybean allergenicity of the products with an impact on protein structure. Fig. 3-3 shows an overview of the classification of the treatments which can impact soybean allergens.

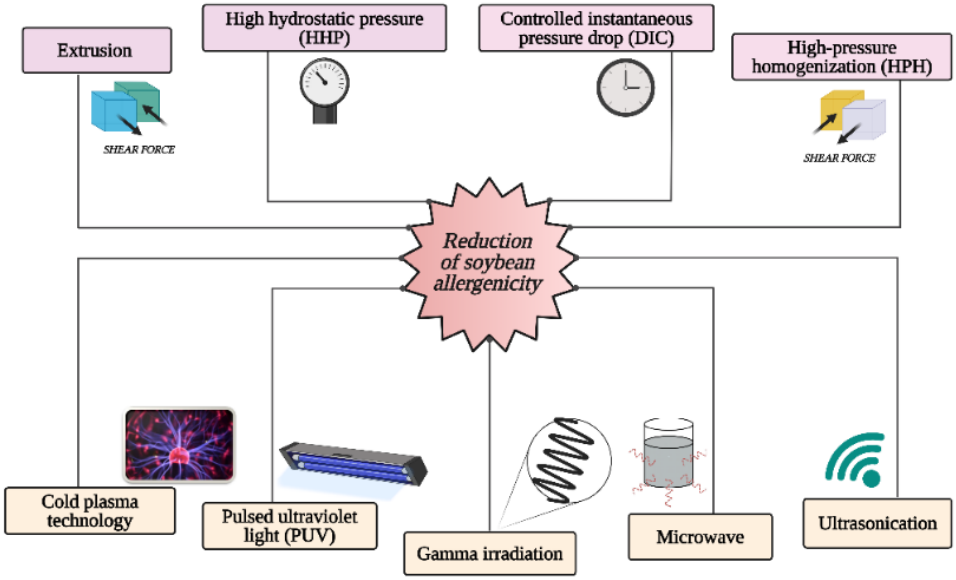


Figure 3-3. Physical treatments used to reduce soybean allergenicity (created with BioRender.com).

The following sections will also describe the treatment history, the principle of the method, its usage, and its effects on soy proteins.

3.4. Physical treatments based on pressure for soy allergy reduction

3.4.1. Extrusion

Extrusion was used in pasta dough for the first time in 1935, followed by animal feed in 1950. In the ‘60, more extensive equipment for extrusion treatment was necessary due to the request for pre-cooked cereals and starches. These machines allowed broadening the spectrum of extruded food, such as snacks, ready-to-eat cereals, infant formula, and dry food for animals (Gray & Chinnaswamy, 1995).

Under high temperature, pressure, and shear forces, the feed material exposed to extrusion melts by changing phase. The mass passes through a die and, in the end, is

bared to the atmospheric conditions. Immediately, the melted feed expands because of the contact with these atmospheric circumstances (Ek & Ganjyal, 2020).

The simultaneous combination of moisture content, external heating, time, pressure, and mechanical shear makes the material porous with an expanded structure. These properties change the material's color, shape, taste, composition, texture, and bulk density (Morantes et al., 2020). Also, a short time and a high temperature induce cooking and a plasticization of the proteins and carbohydrates in the extruder barrel (Moreno et al., 2018; Wilson et al., 2005).

Extrusion impacts soybean allergenicity, particularly in textured soy protein, a vegan meat substitute obtained by extrusion. Parameters such as temperature and pressure can make the ingredients flow by aligning the proteins, followed by expanding and collapsing. In this way, products with a meat-like texture can be obtained (Wilson et al., 2005).

The combination of shear force, temperature, and pressure disintegrates the cell walls, thus affecting the protein structure (Fig. 3-4). Then, an increase in temperature partially breaks disulfide and hydrogen bonds by spreading the spatial structure of the protein and destroying its molecular structure. An additional increase in extrusion temperature breaks the co-ordinate bonds, carbon-nitrogen, and other chemical bonds, destroying the secondary structure. A higher screw rotation and lower feed speed increase the shear force, which affects the epitopes of the antigen, impacting the food allergenicity (Yin et al., 2019; Moreno et al., 2018; Arêas, 2009).

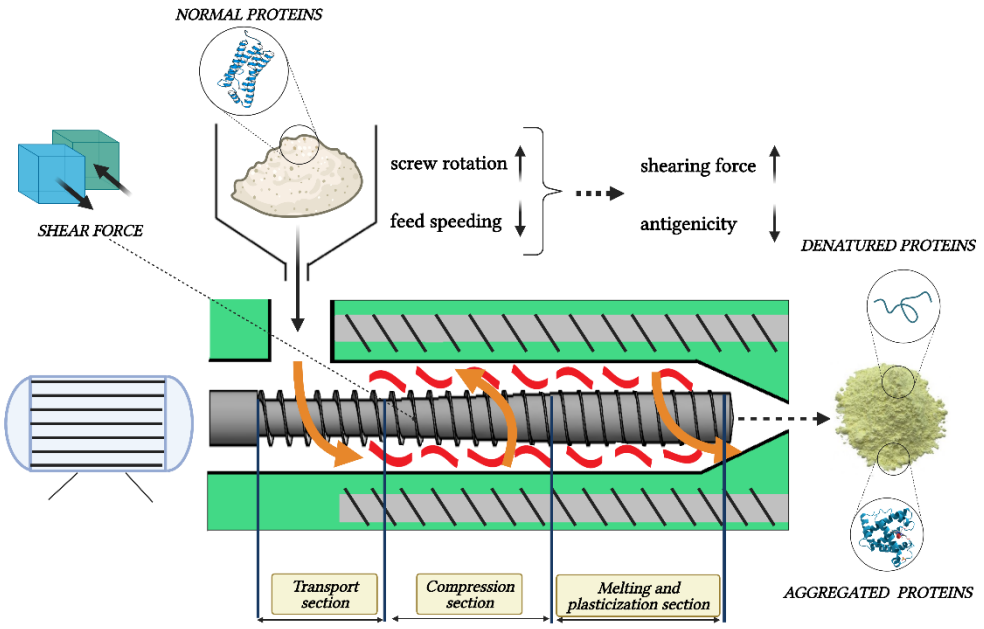


Figure 3-4. The impact of extrusion on proteins (created with BioRender.com).

The aggregation and denaturation of proteins resulting from the thermomechanical effect lead to a decrease in the allergenicity of soy products (Ek & Ganjyal, 2020).

Starting from 1994, Ohishi et al. used a twin-screw extrusion process with a temperature higher than 66°C, which reduced the soybean's antigenicity to 0.1% of the initial value. This decrease was due to the degradation of the molecular structure of proteins (especially fractions with high molecular weight) during the extrusion cooking.

A similar study with a twin-screw extrusion was done by Saitoh et al. in 2000 on milled soybean hypocotyls (~500 μm particle size), where the allergenicity was reduced by 1% of the original activity. A reduction of urease activity and trypsin inhibitor were also observed. The isoflavones were unchanged.

Franck et al. (2002) compared different soybean products and the allergen P34 (Gly m Bd 30K), which can be eliminated through extrusion. Patients sensitive to soybean responded positively to textured soybean proteins with 38 and 50 kDa molecular weight, which can be related to G1 glycinin acidic and basic chains. Also, the 31-34 kDa band did not bind the IgE in the textured product, suggesting that Gly m Bd 30K was removed during the extrusion.

As mentioned earlier, extrusion involves the use of heat, which has caused confusion in the literature, leading to its classification as both a thermal and non-thermal process. In this context, the effect on allergens comes from the combination of shearing, pressure, and temperature. The antigenicity was reported to increase at 100°C. Between 100°C and 120°C, the epitopes were relatively blocked or destroyed by the cross-linking and aggregation of β-conglycinin particles. At 140°C or higher, the antigenicity was lowered by completely breaking the β-conglycinin epitopes and molecular structures without any modifications in the intermolecular connections (Yin et al., 2019).

β-conglycinin antigenicity was only 20.06% under the following optimal extrusion parameters: temperature (130°C), feeding speed (35 g/min), and screw rotation speed (140 rpm). This significant reduction of the allergenicity results from a higher shear force, which can produce depolymerized β-conglycinin polypeptides, breakage of peptide chains, and abolished epitopes (Yin et al., 2019).

The same study presented that an increased water content reduced the antigenicity of soybean (16% water was noted for the lowest antigenicity). The correlation between the last two parameters is related to enhancing viscosity, increasing shearing stress, and reducing antigenicity. Thus, when the temperature increases during extrusion, antigenicity decreases due to protein denaturation (Yin et al., 2019).

In 2020, Zheng et al. reported that the extrusion process decreased allergenic protein activities in two formulas based on corn, one with defatted soybean flour (SF) and the other one with SPI. ELISA technique demonstrated that the extrusion process reduced the immunoreactivities of both SPI-corn (53-68%) and SF-corn products (80%-86%). In addition, extrusion processing decreased the protein solubility of both SPI and SF products by more than 58.7% and 66.3%, respectively, compared to the initial

materials. The products obtained with less than 20% moisture content had a slighter decrease in allergenic proteins.

The temperature conditions impact protein solubility in the extrusion process. A higher temperature leads to a decrease in protein solubility for both products. It was also shown that after the extrusion, the α-helix decreased, and the β-strand structure increased in both formulas. The water hydration capacity of the SPI-corn and SF-corn extrudates was mainly impacted by moisture, which changed from 20% to 40%. As a result, the water hydration capacity was considerably reduced for SPI-corn (from 325.3% to 260.6%) and SF-corn extrudates (from 224.0% to 202.8%). The soy flour and corn formula was less hydrated than the mixture with corn and soybean isolate (Zheng et al., 2020).

In conclusion, it was shown that the extrusion technique combines a mechanical action (shear force) and a thermal effect that induces aggregation and denaturation of the proteins, which lead to the reduction of allergenicity. Further, the product's viscosity is essential because it increases the shearing stress and decreases antigenicity.

3.4.2. High hydrostatic pressure (HHP)

In 1899, Hite was the first researcher to use a hydraulic press to inactivate microorganisms and extend the shelf life for at least one day for milk, compared with untreated milk (Hite, 1899). Starting from there, this technology answered more questions about preserving food by inactivating bacteria.

Later, the effect of pressure on changing the conformational structure was evaluated on some different biomolecules, including proteins. More recently, some scientists had the idea to check the impact on food allergenicity, especially on milk allergens, and broaden the food spectrum (Vanga et al., 2017).

Le Châtelier and isostatic principles are applied in this treatment. It is a discontinuous process used for liquid and solid packed products, with a pressure dispersed continuously, directly, and equally applied in all food directions. Pressurization helps the phenomena (chemical or biochemical reactions, phase transition, and molecular configuration), resulting in reduced volume (Augusto et al., 2018; Muntean et al., 2016).

The pressure applied equally on food products impacts the secondary, tertiary, and quaternary protein structure levels, inducing aggregation (the epitopes can be destroyed or masked) (Rahaman et al., 2016; Jiménez-Saiz et al., 2015).

The primary and secondary bonds suffer an elastic effect (a reversible distortion) under low pressure. Also, the primary bond suffers a compression of the hydrogen bonds, reducing the internal cavity size of proteins. The tertiary structure and intermolecular interactions are destabilized at around 200 MPa. Proteins can unfold at around 500 MPa (depending on the protein type), and the aspect of the structure can be disordered due to pressure (the pressure stabilizes the membranes and disturbs the nucleic acid) (Somkuti & Smeller, 2013).

On the laboratory scale, the equipment chambers can be filled with 0.02-1.5 L, while at the industrial level, the equipment works with volumes up to 320 L by applying pressure up to 1400 MPa (Augusto et al., 2018). In addition, horizontal and vertical installations are used in industrial food applications (Yamamoto, 2017).

Usually, a pressure between 50–1000 MPa is used, with a temperature of between 0°C and 150°C, for a few minutes to hours (Augusto et al., 2018).

The National Advisory Committee on Microbiological Criteria for Foods (NACMCF), United States Department of Agriculture (USDA), and U.S. Food and Drug Administration (FDA) approved the HHP as an alternative to the non-thermal pasteurization (Huang et al., 2014).

No specifications are applied to high-pressure treatment in countries other than the European Union, such as the United States of America. The products obtained through this procedure in the EU fall under the legislation for Novel Foods (Regulation (EU) 2015/2283). Also, the pressure vessels have to follow the Pressure Equipment Directive (PED) regulation (2014/68/EU), which indicates Good Manufacturing Practices (GMP) and security assessment for the maintenance and operation of the vessels (EU, 2015; EU, 2014).

HHP reduces unwanted modifications on the nutritional, physicochemical, and sensory properties of food (Salazar et al., 2021). Also, the sensory characteristics of proteins are modified, and the functional properties of the macromolecules (solubility, gelation, foaming, emulsification, precipitation, hydration, and agglomeration) can vary (Chauvin & Swanson, 2011).

As early as 1996, Omi et al. concluded that soybean seeds immersed in water and pressurized at 300 MPa at 20°C for 25 min showed decreased basic 7S globulin allergenic protein. Ten years later, research conducted on a by-product resulting from the production of tofu (soybean whey) showed a reduction of the allergenicity of the Gly m 1 by applying a treatment of 100-300 MPa for 15 min (Peñas, Préstamo, et al., 2006).

α-helix and β-sheet were damaged with a 10 min treatment at a high pressure of 500 MPa, by changing the conformation into a random coil. Additionally, a complete denaturation of the glycinin was observed when other parameters were applied. In conclusion, it was shown that HHP higher than 300 MPa, caused the denaturation of β-conglycinin, which was fragmented in subunits and other SH groups (Zhang et al., 2009).

It was observed that the allergenicity of germinated soybean treated with high pressure was lowered by using 300 MPa, for 15 min at 40°C in a discontinuous machine. The soybean seeds germinated in darkness for five days, at 20°C. After three days, the antigenicity decreased due to protein degradation, resulting in new peptides (Peñas et al., 2011). Enzymatic hydrolysis occurred during germination, reducing the immunoreactivity because of the increased susceptibility of seed proteins. The obtained nutritional value and the amino acid composition were less affected, the digestibility was higher, and the antigenicity was lower than untreated beans.

However, the HHP treatment did not impact the protein profile or the immunoreactivity of tofu. This clearly shows that this method can lead to variable results depending on the food matrix, its complexity, and the processing parameters. Therefore, further studies should be performed considering different product processing conditions (Peñas et al., 2011).

The addition of NaCl (0.6 mol/L) combined with high pressure at 600 MPa increased the denaturation of the β-conglycinin. At 200 and 400 MPa, the allergen mentioned, and glycinin were protected by the different concentrations of NaCl from high-pressure denaturation (Añón et al., 2011).

The allergenicity of soy protein isolate for infant formula was reduced using HHP, which is a critical application for babies who have allergies to cow milk. The authors noted that SPI treated at 300 MPa for 15 min produced the highest decrease of allergenicity (48.6%) compared to the native SPI (Li et al., 2012).

According to Li et al. (2016), HHP has the highest impact on soybean allergens compared to high-intensity ultrasound (HIU), microwaving, and high-pressure homogenization (HPH). The allergenicity of soybean protein isolate for infant formula was reduced by 46.6% (HHP treatment) at 350 MPa, for 16 min. The allergenicity of the soybean can not be reduced totally, but it was shown that this technique could inactivate some conformational epitopes.

Soy protein isolate was tested at 200, 300, 400, and 500 MPa, with a temperature of 20 ±2°C for 5, 10, 15, and 20 min. It was concluded that pressures ranging between 200 and 400 MPa reduced the antigenicity of β-conglycinin significantly. At 400 MPa for 15 min, the highest decline of antigenicity was 37% (from 92.72% to 55.15%). Additionally, α-helix1 and β-strand1 were reduced significantly, which caused changes in protein structures with an effect on the allergenicity of soybean (Xi & He, 2017).

A recent study from 2018 described that HHP did not result in any visible allergenicity differences with an increase in pressure (300, 400, and 500 MPa) for the soy protein isolates, but the IgE binding dropped with the increase of HHP time. The lowest allergenicity was observed at 300 MPa for 15 min, where the allergenicity of the soy protein isolate for infant formula was reduced by 45.5%. The authors reported that western blotting and mass spectrometry, but not the two-dimensional electrophoresis pattern, produced significant changes in 7S (HHP caused the α and α′ subunits) and 11S globulin (A1 and A1a subunits), hence reducing the allergenicity in infant formula (Li et al., 2018).

Consequently, these treated soy proteins can be utilized as ingredients or sources of peptides with low immunoreactivity in hypoallergenic foods. The decrease or elimination of the protein's antigenicity in soy whey may be due to the combination of the enzymatic hydrolysis and the high-pressure procedure (Lavilla et al., 2020).

In conclusion, besides the ability to partially decontaminate food and keep the organoleptic properties unmodified, it was proved that high pressure produces conformational modifications in soybean proteins, changing some allergen structures and making them less accessible to antibody receptors. The secondary and tertiary

structure of proteins is influenced by the hydrogen, ionic, and hydrophobic bonds affected by the high pressure, decreasing allergenicity, and increasing digestibility (Rahaman et al., 2016).

3.4.3. High-pressure homogenization (HPH)

The milk homogenization machine was patented in 1899 by Auguste Gaulin, whose discovery involved the action of a pressure pump by streaming the milk through some capillary tubes. Then, in 1909, Manton-Gaulin Manufacturing Company launched the first commercial homogenizer. At that time, the process was known as a fixation for milk, as a breakdown of the fat globules was observed, and under the microscope, the view was homogenous and continuous. Later, this observation introduced a new term, homogenization (Osorio-Arias et al., 2021).

Compared with HHP, described before, HPH is a continuous treatment that applies a lower pressure, up to 300 MPa, under shear forces and elongation stress, defined as a mechanical action. Disaggregation and droplets breaking result from passing the sample through a narrow gap (100 μm) with a 200-300 m/s velocity. The main factors that characterize the process are the shear force, turbulence, and hydrodynamic cavitation (Li et al., 2021; Osorio-Arias et al., 2021). This treatment is applied only to fluids (Augusto et al., 2018). Fig. 3-5 represents a schematic overview of HPH.

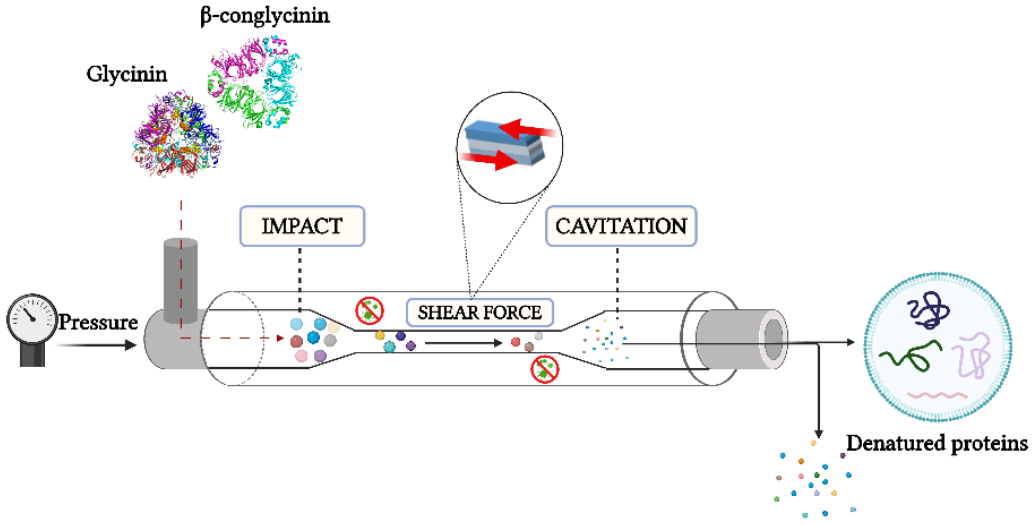


Figure 3-5. The effect of HPH on proteins (created with BioRender.com).

Nowadays, HPH is used to increase product availability by inactivating the microorganisms and inducing a few modifications to the nutritional value and flavor of the treated product. Additionally, it has applications in protein modification, particle size reduction, cell disruption, emulsification, encapsulation, dispersing, and mixing liquids (Tao et al., 2014).

HPH also seems to impact food allergenicity. Cavitation produces a high mass of energy, resulting in high heating values (at each 10 MPa, the temperature increases by 2-3°C) and shear stress. This stress dispersed across the product, and other components (e.g., proteins) can change the spatial structure by deformation and cleavage of protein. High velocity and shear stress can generate free water radicals, modifying protein structure (Augusto et al., 2018; Li et al., 2016).

One study showed this effect, specifically on soybean. In this respect, more studies are available on HHP than HPH. Thus, Li et al. (2016) observed that HPH reduced soybean allergenicity by 29.8%, using a SPI special for infant formula at two steps at 90 MPa, with a homogenization for 1 minute at 10000 rpm.

The mechanical action (shear forces) is responsible for the denaturation or conformation change of the proteins, which reduces the allergenicity of soybeans. Compared with the HHP, which applies the pressure equally on the product, the HPH uses the shear stress tangentially or parallel to the material (Di Stasio & De Cristofaro, 2010).

3.4.4. Controlled instantaneous pressure drop (DIC)

The process of the controlled instantaneous pressure drop (the name in French "Détente Instantané Contrôlée-DIC") was invented in 1988 by the team of Professor Karim Allaf of the University of Technology of Compiègne, France. Decontamination and drying of the food product were the first procedures forming this treatment's basis. Nowadays, DIC has a large spectrum of applications in the food industry and pharmaceutical area. These include steaming, thermal modifications, extraction of volatile and non-volatile components, decaffeination, swell-drying, decontamination, and de-allergenicity (lupin, soybean, lentils, chickpeas, peanuts) (Hamoud-Agha & Allaf, 2020).

Since 2001, DIC treatment was developed on an industrial and pilot scale by the company ABCAR DIC Process (https://www.abcar-dic.com). It is an exciting research topic; the technology is flexible, and powder and solid products are mainly used. At the industrial level, the flow is continuous, and the capacity is 100 kg dry per hour (Abcar DIC Process, 2021).

The product is exposed to steam pressure, up to 0.8 MPa, for a short time of 1-3 min and high temperature, maximum 170°C. It is mainly applied for biological resources and prevents thermal depreciation, producing quality products (Haddad & Allaf, 2007). Although DIC is considered a physical treatment, it is important to note that it also involves the application of temperature, which can lead to confusion about whether it is classified as a thermal or non-thermal treatment.

Food exposure to steam, the short time, and the quick pressure drop (about 0.005 MPa) are responsible for the instantaneous evaporation of water and the destruction of vegetative forms of bacteria. Thus, the treatment has a thermomechanical impact. Additionally, the proteins aggregate, cross-link, and degrade, decreasing allergenicity (Hamoud-Agha & Allaf, 2020).

Also, protein structure can be altered by heat and pressure, by either losing the conformational epitopes or resulting in hidden epitopes. These changes are caused by the damage or reformation of hydrogen bonds and electrostatic and hydrophobic forces. The interactions between disulfide and sulfhydryl bonds are caused by the unfolding of proteins (created under pressure) and produce modifications in their conformation (Boughellout et al., 2013).

Cuadrado et al. (2011) showed a high reduction of the allergenicity of soybean during this treatment. Different pressures and durations were applied (0.3 and 0.6 MPa for 1 and 3 min) for various legumes (peanuts, chickpeas, lentils, and soybeans). A minor decrease of the immunoreactive band was found in the case of soybeans treated for 1 and 3 min at 0.3 MPa. The highest reduction in IgE binding capacity was observed at 0.6 MPa for 3 min. By comparison, the effect created by the DIC treatment is similar to an autoclaving treatment for 30 min at 0.26 MPa.

In a research conducted by Takács et al. (2014), seven spots were found for the control soybean in two-dimensional electrophoresis (2-DE). Two spots were abolished after a 3-minute treatment at 0.3 MPa, and the other became less (24% and 65%) or more intense (156-288%) than the untreated control. The treatment was more effective when applied for an extended time (3 min), at a higher pressure (0.6 MPa), because five spots disappeared and two new ones were present on the gel, but with a lowered intensity (28% and 71%).

In 2007, Haddad & Allaf explained that a 1 and 6 min DIC treatment reduced trypsin inhibitor activity by 94% and 99%, respectively, a new advancement for soybean processing. A variety of soybean with an initial trypsin inhibitor content of 41.6 IU/mg was used that was previously soaked in water and mixed in polyethylene bags at 4°C for a minimum of 12 h.

The combination of steam pressure, high temperature, and short treatment times can reduce the allergenicity of soybeans. This process leads to protein aggregation, cross-linking, degradation, and changes in solubility.

3.5. Physical treatments in the form of waves for soy allergy reduction

3.5.1. Gamma-irradiation (γ-irradiation)

The radiation process is not a new concept. After the discovery of the X-ray, ionizing radiations were proposed for destroying microorganisms in food. The first commercial irradiation for spices (Germany), potatoes, and grain (Soviet Union) occurred in 1958. After a few years, in 2003, the agencies responsible for food safety (International Atomic Energy Agency, Food and Agriculture Organization, World Health Organization) announced that ionized food was safe for consumption without doing any more tests on toxicology. Nowadays, this technique is used for preserving food in more than 60 countries (Bashir et al., 2021).

In the food industry, irradiation serves many purposes. It can be applied in the prevention of foodborne illness, food sterilization, inhibition of sprouting of potatoes

or delaying the ripening of fruits, increasing herbs shelf-life, inactivation of bacteria, and controlling insects (Cătunescu et al., 2019; FDA, 2016).

According to Codex Alimentarius, in "General Standard for Irradiated Foods," the maximum dose permitted for food is 10 kGy, with some exceptions when necessary to use it for a technological reason (Codex Alimentarius, 2003).

Different states in Europe have approved an irradiation dose for the treatment. A few examples are: Belgium (5-10 kGy), Norway, Finland, Germany, Croatia, and Estonia are using 10 kGy, Hungary (2-10 kGy), France (1-10 kGy) and Spain, a more precise dose (8.6-9.5 kGy) (Bashir et al., 2021). The maximum dose for soybean protein isolate in China is 8 kGy (Pi et al., 2021).

The exposure of food products to electron beams, x-rays produced by an electron accelerator, or γ-rays by using a source of radioisotope (cesium 137 or cobalt 60) is called irradiation. 1 to 10 kGy doses are frequently used to cold pasteurize fruits and vegetables. Higher doses between 10 to 50 kGy are employed to sterilize products with a low water activity (i.g. spices). γ-rays irradiation is suitable for manufacturing bulky food-stuffs, while electron beams are suitable for treating surfaces (Chizoba Ekezie et al., 2018; Castagna et al., 2014; Sung et al., 2013).

Besides the preservation of food, some researchers tested the effect of γ-irradiation on the allergenicity of food. Thus, γ-rays have been shown to impact protein structure through reactions such as fragmentation, unfolding, cross-linking, and development of new reactive units, which may decrease allergens (Castagna et al., 2014; Sung et al., 2013).

Moreover, the reactive oxygen species (ROS) and photons break the covalent bond directly or indirectly. Secondary radicals form when ROS react with molecules, but the main denaturation comes after removing the hydrogen from the amino acid side chain, disulfide bonds, and aromatic compounds. Also, if proteins are irradiated in a solution, the allergenicity is reduced compared with proteins dispersed in a dry formulation due to intermolecular aggregation and cross-linking (Chizoba Ekezie et al., 2018).

Using doses between 2.5-30 kGy, the treatment of γ-irradiation on soybeans did not significantly change the intensity of the protein bands of major allergens Gly m 5, Gly m Bd 30 K, Gly m TI, and Gly m 4. Except for a decrease in Gly m TI, irradiation-induced no major modifications (Moriyama et al., 2013).

A minor breakdown of Gly m 5 subunits was observed for irradiation doses between 5-25 kGy. Gly m 6 was reduced at doses higher than 25 kGy and disappeared altogether at 100 kGy. The peptide bond was cleaved at doses of 50 to 100 kGy, allowing the detection of aggregates on SDS-PAGE gels (Meinlschmidt, Ueberham, Lehmann, Reineke, et al., 2016).

Thus, an irradiation dose higher than 25 kGy is required to remove allergens effectively. The legislation allows only absorbed doses below 10 kGy for food products, which is insufficient to degrade the soybean proteins.

To conclude, exposure to the γ-rays causes protein structure changes, namely protein aggregation and conformational changes. Additionally, it was observed that

irradiation has no significant effect on soybean allergenicity at doses allowed for food processing.

3.5.2. Pulsed ultraviolet light (PUV)

The patent for pulsed light used as a sterilization method belongs to Hiramoto (Japan, 1980), who extended it to the United States of America in 1984 (Sterixene, 2018). This discovery is also useful nowadays, being an excellent tool for bacterial inactivation in food manufacturing. Furthermore, the equipment and packaging materials are sterilized using this technique. Starting from 1999, the FDA approved this method for food sterilization (Jan et al., 2017).

Pulsed light (PL) operates with a white light issued as very short and high-power pulses. The light spectrum includes ultraviolet light (54%), infrared light (20%), and visible light (26%) (Shriver et al., 2011).

PL has a higher diffusion potential than UV light, and the pulses with increased energy have better dissipation. A substantial voltage excites xenon (as an inert gas), followed by deexcitation, and then releases photons absorbed by the molecules found in the treated foods. In this way, the products are subjected to several thermal, physical, or chemical modifications (Shriver et al., 2011). Usually, twenty flashes per second are used for food products (Jan et al., 2017).

Because efficient chromophores sustain photoreactions, protein chromophores absorb light at high energy levels. This causes protein modifications to insoluble forms and protein fragmentation through reactions such as oxidation, aggregation, and cross-linking (Chizoba Ekezie et al., 2018).

The heat produced in the product due to the absorption of UV light can contribute to the unfolding of proteins. Also, after the PUV treatment, some residual peptides can re-associate to create protein aggregations. The photothermal, photophysical, and photochemical effects are responsible for the alterations of protein structure, which may reduce allergenicity (Yang et al., 2010; Dong et al., 2020).

In 2010, a study assessed the effect of PUV treatments on soy extracts treated for 2, 4, and 6 min at 13.2 cm from the light source, with energy intensities roughly at 117.6, 235.2, and 352.8 J/cm^2. The study showed that the treatment reduced the IgE binding by about 20%, 44%, and 50% for the 2, 4, and 6 minute-treatment. Treatments of 4 and 6 min had a similar effect on IgE binding. However, 4 min and 235.2 J/cm^2 were optimal because they generated fewer proteins and off-flavors. An increase in time led to aggregation, which could be responsible for the decrease in allergenicity. Thus, further applications can be developed for soybean products and beverages with fewer allergens using the PUV treatment (Yang et al., 2010).

Pulsed ultraviolet light applied at approximately 8 and 10 cm from the food product reduced the immunoreactivity of soy Gly m 5 and Gly m 6. The SDS-PAGE proved

that Gly m 5 and Gly m 6 were no longer visible after 2 and 6 min of treatment, respectively. This phenomenon may be explained by the fact that soy proteins aggregate (by cross-linking) due to exposure to side chains (aliphatic and hydrophobic) (Meinlschmidt, Ueberham, Lehmann, Reineke, et al., 2016).

Furthermore, the same study presented a 91% intense reduction of soy protein immunoreactivity. Modifications of the conformational epitopes were observed for samples treated with PUV for 4 min, at a distance of 8 cm, using the monoclonal antibodies Izimab-Gly m 5-3, Izimab-Gly m 5-4, and Izimab-Gly m 5-5.

On a final note, the PUV treatment causes protein transformation into insoluble forms and fragmentation through oxidation, aggregation, and cross-linking, leading to a decrease in IgE binding. Photophysical, photothermal, and photochemical effects generated by the PUV treatment impact the conformation of soybean allergens.

3.5.3. Cold plasma technology

Originally, cold plasma technology was used to improve the properties of polymers (amelioration of adhesion and printing) and in the electronics field. It is a non-thermal treatment currently used for bacterial inactivation of food materials, food packaging material, and changing the food properties (water absorption, solubility, thermal characteristics, and pasting properties) (Chizoba Ekezie et al., 2017).

When an inert gas is associated with electricity, plasma is produced. The reactive material is charged with photons, different radiations, free radicals, and this formulation represents plasma, the fourth state of matter. The equipment used in this technology contains a power supply (alternating current 60 Hz) and a plasma emitter (a gliding arc system injected with gas). The electrodes are attached to the bottom and top of the system, which operates in the open air. Cold plasma is usually used in the food industry, which operates at 30℃-60℃ (Dey et al., 2016).

A study conducted on the toxicity of edible films treated with cold plasma showed that after 14 days, the subjects did not present any critical toxicity reactions. Male and female rats were administered 1000 mg/kg or 5000 mg/kg/day of edible films. After the ingestion, some modifications in blood appeared, but within typical values (Chizoba Ekezie et al., 2017).

Besides the other applications, cold plasma technology may affect the food allergen reactivity by altering conformational and linear epitopes caused by the generation of insoluble protein aggregates. Furthermore, reactive species may split peptide bonds, and the formed amino acids can be oxidized, impacting the protein integrity (Chizoba Ekezie et al., 2018).

Moreover, gas ionization and excitation produces chemical interactions between the proteins and the reactive species (ions, UV photons, free radicals, excited atoms, electrons) (Bayati et al., 2024). These interactions classified cold plasma as a chemical treatment. Moreover, it is important to mention that cold plasma is often seen in the literature as either a physical or a physico-chemical treatment due to its ability to induce both physical and chemical changes.

The mentioned species can alter the conformation of proteins through different interactions. The cleavage of disulfide bonds and the attack of reactive oxygen species on amino acids through oxidation may reduce allergenicity (Dong et al., 2020; Chizoba Ekezie et al., 2018). Also, a short plasma treatment causes slight oxidation, resulting in the unfolding of proteins and the breaking of peptide bonds. A long-term plasma treatment produces covalent and noncovalent interactions, causing protein aggregation (Zhang et al., 2021).

Meinlschmidt et al. (2016) proved that cold plasma, applied to soy allergens, reduced the visibility of β-conglycinin and glycinin bands and revealed new proteins with 50 kDa molecular weight. A significant reduction (up to 89-100%) in soy protein immunoreactivity was also identified.

A reduction of immunoreactivity between 91-100% was observed when direct cold atmospheric pressure plasma (CAPP) was applied a sinusoidal peak-to-peak voltage at 9 kV_{pp}, 10 kV_{pp}, and 11 kV_{pp} at a frequency of 3000 Hz, for 1-10 min. The operating remote (indirect) CAPP was found to reduce immunoreactivity by 89% after 90 min of treatment, using the monoclonal antibody Izimab-Gly m 5-4. An indirect cold atmospheric pressure plasma is described as microwave-generated plasma, and direct CAPP uses surface dielectric barrier air discharge (SDBD) (Meinlschmidt et al., 2016).

The IgE-binding soybean protein isolate level decreased by almost 75% at 120 Hz for 5 min. At 80 and 120 Hz, the residual allergenicity was reduced slowly with the increasing time of cold atmospheric pressure (Zhang et al., 2021).

In conclusion, cold plasma technology may alter the conformational and linear epitopes caused by the generation of insoluble protein aggregates, which reduces soy allergens.

3.5.4. Microwave

Microwave radiation was discovered as a heating method in 1946. Besides the fact that it was used for a long time for heating, the treatment has many applications nowadays (medical, spectroscopy, electronics, food industry, telecommunications, radar, military, and chemistry). In the food area, the microwave is used for pre-cooking, preheating and reheating, drying potato chips, roasting beans or grains, and so on (Das & Banik, 2021).

The operating principle of the microwave is based on a discontinuous electric field. Due to the rapid alternation of the electric field (2.45 billion times per second), the polar or charged particles of the material subjected to the microwave will rotate back and forth. Also, the repeated movement of the charged particles with the inevitable friction, as well as their collisions, will cause a quick increase in the temperature of the medium, which leads to the heating of the material (Li et al., 2021). A range of $3x10^8$ to $3x10^{11}$ Hz is typical for the frequency applied by electromagnetic waves in the microwave treatment (Ohlsson & Bengtsson, 2001).

The microwave frequency has a particular regulation for preventing interference with other radio waves (Li et al., 2021). Thus, the Industrial and Scientific Medical

(ISM) limited the frequency for food products to $2450x10^6 \pm 50x10^6$ Hz (Ohlsson & Bengtsson, 2001). For the industry, it is usually used at $915x10^6/2450x10^6$ Hz (Li et al., 2021).

The food particles absorb the energy created by microwaves, which produces heating dependent on the product's geometry and water content. This kinetic energy accelerates food protein denaturation by causing conformational modifications, affecting food allergenicity (Vanga et al., 2017; Ohlsson & Bengtsson, 2001). Additionally, protein digestibility increased after microwave treatment, and the secondary structure was altered (losses in β-sheet structures and increases in a random coil) (Li et al., 2021). The same researcher mentioned that conduction heating combined with microwave treatment changed the protein profile by altering the hydrogen and ionic bonds.

A microwave treatment was tested on soybean for 25 min, at 700 W. Only 9 allergic people from a panel of 15 presented a response to the soybean allergen, using the enzyme allergosorbent test (EAST) (Besler et al., 2001).

In the study presented by Li et al. (2016), the antigenicity of SPI for babies was reduced by 24.7% when using a microwave oven heated at the power of 600 W for 10 min. No significant modifications of free SH were reported after the microwave treatment. On the other hand, Guan et al. (2011) mentioned that free SH increases or decreases were possible when employing high-power microwaves, resulting in the breaking of protein isolate disulfide bonds.

Only a few studies have examined the effect of microwaves on soybeans, making this a promising research field. This technique accelerates the denaturation of proteins, impacting the secondary structure.

3.5.5. Ultrasonication

Karl Dussik, a neurologist, studied ultrasound for the first time in 1942 to detect brain tumors. This technology was used more in the following years for medical purposes (Kane et al., 2004). Currently, it is considered an eco-friendly process and has many applications in the food industry. Namely, extraction, drying, emulsification, dehydration of fruits and vegetables, meat tenderization, filtration, inactivation of bacteria, defoaming, and degassing (Zhang et al., 2019; Rahaman et al., 2016). At the industrial level, REUS, a company (www.etsreus.com) from France, developed equipment up to 1000 L (Chemat et al., 2011).

Waves produced by ultrasonic power form gas bubbles in the media, increasing high pressure and temperature, resulting in bubble disruption and shear stress forming in the cavitation zone. The temperature reached can be up to 5500°C and pressure 50 MPa. The waves generate 20000 Hz or more (Jan et al., 2017; Sango et al., 2014). Also, waves applied to the treated material may cause an increase in the chemical and structural transformations. These can be influenced by the temperature, pressure gradient, and additional mechanical fragmentation (Chizoba Ekezie et al., 2018).

The spatial structure changes by modifying the main chain of proteins, with a final impact on physicochemical properties. Some authors state that the hydrophobic effect

produces the conformation change, while hydrogen bonds are affected by cavitation due to the high impact of shear stress. Also, a high-intensity treatment can induce the refolding of proteins (Lin et al., 2021). Also, the secondary structure can be damaged and the disulfide bond restructured (Chizoba Ekezie et al., 2018).

Yang et al. (2015) applied different ultrasound treatments (0 to 300 W) to soybean seeds that germinated at 30°C for 5 days in darkness. At 300 W, the IgE-binding capacity was reduced by about 51.39% in the proteins of sprouted soybeans due to protein disruption or epitope elimination. Additionally, moisture increased from 81.42% to 87.26% was observed in treated sprouts. No significant variations ($p \leq 0.05$) were observed between 0 and 200 W. Therefore, secondary and tertiary protein structures are disrupted by sonication at 300 W and local heat treatment.

In 2016, Li et al. observed a reduction of the antigenicity by 18.9% compared to the untreated soybean protein isolate after applying the ultrasonic treatment at 20000 Hz, 600 W for 15 min.

Furthermore, Zheng et al. (2019) assessed whether ultrasound affected the physicochemical characteristics of the soybean protein isolate for 10 or 25 min at 400 W. In a short time, there were no modifications to the secondary structure of the protein. However, the alteration of secondary and tertiary structure (by increasing the α-helix content and reducing the β-sheet) took place after a treatment of 25 min. Additionally, new aggregates were formed after 10 min. A long time dissociated these new aggregates by reducing the particle size, improving surface hydrophobicity, and increasing solubility. Even if the study did not target allergens, ultrasound impacts the protein structure by altering the secondary and tertiary structures, which may further impact soybean allergenicity.

To conclude, the waves produced by the ultrasonication treatment impact the protein conformation, which is changed by the high intensity and cavitation phenomenon.

To summarize this chapter, Table 3-5 shows a global overview of the physical technologies applied to reduce soy allergens.

Table 3-5. Effect of physical treatments on reduction of soybean allergens.

Treatment	Food formulation	Allergens	Parameters	Observed effect/Allergenicity	References
Physical treatments based on pressure for reduction of soybean allergens					
Extrusion	Defatted soybean meal and other commercial soybean products	Gly m 5 Gly m 6	Twin-screw extrusion T=66℃ to 134℃ Screw speed (20, 40 rpm)	Antigenicity ↓ to 0.1% of the initial value by degradation of protein structures	Ohishi et al. (1994)
	Milled soybean hypocotyls	Gly m 5 (α, α′, β subunits) Gly m 6 (A3)	Twin-screw extrusion Screw speed=280 rpm Feed rate=51.4 kg/h T=72–143°C	Antigenicity ↓ by ~1% of the initial value if soybean hypocotyls are milled ~500 µm (degradation of antigen soybean proteins)	Saitoh et al. (2000)
	Texturized soy protein (Sojatop®)	Gly m Bd 30K	Not mentioned	It might be removed during texturization	Franck et al. (2002)
	Soybean seed	Gly m 5	T=130℃ Feeding speed= 35 g/min Screw speed=140 rpm	Antigenicity of β-conglycinin ↓ to 20.06% (polypeptides depolymerized, the peptide chains damaged, and epitopes abolished)	Yin et al. (2019)
	Soybean seed	Gly m 5	T=100°C and 120°C	Relatively blocked or destroyed epitopes	Yin et al. (2019)
		Gly m 5	T ≥140°C	Lowered antigenicity by a complete breaking of the β-conglycinin epitopes	Yin et al. (2019)
	SPI:corn (1:1, w/w) SF:corn (1.5:1, w/w)	Gly m 5 Gly m 6	T=110°C; 140°C Screw speed (200; 400 rpm) Moisture (20; 40%)	↓ immunoreactivity of SPI-corn and SF-corn (53%–68% and 80%–86%, respectively)	Zheng et al. (2020)

High hydrostatic pressure (HHP)	Soybean seeds	Basic 7S globulin	300 MPa, 25 min	↓ of the allergen	Omi et al. (1996)
	Crude glycinin	Gly m 6	400 MPa, 10 min	Completed glycinin denaturation	Zhang et al. (2003)
		Gly m 6	500 MPa, 10 min	Destroyed α-helix, β-structure and changed into a random coil	Zhang et al. (2003)
	Soybean whey	Gly m 1	100, 200, 300 MPa	Antigenicity was not detected for the sample under 300 MPa (denaturation and dissociation of some proteins)	Peñas et al. (2006)
	Soybean whey	Gly m 1	100-300 MPa, 15 min	↓ immunoreactivity (protein denaturation)	Peñas et al. (2006)
	Soybean	Gly m 5	≥300 MPa	β-conglycinin might be denatured with fragmentation into subunits	Zhang et al. (2009)
	SPI	Gly m 5 Gly m 6	200, 400, 600 MPa + 0.2-0.6 mol/L NaCl	Glycinin protected by NaCl against denaturation	Añón et al. (2011)
			200 and 400 MPa + 0.2-0.6 mol/L NaCl	β-conglycinin protected by NaCl against denaturation	Añón et al. (2011)
			600 MPa + 0.6 mol/L NaCl	β -conglycinin denatured	Añón et al. (2011)
	Soybean sprouts	Gly m 5 Gly m 6	300 MPa, 15 min, 40°C	Lower effect of IgE immunoreactivity	Peñas et al. (2011)
	Commercial tofu	Gly m 5 Gly m 6	300 MPa, 15 min, 40°C	No impact on the immunoreactivity	Peñas et al. (2011)
	SPI (infant formula)	Gly m 5 Gly m 6	300 MPa, 15 min	↓ allergenicity by 48.6% (changing in allergen structure)	Li et al. (2012)

	SPI (infant formula)	P34, LOX, 130 kDa, 115 kDa, 30 kDa, 28 kDa, α and γ subunit of β-conglycinin, Gly m 6 (Chain A and B)	350 MPa, 16 min	↓ allergenicity by 46.6% (conformational epitopes inactivated)	Li et al. (2016)
	SPI	Gly m 5	400 MPa, 15 min	↓ allergenicity by 37% (α-helix1 and β-strand1 reduced)	Xi & He (2017)
	SPI	Globulins 7S (α and α′ subunits) 11S (A1 and A1a subunits),	300 MPa, 15 min	↓ allergenicity by 45.5% (alteration of subunits protein)	Li et al. (2018)
High-pressure homogenization (HPH)	SPI (infant formula)	P34, LOX, 130 kDa, 115 kDa, 30 kDa, 28 kDa, β-conglycinin (α γ subunit), Gly m 6 (chain A and B)	90 MPa in two steps	↓ allergenicity by 29.8% (effect on protein conformation)	Li et al. (2016)
Controlled instantaneous pressure drop (DIC)	Soybean seeds	Not mentioned	1 and 3 min, 0.3 MPa	Minor decrease of the immunoreactive band from soybean	Cuadrado et al. (2011)
			3 min, 0.6 MPa	Higher ↓ of immunoreactivity (degradation of proteins)	Cuadrado et al. (2011)
	Soybean seeds	Not mentioned	3 min, 0.3 MPa	2 IgE spots from 7 eliminated on the immunoblot	Takács et al. (2014)

			3 min, 0.6 MPa	5 IgE spots disappeared from immunoblot and 2 small persisted noticeable on the gel	Takács et al. (2014)
Physical treatments in the form of waves for reduction of soybean allergens					
Gamma-irradiation (γ-irradiation)	Soybean	Gly m 5 Gly m Bd 30K Gly m 4	30 kGy	No major modifications	Moriyama et al. (2013)
	SPI	Gly m 5	5-25 kGy	Minor breakdown of Gly m 5 subunits	Meinlschmidt et al. (2016)
		Gly m 6	> 25 kGy	Gly m 6 reduced	Meinlschmidt et al. (2016)
			100 kGy	Gly m 6 vanished (cleavage of peptide bond)	Meinlschmidt et al. (2016)
Pulsed ultraviolet light (PUV)	Soybean extract	Gly m 5 Gly m 6	PUV at 2 min, 13.2 cm distance, 117.6 J/cm^2 energy intensity	↓ immunoreactivity by 20% (protein aggregation)	Yang et al. (2010)
			PUV at 4 min, 13.2 cm distance, 235.2 J/cm^2 energy intensity	↓ immunoreactivity by 44% (protein aggregation)	Yang et al. (2010)
			PUV at 6 min, 13.2 cm distance, 352.8 J/cm^2 energy intensity	↓ immunoreactivity by 50% (protein aggregation)	Yang et al. (2010)
	Untoasted soybean/SPI	Gly m 5	1-6 min, distance 10 cm	Gly m 5 not detected after 2 min	Meinlschmidt et al. (2016)
		Gly m 6	1-6 min, distance 10 cm	Gly m 6 vanished after 6 min	Meinlschmidt et al. (2016)
			4 min, distance 8 cm	↓ immunoreactivity up to 91% (soy proteins aggregation and crosslinking)	Meinlschmidt et al. (2016)

Cold plasma technology	Untoasted soybean/SPI	Gly m 5	Direct cold atmospheric pressure plasma (9 kv_{pp} -11 kv_{pp}) for 1-10 min, frequency of 3000 Hz	↓ immunoreactivity 91-100% (protein denaturation)	Meinlschmidt et al. (2016)
			Remote cold atmospheric pressure plasma, 90 min, using the monoclonal antibody Izimab-Gly m 5-4	↓ immunoreactivity by 89% (protein denaturation)	Meinlschmidt et al. (2016)
	Commercial SPI	Gly m 5 Gly m 6	120 Hz, 5 min	The level of IgE-binding SPI diminished by 75% (changing the conformational structure)	Zhang et al. (2021)
Microwave	SPI (infant formula)	P34, LOX, 130 kDa, 115 kDa, 30 kDa, 28 kDa, Gly m 5 (α, γ subunits), Gly m 6 (chain A, B)	600 W, for 10 min microwave oven	↓ antigenicity by 24.7% (protein denaturation)	Li et al. (2016)
Ultrasonication	Soybean sprouts	Main soybean allergens during germination	0–300 W	IgE-binding ↓ by about 51.39% (protein disruption/epitopes elimination)	Yang et al. (2015)
	SPI (infant formula)	P34, LOX,130 kDa, 115 kDa, 30 kDa, 28 kDa, Gly m 5 (α and γ subunit), Gly m 6 (chain A and B)	20000 Hz, 600 W, 15 min	↓ antigenicity by 18.9% (protein denaturation)	Li et al. (2016)

3.6. Benefits and drawbacks of physical treatments

After identifying all the physical treatments that impact soybean allergenicity, we compared all proposed techniques to see which may be the best or the worst for reducing soy allergens. In this way, we summarized the advantages and limitations of those techniques in Table 3-6.

Cold plasma, γ-irradiation at high intensities, and PUV seem the most efficient in reducing soy allergenicity. Cold plasma reduces immunoreactivity of Gly m 5 up to nearly 100% by using mouse monoclonal anti-Gly m 5 antibodies (Meinlschmidt, Ueberham, Lehmann, Reineke, et al., 2016). A similar level of reduction is found in γ-irradiation at 100 kGy for the Gly m 6. Unfortunately, the legislation allows only absorbed doses below 10 kGy for food products. At this level, allergens are not removed effectively (Chizoba Ekezie et al., 2018). PUV treatment seems to cause protein crosslinking and aggregation or alter the allergen conformation (for the Gly m 6, a reduction up to 91% of immunoreactivity was observed for samples treated at 8 cm distance for 4 min) (Meinlschmidt, Ueberham, Lehmann, Reineke, et al., 2016). However, extended exposure to PUV can be harmful to industrial workers. Regarding future perspectives, these technologies are also quite expensive to upscale to an industrial level, with limited productivity.

DIC and extrusion present a good potential to reduce the allergenicity of soybeans (> 70%). Nevertheless, at this moment, allergen removal by DIC is at a research level, not an industrial application. Additionally, an 80-86% decrease of β-conglycinin and glycinin was observed with the extrusion technique for a combination of soybean protein isolate with corn flour. It might be considered a potential treatment in terms of high productivity, low cost of equipment, and improved protein digestibility. In terms of bioaccessibility, some components might be affected due to the temperature involved (> 200°C). However, regarding all advantages, extrusion seems to be today the most convenient technology to reduce soy allergenicity.

Further, HHP and ultrasonication express moderate effects (about 50%) regarding allergenicity reduction. HHP seems to be the most studied treatment. This method better preserves the nutritional and sensory characteristics of the product. It can be used for liquids and solids, but some disadvantages, such as the discontinuous process and the high cost of the pressure vessel, must be considered. Ultrasonication has a similar effect on allergenicity but is still uncertain about reducing the allergenicity to an industrial level, as is the case for other physical treatments.

Finally, HPH and microwave were shown to have less potential (allergenicity reduction < 30%). However, these technologies could have an interest if combined with other technologies. In this way, some studies showed that a combination of different techniques (particularly fermentation and enzyme in combination with others) might be optimal to reduce the level of allergenicity because, at this moment, no treatment alone can reduce 100% all the allergens (Dong et al., 2020; Pi et al., 2021; Chizoba Ekezie et al., 2018; Rahaman et al., 2016; Pi et al., 2019).

Table 3-6. The advantages and limitations of physical treatments.

Treatment	Advantages	Limitations	Future perspectives	References
Physical treatments based on pressure for reduction of soybean allergens				
Extrusion	•Good reduction of allergenicity (higher than 70%) •Protein digestibility improved (by protein denaturation) •Trypsin inhibitors, hemagglutinins, undesirable enzymes destructed •Natural food colors and flavors maintained •Continuous process and high productivity •Low cost	• The Maillard reaction can reduce protein nutritional values •The stability of vitamins A and E is affected •Several heat-labile vitamins and amino acids are lost •Carotenoids and isoflavones are affected •Temperature > 200°C affects the nutritional quality of the product	•The high potential process to reduce soy allergenicity, but the relationship between nutritional and sensory aspects of the product and the interaction between nutrient retention and extrusion conditions must be considered	(Moreno et al., 2018; Navale et al., 2016; Arêas, 2009)
High hydrostatic pressure (HHP)	•Preservation of intrinsic and natural properties of foods •Inactivation of microorganisms •Minimal thermal exposure and reduced processing time •Suitable for liquid and solid packed products •Novel food development •Food safety improvement of SPI in the manufacture of baby food •Positive feedback from the consumer as a physical and environmentally friendly process	•Moderate (about 50%) reduction of allergenicity •Oxidation of vitamins (vitamin C) and colorants •Discontinuous process •Limited throughput for fluid products •High cost of pressure vessel (0.5 to 4 million €)	•Moderate effect on the reduction of food allergenicity •Further studies are needed to assess the effects of food allergens on characteristics like functionality, structure, and digestibility, as well as their clinical implications for developing foods that ensure true immunological tolerance and address hypersensitivity	(Augusto et al., 2018; Muntean et al., 2016; Balasubramaniam et al., 2015; Huang et al., 2014; Lavilla et al., 2020)

High-pressure homogenization (HPH)	•Low-temperature process •Preservation of protein's nutritional values •Inactivation of microorganisms •Continuous process	•Weak reduction of allergenicity (< 30%) • Pressure/shearing at 200-300 MPa reduces vitamin C and affects bioactive component preservation •Decrease the total tocopherol content (soymilk) at 200-300 MPa •Cannot be used alone to guarantee the reduction of allergenicity (complementary technology is needed) •High cost of maintenance •Increased lipid oxidation	•This technology has a limited effect on reducing soy allergenicity when used alone and may only be beneficial when combined with other processes	(Osorio-Arias et al., 2021; Augusto et al., 2018; Hogan et al., 2005; Chauhan et al., 2018)
Controlled instantaneous pressure drop (DIC)	•Good reduction of soy allergenicity (> 70%) at a pressure > 0.6 MPa •Higher sensory properties (flavor, color, texture) •Inactivation of microorganisms •Meets the highest requirements in terms of food safety •Few thermal degradations •Quick processing time •Environmental preservation	•High cost of the technology •The early state of use at the industrial level	•DIC seems to have a good impact on soy allergenicity •Nevertheless, *in vitro* tests and *in vivo* clinical data are needed to check the impact of DIC on the allergenicity of different legumes (soybean, lupine, chickpea, peanut, lentil) •Few possibilities, at present, to process large volumes of production	(Hamoud-Agha & Allaf, 2020; Burbano & Cuadrado, 2014)

Physical treatments in the form of waves for reduction of soybean allergens				
Gamma-irradiation (γ-irradiation)	•Excellent reduction at a higher dose (100 kGy) (> 90%) •Inactivation of microorganisms •Successful for different products •Availability of different sources (γ-rays, electron beam)	•Weak reduction of allergenicity at a lower dose (25 kGy) (< 30%) •Long exposure to radiation can be harmful to industry workers •Legislative limitation – maximum permitted dose for food is 10 kGy •Limited consumer acceptability	•γ radiation allows for reducing allergenicity effectively at high intensities. The level of energy is, unfortunately, 10 times superior to what is authorized by the legislation •Low intensity treatment reveals a low impact on allergenicity •Further risk assessment studies on irradiated food must also be performed	(Meinlschmidt et al., 2016; Chauhan et al., 2018; Moriyama et al., 2013)
Pulsed ultraviolet light (PUV)	•Excellent reduction of allergenicity (> 90%) •Continuous treatments •No chemicals are used •Non-heat process •Inactivation of microorganisms •Few impacts on flavor, color, aroma •Few impacts on the environment	•The investment cost is very high (300000-800000 €) •Long exposure to the radiation can be harmful to industry workers	•High potential technology to reduce soy allergenicity, but at a very high cost •Consequently, more research is required on the optimization of UV light use	(Meinlschmidt et al., 2016; Chauhan et al., 2018)
Microwave	•Uniform heating of products •No solvent is needed •Energy saving •Shorter time of equipment starting and duration of the process	•Weak reduction of allergenicity (< 30%) •May impact appearance, aroma, color, texture, and reconstitution capacity of dried herbs •Expensive equipment •Unsuitable for upscaling (large scale)	•Studies show that microwave has a limited impact on soy allergenicity •The treatment could also affect the product, and high productivity is not particularly easy to achieve	(Brewer, 2005; Das & Banik, 2021)

Ultrasonication	•Faster mass and energy transfer •Inactivation of microorganisms •Less environmental impact •Small batch continuous equipment available	•The reduction of allergenicity is moderate (about 50%) •Off-flavors could appear after treatment •Impact on food properties at high power intensity (flavor, color, pH, antioxidant capacity, cloudiness) •Technology scale-up needed due to low production volume	•Ultrasonication shows a moderate effect on the reduction of soy allergenicity •Scale-up research on energy-efficient ultrasound processors for economic feasibility	(Ojha et al., 2018; Li et al., 2021; Chemat et al., 2011)

3.7. Conclusions

All of the treatments reviewed hereby were found to reduce soy allergenicity at different levels. In this way, PUV, γ-irradiation at a high level, cold plasma, extrusion, and DIC have a good potential (reducing allergenicity > 70%). The effect of ultrasonication, microwave, HPH, and HHP is much more moderate. As mentioned in this chapter, it is important to notice that although all of these are considered physical treatments, some, such as extrusion, HHP, DIC, or microwave, also involve the application of temperature. Regarding proteins, the most identified modifications are denaturation, inactivation of conformational epitopes, degradation of structures, alteration of protein subunits, aggregation, and cross-linking. Besides the potential use of these technologies to lower allergenicity, physical treatments better impact the product, nutritional and organoleptic characteristics. These advantages can then increase the shelf-life and added value of the product compared to the conventional methods.

However, some challenges and limitations must be addressed for all technologies before replacing conventional methods entirely. For example, the minimal effect on nutritional and sensory characteristics by HHP, the presence of off-flavors produced after ultrasonication, the negative impact of extrusion on carotenoids and isoflavones, the decrease in the bioaccessibility of different compounds for HPH, the decrease in protein digestibility when employing cold plasma, and the negative effect on product texture produced by microwave processing. Besides product challenges, implementation, health, and production challenges must also be addressed. Such are the cases of PUV and γ-irradiation where workers and consumer health could be impacted. Therefore, manufacturers and governments need to construct and follow regulations that aim to reduce their risks while maximizing their advantages (Moreno et al., 2018; Augusto et al., 2018; Zhang et al., 2021; Meinlschmidt et al., 2016; Ojha et al., 2018).

To drive forward the aforementioned physical treatments for soybean processing, it is crucial to understand the relationship of allergens with the immune system at a molecular level. This insight will help us understand the complexity of soybean allergenicity since it may increase, decrease, or persist unchanged depending on several method parameters such as intensity, time, temperature, pressure, and mechanical force. Therefore, scientists and engineers could have more tools for the design and selection of appropriate process conditions to improve the efficiency of the reduction of immunoreactivity in soybean products.

This part of the literature review hopes to bring more attention to the field by compiling the most recent advances in physical treatments focusing on soybean allergen reduction. Even if thermal processing was the first treatment applied to this intent, promising alternative technologies such as extrusion, high hydrostatic pressure, ultrasonication, controlled instantaneous pressure drop, cold plasma, pulsed ultraviolet light, and gamma-irradiation will have relevant and exciting applications in the food industry in the coming years.

Moreover, in this chapter, we described HPH, but recently, there has been an interest in another treatment, which is very close, dynamic high-pressure microfluidization (DHPM). Due to the novelty of this technique in soybean allergen research, it will be presented separately in Chapter 4 to highlight its unique contributions.

4

Modification of proteins using microfluidization

Description of Chapter 4

This chapter will explore the effects of dynamic high-pressure microfluidization (DHPM) on proteins and their allergenicity. Additionally, it will provide an overview of the treatment process, discuss various applications of microfluidization in the food industry, and compare DHPM with high-pressure homogenization (HPH).

Chapter 4. Modification of proteins using microfluidization

4.1. Introduction

In the previous chapter, we discussed high-pressure homogenization (HPH), but another technique that has recently gained attention is microfluidization. While similar to HPH, microfluidization has distinct features, which will be explored in this section.

This treatment, often named "dynamic high-pressure microfluidization (DHPM)" or "high-pressure microfluidization," is an environment-friendly physical treatment and emerging technology. It transports the fluids through microchannels of fixed geometry, which are exposed to intense shear forces, high velocity, high-frequency vibrations, cavitation, and oscillation (Sahil et al., 2022; Ozturk & Turasan, 2021; Guo et al., 2020). This unique combination of forces makes the products more homogeneous than conventional methods. Breakdown and homogenization occur primarily in the interaction chamber, driven by inertial forces in turbulent flow and cavitation. The Reynolds number, as illustrated in the following equation, indicates the presence of turbulent flow (Kavinila et al., 2023).

$$Re = \frac{\rho U D}{\mu}$$

Where ρ and U represent the liquid's density and velocity, D is the microchannel's diameter, and μ is the dynamic viscosity.

Different interaction chamber geometries, such as Y and Z types, are used depending on the target application. The Y-type chamber is typically for liquid-liquid dispersion processing (emulsions and liposomes), while the Z-type is for solid-liquid dispersions (the formation of nano-dispersions and disruption of solid structures) (Ozturk & Turasan, 2021). In the Y-chamber, the pressurized liquid flow divides into two micro-channels that converge to create the necessary forces. In the Z-type, these forces are generated by the pressurized liquid flowing through micro-tubes in the shape of Z (Ozturk & Turasan, 2021).

The choice of chamber type (Z or Y type) depends on the desired outcome of the process. The operating pressure determines the force applied to the product within the interaction chamber, which is crucial for achieving particle size reduction and the desired changes. Multiple cycles through the chamber can be employed at similar conditions to achieve the desired particle size reduction (Sethi et al., 2022). However, increasing the number of processing cycles can lead to a rise in product temperature. A pressure increase of approximately 6.9 MPa is estimated to result in a 1.7°C temperature rise during the 1-5 millisecond residence time in the interaction chamber (applied to water) (Microfluidics, 2014). The temperature increase resulting from the process will vary depending on the material being processed. This rise in temperature

occurs almost instantaneously but can be rapidly reduced if necessary (Microfluidics, 2014). Fig. 4-1 shows an overview of the two chambers.

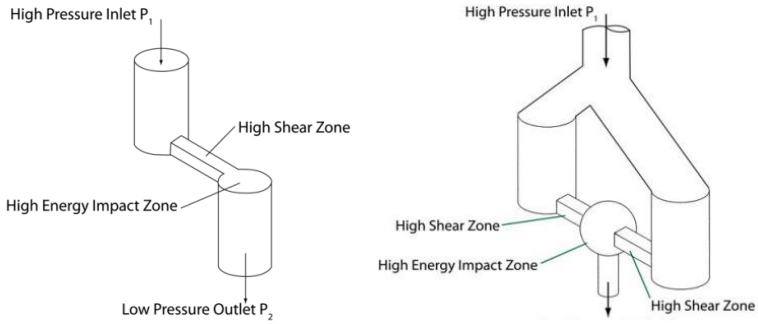


Figure 4-1. Flow path of Z (left) and Y (right) type interaction chambers of microfluidization system (Microfluidics, 2014).

To control temperature for heat-sensitive products, the product can be circulated through a cooling coil between cycles. Also, pressures can reach up to 200 MPa, and velocities can go up to 400 m/s due to the very small size of the channels (usually in µm) (Sahil et al., 2022).

Figure 4-2 provides an overview of the high-shear treatment mechanism adapted for our experiment. Microfluidizer Processor M-110EH from Microfluidics International Corporation was used, which consists of two Z chambers with sizes of 200 µm and 100 µm. Additionally, the figure illustrates the system's recirculation capability, as indicated by the arrows.

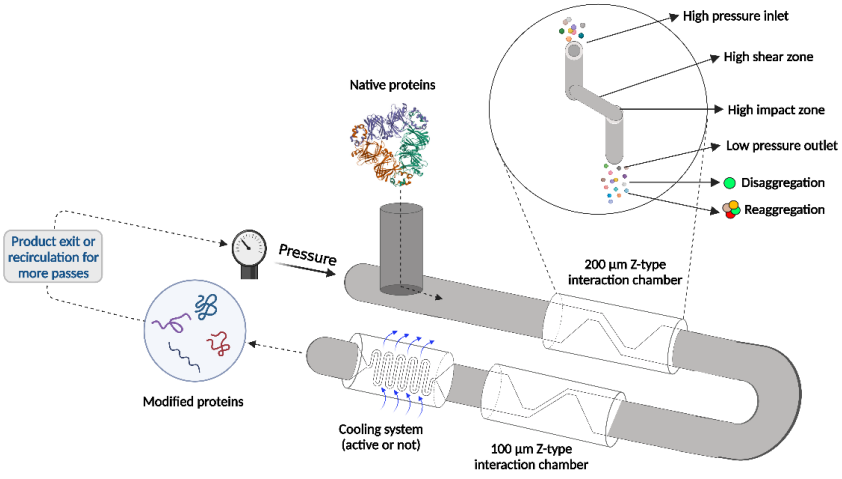


Figure 4-2. The schematic representation of the Z interaction chamber (created with BioRender.com).

Moreover, the shape of the geometrical chamber (Z or Y type), diameter, number of passes, temperature, pressure, and the nature of the treated sample are the most essential treatment conditions to have an efficient process (Li et al., 2022).

As mentioned in the introduction, high-pressure homogenization (HPH) and microfluidization operate on similar principles. To conclude, the following table will outline the key similarities and differences between these techniques, highlighting the most relevant information (Sahil et al., 2022; Osorio-Arias et al., 2021; Guo et al., 2020; Tobin et al., 2015; Microfluidics, 2014).

Table 4-1. Similarities and differences between HPH and DHPM.

Parameters	*High-pressure homogenization (**HPH**)*	*High-pressure microfluidization (**DHPM**)*
Pressure	Up to 300-400 MPa	Up to 200 MPa
Velocity	200-300 m/s	400 m/s
Valve/ interaction chamber	Microchannel valve Jet to wall (Z-chamber) Jet to jet (Y-chamber) Multi-slotted Y-chamber	Geometric interaction chamber (Y and Z)
Process	Continuous	Continuous
Volume stream	<10-20, 50–160 L/h Industrial up to 5000 L/h	7.2, 27, 30 L/h Industrial 4.998 L/h
The increase in temperature with pressure	0.15–0.20°C/MPa	~0.24°C/MPa
Cooling system	Yes	Yes
Products	Fluids	Fluids
Mechanical effects	Cavitation High shear Turbulence	High velocity High frequency vibration Instantaneous pressure drop Intense shear Oscillation Cavitation
Applications	Protein modification Particle size reduction Cell disruption Emulsification Encapsulation Dispersing Mixing liquids	Protein modification Emulsification (food industry, pharmaceutical, cosmetics) Homogenization (milk) Microbial inactivation

4.2. Applications of microfluidization in the food industry

Microfluidization can be used in various applications in the food industry, including emulsion and suspension stabilization, encapsulation, bioactive compound extraction enhancement, particle size reduction, and enhancement of food products' physicochemical properties and sensory qualities (Fig. 4-3) (Y. Li et al., 2022; Kumar et al., 2022; Ozturk & Turasan, 2021). Microfluidization can modify macromolecules such as protein, starch, dietary fiber, and non-starch polysaccharides (Guo et al., 2020a). It also plays a role in inactivating microorganisms and enzymes in food products (Y. Li et al., 2022; Kumar et al., 2022; Ozturk & Turasan, 2021). Figure 4-3 illustrates the effects of microfluidization on various biomacromolecules and highlights its potential applications in the food industry.

For instance, the total plate count in sugarcane juice decreased significantly from 2.24×10^6 CFU/mL (68–69 MPa for 1 pass) to 148 CFU/mL following treatment at 206–207 MPa for 3 passes (Kohli et al., 2019). Additionally, microfluidization as a pre-treatment method enhanced the recovery efficiency of soluble fiber from cellulase-hydrolyzed peach pomace after 4 passes at 140 MPa (Xu et al., 2015). Moreover, vitamin C nanoliposomes, prepared using film evaporation and microfluidization, exhibited enhanced antioxidant activity, storage stability (37°C for 24 hours and 4°C for 60 days), sustained drug release, and skin penetration compared to conventional Vitamin C liposomes without losing biological activity (S. Yang et al., 2012).

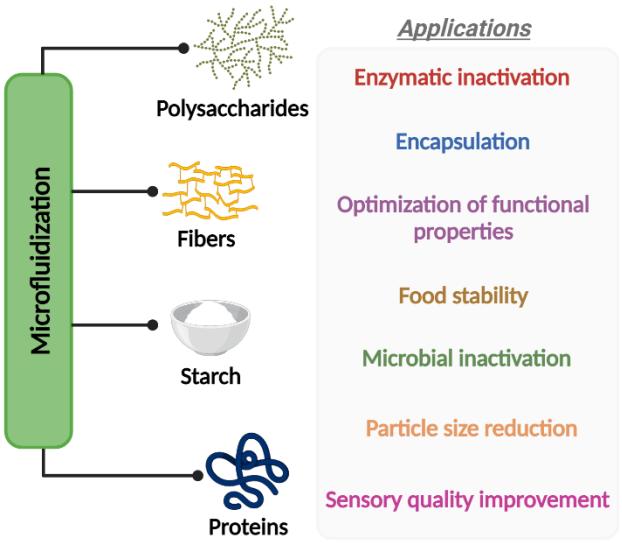


Figure 4-3. Impact of microfluidization on biomacromolecules and different applications in the food industry (created with BioRender.com).

4.3. Protein structure and allergen modification through microfluidization

4.3.1. Impact of microfluidization on protein structure

In the context of proteins, microfluidization disrupts protein aggregates, enhances solubility, and modifies protein structures (Guo et al., 2020). Regarding the functionality of proteins, different researchers studied the impact of microfluidization on different protein structures. For instance, SPI was treated at 120 MPa for 3 passes, and an increase in surface hydrophobicity, solubility, and disulfide bonds were observed. Also, stability against creaming and emulsifying efficiency were improved. Related to protein structure, this treatment disrupted the insoluble aggregates into small solubles, unfolded and denatured the proteins (Shen & Tang, 2012).

Research on edible birds' nests recently revealed that water-insoluble protein fraction experienced partial solubilization (26–27%) following microfluidization treatment at 120 MPa. This increase in solubility could be linked not only to the reduction in particle size but also to the modifications in the protein's secondary structure (increase in α-helix 11.63% (0 MPa) to 13.43% (120 MPa) (Chok et al., 2021).

Microfluidization can also be applied to other proteins, resulting in a modified structure for peanut protein isolate, ovalbumin, β-lactoglobulin, pea globulin, potato protein isolate, and more. For example, significant changes were observed in the solubility (increasing from 30% to 59%) and foaming properties (20% to 65%), with increasing pressure (from 40 to 160 MPa) on whey protein (Liu et al., 2011). A recent review (Ozturk & Turasan, 2021) described the latest developments in the applications of microfluidization with an overview of research articles using this technique on protein structure.

4.3.2. Impact of microfluidization on food allergens

Concerning allergens, this technique has been tested mostly for peanut or milk proteins (H. Chen et al., 2019; X. Hu et al., 2011). In this way, it was shown that the high shear treatment reduced the immunoreactivity of the Ara h 2 (the main allergen in peanuts) due to conformational changes in the allergen, with the increasing pressure from 90 MPa to 120 MPa (C. qiu Hu et al., 2011). Chen et al., 2019 also demonstrated that the antigenicity of β-lactoglobulin after gastrointestinal digestion *in vitro* decreased when the proteins were pretreated by microfluidization. This decrease was more significant with increased pressure applied during treatment (from 0.1 to 160 MPa).

Other researchers obtained similar results for β-lactoglobulin if the pressure exceeded 80 MPa. On the other hand, the same study showed that a pressure of up to 80 MPa increases the antigenicity of β-lactoglobulin (J. Zhong et al., 2011). This

increase might be attributed to structural modifications (protein unfolding or disaggregation) resulting from high-shearing treatment, leading to exposure of previously masked epitopes (Ozturk & Turasan, 2021; H. Chen et al., 2019; Zhong et al., 2011).

Another example is, following a 60 MPa microfluidization treatment, Ara h 2 experienced changes in the secondary structure. The percentages of α-helices and β-turns notably decreased, while the percentage of β-sheets increased, all correlated with a decrease in the antigenicity of peanuts (C. Qiu Hu et al., 2011).

As observed, the impact of microfluidization on soy protein structure and the allergenicity of other proteins has been demonstrated. However, there is no specific information available on how this treatment affects soybean allergens. For this reason, these aspects were discussed separately in this chapter.

PART TWO

Experimental work

Chapter 5

Impact of microfluidization on soy protein structure in powder and solution

Chapter 6

Effect of microfluidization, enzymatic hydrolysis, and their combination on soy allergens

5

Impact of microfluidization on soy protein structure in powder and solution

Description of Chapter 5

Following the discussion of the relation between protein structure and allergenicity in earlier chapters, this chapter presents preliminary results on the effect of microfluidization on soybean protein structure. These studies will help identify suitable samples for testing allergenicity and provide insights into the impact of microfluidization on primary, secondary, and tertiary protein structures under various processing conditions, including different cycle numbers and heat induced by the process itself. Understanding the behavior of soybean proteins under these conditions is crucial for optimizing the microfluidization process and its potential applications in reducing allergenicity.

This work is an original contribution adapted from Kerezsi, A. D., Lelia Pop, O., Othmeni, I., Figula, A., Francis, F., Karamoko, G., Karoui, R., & Blecker, C. (2024)., "Impact of pilot-scale microfluidization on soybean protein structure in powder and solution." This paper was published in *Food Research International, 188, 114466*, on 3 May 2024 (https://doi.org/10.1016/J.FOODRES.2024.114466).

Chapter 5. Impact of pilot-scale microfluidization on soy protein structure in powder and solution

5.1. Introduction

In recent years, researchers and consumers have shifted their attention more to plant-based proteins (Islam et al., 2023; Rodrigues et al., 2012), driven by their multiple health benefits and extensive utility within the food industry (Sui et al., 2021), economy and sustainability concerns (Aschemann-Witzel et al., 2021; Santo et al., 2020; De Boer & Aiking, 2011).

Among plant-based proteins, soybean (*Glycine max*) is well recognized for oil extraction and represents more than 50% of oil production (Rodrigues et al., 2012). Besides this, the by-products obtained are valorized. They are also very important for human health (reduction of cholesterol and cardiovascular disease, improvement of bone health) due to the amino acid profile and high quality of proteins (Qin et al., 2022).

Progress in the food industry has led to the development of a range of soybean products such as flour, defatted meal, concentrates, isolates, and texturized products (Jideani, 2011). As a result of multiple varieties, soybean product consumption has increased in the past years. Soybean protein isolate is one of the most discussed soybean ingredients obtained by isoelectric precipitation (Lee et al., 2016; Jideani, 2011) and contains 90% or more protein (Codex Alimentarius, 2022).

In order to enhance the functional properties given by soybean proteins, covering solubility, foaming ability, emulsification, gel formation, and more, different physical treatments have been investigated for their advantages, such as improving the organoleptic properties (texture, flavor, color, aroma) and maintaining the levels of antioxidants and phenolic compounds in the final product (Dong et al., 2020).

To date, high-pressure homogenization, high hydrostatic pressure, microfluidization, ultrasonication, and similar techniques, already described in the previous chapters, play a pivotal role. These processes are applied to improve functional properties and induce structural changes in proteins.

Recently, Hu et al., 2023 reported an increase in emulsification activity (5.81–29.6%) and emulsion stability (5.31–25.9%) at pressure from 20 to 100 MPa for the high-pressure homogenization, with a heat treatment was applied beforehand. This treatment increases the surface hydrophobicity of the SPI and reduces the random coils and β-sheets, forming soluble aggregates and improving the emulsifying properties. Additionally, Martínez et al., 2011 highlighted that subjecting the SPI-hydroxypropylmethylcellulose mixtures to dynamic high-pressure treatment (up to 300 MPa) will enhance foaming properties. It is observed that these processes can be applied directly to protein ingredients, such as SPI, to modify their functionality.

Further, their functionality can be used in food formulations to obtain a final product. Moreover, besides improving functional properties, some of these treatments

were recognized to reduce soybean allergens. All the physical treatments that showed a decrease in soybean allergenicity are detailed in Chapter 3.

Among the treatments discussed, we focus on microfluidization and its impact on the protein structure. More details about this technique and its outcomes regarding the proteins are described already in Chapter 4.

To our knowledge, no investigations have yet considered the impact of microfluidization on the structure of soybean protein isolate by combining different numbers of passes, either with or without temperature control during the process. In temperature-controlled treatments, the temperature of solution remains stable, ensuring that only shear forces and pressure influence the process, avoiding any thermal effects. In contrast, when temperature is not controlled, the temperature of the product increases with each pass, meaning that the process is influenced not only by shear forces and pressure but also by the heat generated naturally due to high shear and pressure within the interaction chamber.

Consequently, this part will describe the effect of the microfluidization process on the structural aspect of soybean protein isolate, both in its powdered and solution forms. Moreover, it differentiates the impact of heat induced during shearing and pressure by testing different parameters regarding the number of passes and temperature. The first part of the results focuses mainly on protein profile, mid-infrared and fluorescence spectroscopy techniques. Figure 5-1 shows the experimental flow of this part of results.

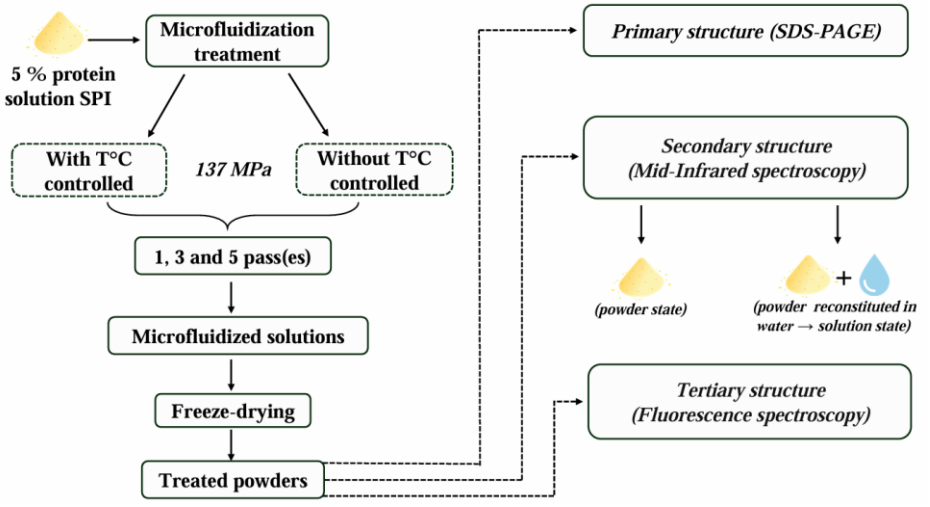


Figure 5-1. Roadmap of the experimental plan.

5.2. Material and methods

5.2.1. Materials

Soybean protein isolate was purchased from SEAH International (France). The protein content is 90.0% (not related to the dry matter). It was determined by the Dumas method (N×6.25), according to Serrano et al., 2013 and using Elementar (Rapid N Exceed). 1-anilinonaphthalene-8-sulfonic acid (ANS) was used to assess protein surface hydrophobicity (PSH), and it was obtained from Cayman Chemical Company (supplier − Sanbio B.V, Netherlands).

5.2.2. Processing

Microfluidization (high shearing treatment)

A 5% w/v protein solution of SPI was prepared by dispersing SPI in Milli-Q ultrapure water at neutral pH (close to 7). The solution was stirred for 4 hours at ambient temperature (25°C) and left overnight at 4°C for complete hydration. Subsequently, the SPI solution was subjected to microfluidization treatment using Microfluidizer Processor M-110EH from Microfluidics International Corporation. The solution was adapted to this condition as we used a pilot scale process.

During this treatment, the liquid was pumped through two interaction chambers composed of a fixed geometry chamber (Z-type) — the first chamber having 200 µm, followed by the second with 100 µm. The microfluidization was conducted with and without temperature control during the process, the solutions being treated at a pressure of 137 MPa (the maximum pressure reached with the concentration used). The solutions prepared were circulated in the system for 1, 3, and 5 passes. The microfluidization system used for our experiments and the mechanism were already described in Chapter 4.

During processing with the cooling system, temperature regulation was achieved using a heat exchanger (SC5000 Recirculating cooler, JULABO GmbH), where the temperature was set at 10°C.

When temperature was controlled, the SPI solutions remained stable, with only minor increases. After one pass, the temperature reached 26°C, rising to 27.7°C after three passes, and concluding at 27.1°C after five passes. On the other hand, when temperature was not controlled, the impact of temperature was visible. In this way, after 1 pass, the temperature reached 41.9°C, then increased to 65.9°C after 3 passes, and it ended with 75°C passing 5 times in the microfluidizer.

The samples where the cooling system was applied are named 1PW, 3PW, and 5PW, where 1, 3, and 5 represent the number of passes used for this treatment. The microfluidized samples where the temperature was not controlled were also denoted as 1PN, 3PN, and 5PN. A non-microfluidized sample was used as a control for the following experiments, named “C.” Frozen solutions from microfluidization treatment were freeze-dried (Christ Gamma 2-16 LSC Plus) for 96 h. Primary and secondary desiccations were performed during 72 and 24 hours under 1.00 and 0.005

mbar pressures. Freeze-dried samples were ground until a homogeneous powder was obtained and stored at 15°C before analysis.

5.2.3. Sample analysis

Because we were interested in the behavior of the proteins for the secondary structure, the following sections will focus on contrasting the mentioned samples in their different states; either as a powder or as a solution.

The microfluidized powders obtained after freeze-drying and the control were used in their initial state to elaborate the secondary structure.

Microfluidized powders and control were also used to reconstitute the samples in Milli-Q ultrapure water for a final 5% protein solution (w/v) concentration. The solutions were stirred for 2 h at 250 rpm, maintaining an ambient temperature of 25°C at their natural pH (close to 7). These solutions were designed to analyze the secondary structure, intrinsic fluorescence, and protein surface hydrophobicity. To analyze the primary structure, 20 mg of samples were solubilized with 1 mL of Tris-SDS buffer for 2 h at room temperature.

5.2.3.1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated according to Laemmli, 1970 using 5% stacking gel and 12% separating gel (Blancher & Jones, 2001). Protein samples were solubilized in Tris-SDS buffer, containing 4% (w/v) SDS, 10 mM Tris HCl (pH=7.4), and protease inhibitor. They were quantified using the DC Protein assay kit for microplates. After protein solubilization and quantification, the samples were heated for 10 min at 70°C. Then, 20 µL of the solution was loaded per well, having 20 µg of proteins. Electrophoresis under reducing conditions was performed at room temperature at a constant voltage of 60 V for 30 min, followed by 90 min at 150 V. Precision Plus Protein Unstained Protein Standards, Bio-Rad (250-10 kDa) was used to estimate protein size. After electrophoresis, the gels were stained in a Silver Blue solution for one night. The gels were read using the ChemiDoc MP imaging system and analyzed by the Image Lab 6.1 Software, both from Bio-Rad Laboratories.

5.2.3.2. Mid-infrared (MIR) measurement in powder and solution

The soybean protein secondary structure was analyzed using the Fourier Transform Infrared Spectroscopy (FTIR) method on the spectrometer IRTracer-100 (Shimadzu, Duisburg, Germany). The analysis was performed on powders and solutions described in the previous section.

The spectra of solutions and powders were recorded in a horizontal attenuated total reflectance (ATR) cell with zinc selenide crystal, using 20 reflections over the wavenumber range of 900 to 3000 cm^{-1}. Sixty-four scans were accumulated for each spectrum with a resolution of 16 cm^{-1}. Milli-Q ultrapure water was used as a background for the solutions analysis, and air for the powders. The second derivative analysis was applied to the MIR spectra for the amide I, II, and III regions to quantify the secondary structure proportions. The analysis was performed in triplicate for both

powders and solutions. LabSolutionIR software (Easy Macro function) determined the estimation of protein secondary structure.

5.2.3.3. Intrinsic fluorescence measurement in the solution

The intrinsic fluorescence of the solutions at their natural pH was determined using a Fluoromax-4 spectrofluorometer (Jobin Yvon, Horiba, NJ, USA) equipped with a temperature controller (T Haake A25 AC200) and a thermostatically controlled quartz cell. The analysis was carried out at 25°C, based on the method of (Nahimana et al., 2023), with slight modifications for the protein concentration. For this study, we used 2 ml of the 5% protein solutions (w/v), incubating for 3 minutes in a dark place. The excitation wavelength was set for 290 nm and 305–450 nm for emission. The analysis was performed in triplicate.

5.2.3.4. Protein surface hydrophobicity (PSH)

The protein surface hydrophobicity of the microfluidized samples was evaluated by using a Fluoromax-4 spectrofluorometer (Jobin Yvon, Horiba, NJ, USA) equipped with a temperature controller (T Haake A25 AC200) and a thermostatically controlled quartz cell. The samples were treated with ANS− solution (8 mM) as a fluorescence probe in different concentrations (0 to 150 µM). Depending on the concentration, 5 µl or 7.5 µl of ANS was added to each 2 ml of the 5% protein solutions (w/v), incubating for 3 minutes in a dark place. The excitation wavelength was set at 350 nm, while the emission range was between 400 and 650 nm. The analysis was performed in triplicate. The PSH of the samples was calculated using Benesi-Hildebrand double inverse linearization equation: $1/\Delta F = 1/\Delta F_{max} + Kd/[ANS] \times \Delta F_{max}$, which is explained by (Miriani et al., 2011), and it is expressed in if/g × µM. After calculating all the parameters, the PSH index was determined by the formula $PSH = [\Delta F_{max}/ Kd]/[P]$. In the following paragraph, each element of the equation is individually outlined.

- ΔF: difference in fluorescence intensity between the sample containing ANS and the sample without ANS
- ΔF_{max}: the maximum fluorescence that can be achieved under ANS saturated concentration and, therefore, the maximum number of binding sites where ANS could bind
- ANS: concentration of ANS expressed in µM
- Kd: apparent dissociation constant of a supposed monomolecular complex (protein-ANS) expressed in µM ($K_d = 1/K_b$)
- P: protein concentration in the solution (g/L)

5.2.3.5. Statistical analysis

Statistical analysis was performed with IBM SPSS Statistics (V 28, 2021). All data were analyzed using univariate analysis, and differences between means were evaluated using the Tukey test with a p-value < 0.05 for significant differences.

5.3. Results and discussion

5.3.1. Impact of microfluidization on the SPI primary structure

SDS-PAGE was performed to analyze the molecular weight distribution of the native and microfluidized SPIs. Fig. 5- 2 shows the electrophoretic profile obtained after the treatment, and the proteins based on their size were identified from the literature (J. Wang et al., 2022; L'Hocine & Boye, 2007).

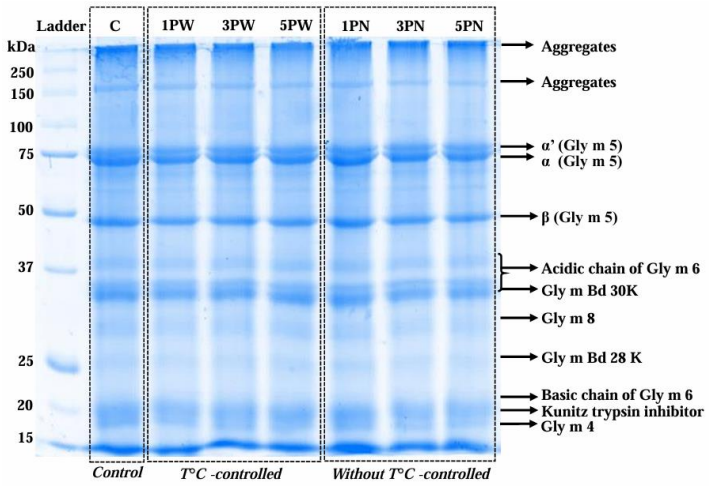


Figure 5-2. SDS-PAGE (12 % separating gel) profile under reducing and denaturation conditions of native and microfluidized SPIs. Lane 1: unstained marker (kDa). Lane 2: non-treated SPI as a control. Lanes 3, 4, and 5: microfluidized samples where the temperature was controlled. Lanes 6, 7, and 8: microfluidized samples without temperature control.

SDS-PAGE showed that the protein bands remained unchanged when exposed to microfluidization treatment, indicating no protein fragmentation. This phenomenon was previously observed for whey and hazelnut proteins (Saricaoglu et al., 2018; Bouaouina et al., 2006). These results suggest that microfluidization does not impact the primary structure of soy proteins, which is consistent with the literature (Gong et al., 2019; H. Chen et al., 2019).

After electrophoresis, different proteins were detected in our native and microfluidized SPIs: the α' subunit (~76 kDa), α subunit (~72-74 kDa), and β subunit (~48-50 kDa) of β-conglycinin (Gly m 5); the acidic chain (~33-40 kDa) and basic chain (~20-22 kDa) of glycinin (Gly m 6); the Kunitz trypsin inhibitor (~20 kDa) and the pathogenesis-related protein (~17 kDa) (Gly m 4). Other minor bands were identified corresponding to the P34 soybean vacuolar protein (~30-34 kDa) (Gly m Bd 30K), 2S albumin Gly m 8 (~28 kDa), and 7S globulin Gly m Bd 28K (~26 kDa). Moreover, according to the literature, the bands presented at the top of the gel and those > 150 kDa seem to be aggregates (J. Hu et al., 2023; Shen & Tang, 2012). In addition, Table 5-1 presents the main proteins identified by SDS-PAGE, along with

their molecular weights in kDa, for samples treated and untreated with microfluidization.

Table 5-1. Protein fractions of soybean identified by SDS-PAGE with corresponding molecular weight (kDa).

Protein fraction		Molecular weight (kDa)
β-conglycinin/ Gly m 5/7S	α'	~76
	α	~72–74
	β	~48–50
Glycinin/ Gly m 6/ 11S	Acidic chain	~33–40
	Basic chain	~20–22
Gly m Bd 30 K (P34)		~30–34
Gly m 8		~28
Gly m Bd 28 K		~26
Kunitz trypsin inhibitor		~20
Pathogenesis-related protein Gly m 4		~17

5.3.2. Mid-infrared analysis

5.3.2.1. Mid-infrared spectra

Mid-infrared (MIR) spectroscopy provides insight into the characteristic vibration patterns of covalent bonds within molecules. As a result, it offers quantitative data about all the components, including proteins, that can absorb IR radiation (Etzion et al., 2004). Also, it provides information about the changes in protein structure (T. Zheng et al., 2019).

To assess the impact of the microfluidization process with and without temperature control on the SPI secondary structure under different cycles, we conducted MIR spectroscopy scans within the range of 3000–900 cm^{-1}. FTIR-ATR spectra are divided into 3 regions, which correspond to fat (3,000–2,800 cm^{-1}), protein (1,700–1,500 cm^{-1}), and fingerprint (1,500–900 cm^{-1}) (Boubellouta & Dufour, 2012).

The results of this method are presented in Fig. 5-3 (a) and (b) for the powders and Fig. 5-4 (a) and (b) for the solutions, where different major bands were found.

According to Türker-Kaya & Huck, 2017, an essential peak for determining the secondary structure of proteins in the MIR spectrum is the amide I band, between 1650 and 1630 cm^{-1} (C=O stretching vibration), followed by the amide II, which contains mainly protein in the range of 1560−1540 cm^{-1} (N–H stretch and C=N). In plants, lignin, cell wall polysaccharides, protein, and lipids can be observed between 1515 and 1150 cm^{-1} (C–H asymmetric and symmetric bending, C=C aromatic stretch, C–O) (Türker-Kaya & Huck, 2017).

The peaks associated with the secondary structure are linked to α-helix (1650–1660 cm^{-1}), β-sheet (1620–1641 cm^{-1}), β-turn (1660–1690 cm^{-1}), random coil (1641–1650 cm^{-1}), aggregates A1 (1610–1620 cm^{-1}) and A2 (1690–1695 cm^{-1}). Aggregates A1 and A2 represent the intermolecular and intramolecular between β-sheets (Long et al., 2015; Carbonaro & Nucara, 2010).

In the following sections, we present the spectra obtained in MIR for the microfluidized samples in both states – powder and solution and the band assignments for the different regions.

5.3.2.1.1. Protein as a dry powder - Spectra analysis of microfluidized SPI, with and without temperature-controlled

The bands found within the range of 3,000 to 2,800 cm^{-1}, which are linked to C–H stretching vibration, are recognized for their sensitivity to the physical condition of lipids (Boubellouta & Dufour, 2012). The absence of the peak in this region (Fig. 5-3 (a) and (b)) illustrates the very low amount of fat in our samples.

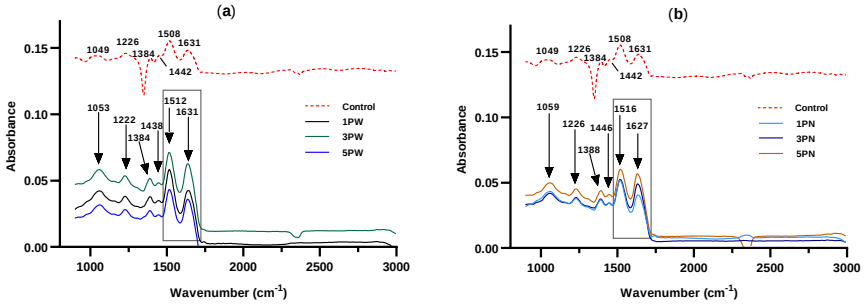


Figure 5-3. Mid-infrared spectra of native and microfluidized SPIs in the powder. The spectral region considered in this study is from 900 to 3000 cm^{-1}. **(a)** Microfluidized samples where the temperature is controlled and **(b)** without a temperature controlled.

For the microfluidized powders where the temperature is controlled (Fig. 5-3 (a)), the prominent peak observed related to proteins is 1631 cm^{-1} (C=O stretching). This is assigned to the β-sheet, corresponding to a low-frequency range (Carbonaro & Nucara, 2010). According to Pelton & McLean, 2000, the peak observed at 1512 cm^{-1} belongs to Amide II (CN stretching, NH bending) and is a structure of the antiparallel β-sheet.

The same trend was noticed for the microfluidized powders where the temperature was not controlled (Fig. 5-3 (b)). β-sheet is observed at 1627 cm^{-1} (C=O stretching) and antiparallel β-sheet at 1516 cm^{-1} (CN stretching, NH bending). Moreover, when the control is compared with the microfluidized powders, it can be observed that the absorbance is about 3 times higher.

The small peaks represent the low fingerprint contamination of the samples. In addition, the two highest peaks correspond to the high amount of protein present in the SPI.

5.3.2.1.2. Solubilized protein - Spectra analysis of microfluidized SPI, with and without temperature-controlled

Regarding the samples in their solution form (Fig. 5-4 (a) and (b)), the findings obtained showed a similarity in the case of both conditions (with and without controlling the temperature) when compared with the control. The peak corresponds to the β-sheet absorbed at 1627 cm^{-1} (C=O stretching) in the Amide I region. In the amide II region of the spectrum, a parallel β-sheet structure was found at a higher frequency of 1546 cm^{-1}. The graphs show that when the powders are reconstituted in water, the absorbance is similar for all the samples, including the control. The protein adopts more energetically stable conformations as the water content increases. This might occur because of the increased mobility of protein segments and the occupation of empty spaces within these configurations (Abbott et al., 1996)In this way, particles are more electrically charged when they come into contact with other surfaces or particles in the absence of water (Matsusaka et al., 2010).

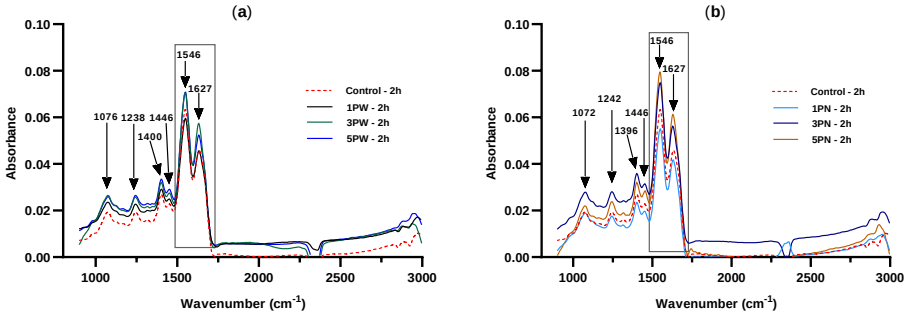


Figure 5-4. Mid-infrared spectra of native and microfluidized SPIs in the solution. The spectral region considered in this study is from 900 to 3000 cm^{-1}. **(a)** Microfluidized samples where temperature is controlled and **(b)** without temperature controlled.

Also, in this case, the small peaks represent the low fingerprint contamination of the samples, and the two highest peaks correspond to the high amount of protein present in the SPI.

The findings presented for the powder and solution forms, provided information regarding the SPIs during high-intense shear treatment, allowing us to visualize the structural changes of the protein.

5.3.2.2. Secondary structure analysis

The second derivative analysis was applied to study the changes in the soybean protein structure. This analysis determined the percentages of α-helix, β-sheet, β-turn, random coil, aggregates A1, and aggregates A2, presented in Tables 5-2, 5-3, 5-4, and 5-5. Thus, the following sections will present different properties of the protein linked to their secondary structure. These will be related to the techno-functional properties (L. Zheng et al., 2021; Djemaoune et al., 2019), allergenicity (Pi et al., 2021; Jideani,

2011), digestibility (Melchior et al., 2022; Pan et al., 2022; Carbonaro et al., 2012) and flexibility of proteins (Yan et al., 2021; Zhu et al., 2020).

5.3.2.2.1. Protein as a dry powder - Spectra analysis of microfluidized with temperature-controlled SPI

Table 5-2 presents the percentages of protein secondary structure for the SPIs microfluidized powders where the temperature is controlled. Compared to the control (a non-treated sample), we notice that the SPI treated by microfluidization showed a significant decrease in the quantities of β-sheet, α-helix and random coil ($p < 0.05$). On the other hand, the proportions of β-turn, A1, and A2 aggregates increased significantly ($p < 0.05$).

Table 5-2. Secondary structure (%) of the native and microfluidized treated SPI (temperature controlled) in the powder.

Structural elements	*MF-treated samples with temperature-controlled – in powder*			
	C	**1PW**	**3PW**	**5PW**
α-helix	8.93 ± 0.15^{c}	8.17 ± 0.06^{a}	8.40 ± 0.00^{a}	8.23 ± 0.06^{a}
β-sheet	20.03 ± 0.21^{e}	15.40 ± 1.61^{d}	9.00 ± 0.79^{b}	8.43 ± 0.90^{b}
β -turn	38.67 ± 0.06^{a}	40.63 ± 0.86^{b}	45.80 ± 0.17^{c}	46.03 ± 0.06^{c}
Random coil	11.87 ± 0.06^{e}	10.20 ± 0.10^{cd}	8.57 ± 0.12^{a}	8.37 ± 0.15^{a}
A1	15.13 ± 0.06^{a}	19.27 ± 0.25^{c}	20.43 ± 0.25^{d}	21.00 ± 0.40^{d}
A2	5.37 ± 0.06^{a}	6.40 ± 0.56^{b}	7.73 ± 0.32^{c}	7.97 ± 0.40^{c}
A1+A2	20.50 ± 0.12^{a}	25.67 ± 0.81^{c}	28.17 ± 0.57^{d}	28.97 ± 0.80^{d}

The data refers to the mean ± standard deviation of triplicate measurements. Values in the same column with different superscripts (a-e) represent significant differences ($p < 0.05$) between the treated and non-treated samples.

Interestingly, it was also observed that the most significant changes ($p < 0.05$) are for β-sheet, the proportion decreasing from 20.03% for native SPI (control) to 15.40%, 9.00%, and 8.43% for 1, 3, and 5 passes, respectively. This reduction can have an impact on protein properties such as digestibility. Carbonaro et al., 2012 have demonstrated an inverse correlation between the proportion of β-sheet and protein digestibility. In this case, decreasing the β-sheet ratio could increase their digestibility. This could also impact allergenicity. Related to this, different researchers explained that an increase in digestibility could be linked with a reduction in allergenicity during the digestive process (H. Chen et al., 2019; Rahaman et al., 2016; Zhong et al., 2014).

The observed decrease in β-sheet and the increase in β-turns in treated samples indicated that microfluidization may lead to protein unfolding, with the hydrophobic regions opening and becoming exposed (Fang et al., 2020). This will increase the protein hydrophobicity, which is detailed in the following section.

Furthermore, another aspect impacted by microfluidization is related to the α-helix. A decrease in the α-helix was observed for the microfluidized SPIs and can be

connected with increased flexibility. According to Tang, 2017, flexibility is related to how proteins adapt to changes in their external environment, affecting soy proteins' emulsion stability. In this way, a lower amount of α-helix suggests a higher protein flexibility (Zhu et al., 2020).

If the number of cycles is compared, we can notice a significant difference ($p < 0.05$) between the SPI passed 1 and 3 times (when the temperature is controlled) for all secondary structures (except for α-helix). However, no significant differences were observed between 3 and 5 passes for any of the secondary structures studied. This suggests that the changes during microfluidization treatment depend on the number of passes and the heating resulting from the shearing effect. It seems that 1 or 3 passes are enough to see a modification in the secondary structure. At higher passes, the resulting mechanical forces might be responsible for the protein rearrangement and aggregation, forming new electrostatic interactions and disulfide bonds (Melchior et al., 2022).

5.3.2.2.2. Protein as a dry powder - Spectra analysis of microfluidized without temperature-controlled SPI

Similar trends are observed for microfluidization treatment without controlling the temperature but with a few differences (Table 5-3). When microfluidization is applied for the SPIs and the temperature is not controlled, there is a significant decrease in β-sheet and an increase in β-turn ($p < 0.05$). A minor decrease in random coil content was observed, while A2 increased with the number of passes (between 1 and 3, but not for 5). Regarding the impact of temperature, when this is not controlled, it increases with each pass. In this way, after 1 pass, the temperature reached 41.9°C, then increased to 65.9°C after 3 passes, and it ended with 75°C passing 5 times in the microfluidizer.

Table 5-3. Secondary structure (%) of the native and microfluidized treated SPI (without temperature controlled) in the powder.

Structural elements	*MF-treated samples without temperature-controlled – in powder*			
	C	**1PN**	**3PN**	**5PN**
α-helix	8.93 ± 0.15^{c}	8.67 ± 0.06^{b}	9.17 ± 0.15^{c}	9.53 ± 0.06^{d}
β-sheet	20.03 ± 0.21^{e}	11.53 ± 0.38^{c}	3.73 ± 0.06^{a}	3.87 ± 0.06^{a}
β -turn	38.67 ± 0.06^{a}	45.57 ± 0.21^{c}	51.57 ± 0.12^{d}	52.43 ± 0.06^{d}
Random coil	11.87 ± 0.06^{e}	10.00 ± 0.10^{c}	9.63 ± 0.21^{b}	10.37 ± 0.06^{d}
A1	15.13 ± 0.06^{a}	17.00 ± 0.00^{b}	16.87 ± 0.12^{b}	14.70 ± 0.10^{a}
A2	5.37 ± 0.06^{a}	7.17 ± 0.12^{bc}	9.10 ± 0.00^{d}	9.10 ± 0.00^{d}
A1+A2	20.50 ± 0.12^{a}	24.17 ± 0.12^{b}	25.97 ± 0.12^{c}	23.80 ± 0.10^{b}

The data refers to the mean ± standard deviation of triplicate measurements. Values in the same column with different superscripts (a-e) represent significant differences ($p < 0.05$) between the treated and non-treated samples.

On the other hand, the data for α-helix showed a different trend from the one observed in the treatment where the temperature was controlled. The increase of the internal heating can explain the increase in α-helix for 3 and 5 passes due to the shearing effect, and this was demonstrated when heat treatment was applied to soy proteins (T. Li et al., 2021; Long et al., 2015; Carbonaro et al., 2012). Considering the information described above regarding the flexibility of proteins (Zhu et al., 2020), it seems that 3 and 5 passes are less suitable for forming a stable emulsion.

Moreover, it has also been demonstrated that applying heat treatment to soy proteins increases the proportions of β-turn and A2 aggregates and decreases β-sheet proteins. These results were also obtained for the heat treatment at 65°C and 75°C for 1 h (T. Li et al., 2021; Long et al., 2015; Carbonaro et al., 2012), compared with microfluifization treatment, were for 5 passes were registered around 46 min (flow rate 26l/h). It was observed that both temperature and shearing treatment contribute to the modification of the structure when the cooling system is not used, leading to more significant changes in the proportions of these structures than using the cooling system.

On the contrary, a decrease in A1 aggregates was shown when heat treatment was subjected to soy proteins. This results in the unfolding of proteins and increased exposure of hydrophobic groups on their surfaces. These exposed hydrophobic groups can then interact, potentially forming aggregates. The unfolding of polypeptide chains and the formation of aggregates often happened simultaneously, indicating that these changes occurred at various levels (Long et al., 2015).

In the same way, as for microfluidization with the cooling system, there were no significant differences between 3 and 5 passes for most secondary structures. However, this is not the case for α-helix, random coils, and aggregates A1, where we have a statistical difference ($p < 0.05$). Furthermore, compared with the control, a drastic decrease can be observed for β-sheet for 3 and 5 passes, from 20.03% to 3.73% and 3.87%, respectively. Similar findings are reported by Gong et al., 2019 for a pressure higher than 120 MPa. They explained that exposing the sample to 120 MPa resulted in the most significant disruption of the aggregated structure, and applying higher pressures led to a reformation of the aggregates.

Additionally, if we consider the results obtained by Y. Chen et al., 2016, treated samples 3PN and 5PN are potentially suitable for reducing soybean allergens. The researchers explained that decreasing in β-sheet and increasing in α-helix led to a notable improvement in binding capacity, resulting in a decrease in allergenic potential. However, the correlation between this protein's secondary structure and binding capacity requires more studies to prove if there is a relation between them.

5.3.2.2.3. Solubilized protein - Spectra analysis of microfluidized with temperature-controlled SPI

The influence of microfluidization in the solutions with temperature control is presented in Table 5-4. Compared to the dry state, results show that the protein's secondary structure changes completely when the powders are reconstituted in water. These observations could be due to the hydrogen bond variation induced by pressure,

the number of passes (Melchior et al., 2022), and/or the freeze-drying process (Arakawa et al., 2001). When microfluidization was applied, no significant difference was observed for α-helix, random coil, aggregates A1 and A2. However, a considerable difference was highlighted for β-sheet and β-turn. For α-helix, an increase from 9.07% to 9.13%, 9.37%, and 9.23% for 1, 3, and 5 cycles is observed when compared to the control. Regarding β-sheet, between the microfluidized samples, there is a significant difference ($p < 0.05$); the highest content of β-sheet is represented by one treatment cycle.

Table 5-4. Secondary structure (%) of the native and microfluidized treated SPI (temperature controlled) in the solution.

Structural elements	*MF-treated samples with temperature-controlled – in solution*			
	C-2h	**1PW-2h**	**3PW-2h**	**5PW-2h**
α-helix	9.07 ± 0.06^{a}	9.13 ± 0.06^{ab}	9.37 ± 0.06^{bc}	9.23 ± 0.06^{bc}
β-sheet	21.53 ± 0.32^{bc}	21.83 ± 0.15^{d}	20.27 ± 0.12^{a}	21.10 ± 0.17^{b}
β -turn	36.70 ± 0.10^{a}	36.70 ± 0.20^{a}	37.77 ± 0.12^{c}	36.90 ± 0.00^{ab}
Random coil	13.40 ± 0.10^{a}	13.50 ± 0.10^{a}	13.40 ± 0.10^{a}	13.53 ± 0.15^{ab}
A1	14.30 ± 0.30^{b}	14.03 ± 0.06^{ab}	14.03 ± 0.06^{ab}	14.23 ± 0.15^{b}
A2	5.00 ± 0.10^{ab}	4.83 ± 0.06^{a}	5.17 ± 0.06^{b}	5.03 ± 0.06^{ab}
A1+A2	19.30 ± 0.40^{b}	18.86 ± 0.12^{ab}	19.20 ± 0.12^{b}	19.26 ± 0.21^{b}

The data refers to the mean ± standard deviation of triplicate measurements. Values in the same column with different superscripts (a-d) represent significant differences ($p < 0.05$) between the treated and non-treated samples.

These results could be explained by the fact that protein molecules interact directly in the powders. Without the presence of excess bulk water molecules, intermolecular interactions become more energetically favorable. In contrast, after the reconstitution of the samples in water, the protein configuration changes, inducing reversible denaturation (Liao et al., 2002).

Further, after the hydration, the total number of aggregates (A1+A2) is decreasing, and the content of β-sheet is increasing. This might be due to the disruption of the soluble aggregates, which are dissolved in water and then converted back to β-sheet. This observation can not be applied to the powders (see previous section), where is observed the opposite trend (increasing aggregates and decreasing in β-sheet). In this case, the interaction between β-sheets forms the aggregation phenomenon (Luo et al., 2022).

Lyophilization applied after the microfluidization treatment could also explain the difference in results compared with the powder state. Very interestingly, the lyophilization process alone can subject protein and peptide molecules to stress, causing considerable modifications in conformation when stabilizing excipients (such as sugars, sugar alcohols, surfactants, specific amino acids, buffers, and polymers) are

absent (Thakral et al., 2021; Carpenter et al., 1997). One exciting aspect of these findings is that the interactions between proteins and sugars through hydrogen bonding are important for stabilizing proteins (Carpenter et al., 1992; Carpenter & Crowe, 1989).

Regarding stability, most proteins are quite delicate under neutral pH and room temperature conditions, making them easily susceptible to denaturation when subjected to temperature and pressure variations, changes in pH, and adding substances such as chaotropic salts, guanidine, HCl, or urea (Arakawa et al., 2001; Pace, 1990). Besides freeze-drying, freeze-thawing is also responsible for the stress described above. Many proteins are not stable to these, even if they are one of the most common methods for the long-term storage of proteins (Arakawa et al., 2001).

Furthermore, it is noteworthy that microfluidization can modify the structure of globular protein aggregates accompanied by a decrease in particle size (Shen & Tang, 2012; Liu et al., 2011; Dissanayake & Vasiljevic, 2009). This size reduction will make more molecules available, which will be subjected to additional stress due to the formation of ice crystals. This process will form ice-water and ice-air interfaces, which can destabilize the protein. Additionally, the solution will become more concentrated when removing water (as ice), possibly altering the samples' pH, viscosity, and ionic strength (Thakral et al., 2021).

5.3.2.2.4. Solubilized protein - Spectra analysis of microfluidized without temperature-controlled SPI

Results for the solution samples without temperature control are presented in Table 5-5. The samples in their solution form have a similar behavior in the spatial structure of the protein as samples where the temperature was controlled. However, it seems that β-sheet and β-turn are not changed during the reconstitution in water (no statistical differences). Interestingly, the intrinsic heat produced by the high shear and cavitation is responsible for this modification in the structure.

Table 5-5. Secondary structure (%) of the native and microfluidized treated SPI (without temperature controlled) in the solution.

Structural elements	*MF-treated samples without temperature-controlled – in solution*			
	C-2h	**1PN-2h**	**3PN-2h**	**5PN-2h**
α-helix	9.07 ± 0.06^{a}	9.17 ± 0.06^{ab}	9.27 ± 0.06^{bc}	9.23 ± 0.06^{c}
β-sheet	21.53 ± 0.32^{bc}	21.40 ± 0.35^{bc}	21.17 ± 0.35^{bc}	21.37 ± 0.21^{bc}
β -turn	36.70 ± 0.10^{a}	37.10 ± 0.26^{ab}	36.93 ± 0.12^{ab}	37.17 ± 0.21^{b}
Random coil	13.40 ± 0.10^{a}	13.47 ± 0.06^{a}	13.80 ± 0.10^{b}	13.60 ± 0.00^{ab}
A1	14.30 ± 0.30^{b}	13.97 ± 0.12^{ab}	13.80 ± 0.00^{a}	13.70 ± 0.10^{a}
A2	5.00 ± 0.10^{ab}	4.93 ± 0.06^{a}	5.00 ± 0.10^{ab}	4.93 ± 0.06^{a}
A1+A2	19.30 ± 0.40^{b}	18.90 ± 0.18^{ab}	18.80 ± 0.10^{ab}	18.63 ± 0.16^{a}

The data refers to the mean ± standard deviation of triplicate measurements. Values in the same column with different superscripts represent (a-c) significant differences ($p < 0.05$) between the treated and non-treated samples.

Furthermore, a difference between 1 and 5 cycles ($p < 0.05$) is observed for α-helix. Compared with the control, α-helix is increasing from 9.07% to 9.17%, 9.27%, and 9.23% for 1, 3, and 5 passes, respectively. C. Wang et al., 2020 reported that an increase in α-helix might be due to the complete solubilization of the proteins under the microfluidization treatment. Moreover, after the hydration, we can observe the same trend for the aggregates and β-sheet. The total number of aggregates is lower than the β-sheet, which is higher. This observation is due to the disruption of the soluble aggregates, which are dissolved in water and then converted back to β-sheet (Luo et al., 2022).

Similar to the samples obtained with temperature control, lyophilization might explain the difference in results by adding additional stress related to the formation of ice crystals and their consequences.

Finally, differences in the behavior of the protein in powder and solution states are observed. By changing their conformation, the freeze-drying process seems to impact the treated samples when reconstituted in water. Moreover, microfluidization also contributed to the difference between the two states (powder and solution) by decreasing the particle size and opening more sites.

5.3.3. Intrinsic fluorescence spectra analysis

Fluorescence spectra analysis is a valuable technique that elucidates the changes in the tertiary structure of proteins, such as folding-unfolding and dynamics. Intrinsic fluorescence means the aromatic amino acids from proteins, such as tryptophan, tyrosine, and phenylalanine residues, have natural fluorescence that can signal protein conformational changes.

Tryptophan gives the highest intensity when compared with the other mentioned aromatic amino acids, which is why it is most used in the intrinsic fluorescence methods (He et al., 2021; Vera et al., 2019; Turoverov & Kuznetsova, 2003). The maximum emission wavelength (λ_{max}) and the difference in the fluorescence intensity (FI) are the parameters to evaluate the modifications occurring in the proteins' tertiary structure (Nahimana et al., 2023).

5.3.3.1. Intrinsic fluorescence spectra analysis of microfluidized with temperature-controlled SPI in the solution

Fig. 5-5 presents the results of the maximum emission wavelength and the difference in the fluorescence intensity for the microfluidized SPI, where the temperature was controlled. As we can observe, the λ_{max} of the non-treated sample is 377 nm. The variation of microfluidized samples increased for 1 and 3 passes (377.67 nm for both). If they are compared together, there is no difference between 1 and 3 passes. The highest number of passes (5) led to a higher emission wavelength of 378 nm.

However, there is no statistical difference between the samples treated under this condition, except between 5PW and 3PN, which can be due to the high shear forces. A similar λ_{max} was observed for the white lupin protein isolate (Nahimana et al., 2023). When a pretreatment at 95°C was applied before microfluidization for soybean protein isolate, the maximum emission wavelength was 337.4 nm, indicating an increase in hydrophilicity in the microenvironment surrounded by tryptophan chromophores (Shen & Tang, 2012).

The observed increase in maximum emission wavelength for our samples could also suggest that the microenvironment of the tryptophan becomes more hydrophilic with an increased number of passes. It signifies an increasing polarity around tryptophan residues caused by the unfolding of the protein and an enhanced interaction of the fluorophore with the aqueous medium (He et al., 2021; Ling et al., 2019).

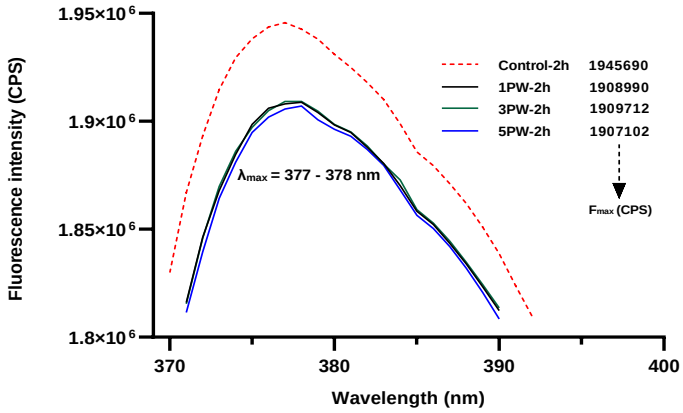


Figure 5-5. Intrinsic tryptophan emission spectra of microfluidized samples at 137 MPa in the solution, where the temperature was controlled for 1, 3, and 5 passes compared to the non-treated SPI.

On the other hand, when we look at the maximum fluorescence (F_{max}) for the 1PW, 3PW, and 5PW, F_{max} is decreasing compared with the control (Fig. 5-5). In our case, cross-linking of protein molecules might be responsible for the reduced fluorescence intensity, which indicating that tryptophan residues are less exposed for 5 passes (Ling et al., 2019). These findings suggest that the emission wavelength increases, and the fluorescence intensity decreases differently for each number of passes. Moreover, when microfluidization is applied, we observe modifications in the tertiary structure.

5.3.3.2. Intrinsic fluorescence spectra analysis of microfluidized without temperature-controlled SPI in the solution

The obtained results are different in the samples where the temperature was not controlled during the microfluidization treatment. As we can observe in Fig. 5-6, the λ_{max} increases after 1 pass (378 nm) compared to the non-treated SPI (377 nm). Moreover, there is a decrease for 3 passes (376.67 nm), and then for the most drastic treatment (5 passes), it returns at 377 nm. A statistical difference ($p < 0.05$) was noted

between 1 and 3 passes, whereas this was not the case for the 5-pass cycle. Even if the temperature is increasing during the process (see materials and methods 5.2.2), due to the intrinsic high shear forces, the fact that the temperature is not controlled could be responsible for these oscillations in λ_{max}. The increase-decrease of the maximum emission wavelength was already described in the previous section (see 5.3.3.1.).

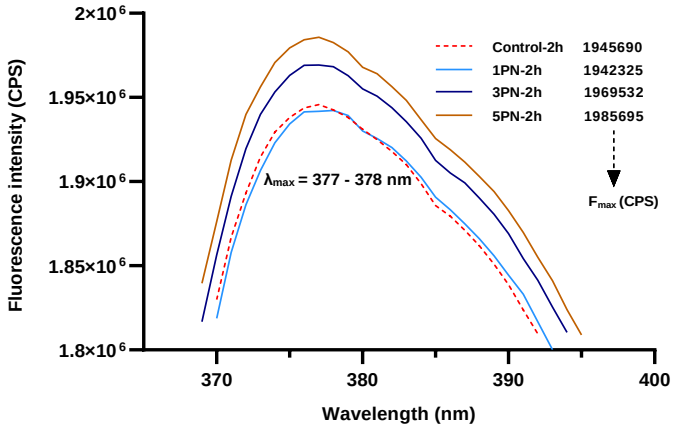


Figure 5-6. Intrinsic tryptophan emission spectra of microfluidized samples at 137 MPa in the solution, where the temperature was not controlled for 1, 3, and 5 passes compared to the non-treated SPI.

Interestingly, the results from the maximum fluorescence reveal the opposite compared to temperature-controlled samples. Namely, for 3 and 5 passes, F_{max} was increasing, compared to the control (Fig. 5-6). This result might be due to the protein unfolding caused only by high shear forces, cavitation, and pressure (Wu et al., 2019). In contrast, F_{max} decreased for one pass, which means a cross-linking or aggregation when soybean proteins are exposed to these conditions (Ling et al., 2019). If the samples are compared between them, we can assume that the F_{max} increases with the number of passes. Moreover, increased fluorescence intensity enhances the stability and emulsification properties (Zhu et al., 2020). Based on this, 3 and 5 passes without temperature control are suitable for further food applications. We also observe modifications in the tertiary structure configuration of soybean proteins for this treatment.

5.3.4. Protein surface hydrophobicity

Protein surface hydrophobicity evaluates different modifications in protein tertiary structure using ANS as an extrinsic fluorescence probe. Typically, ANS binds to exposed hydrophobic clusters within protein molecules. Consequently, PSH serves as an indicator of their surface hydrophobic characteristics, being essential for the functional properties of proteins, such as foaming, gelation, and emulsion (He et al., 2021; Z. Wang et al., 2014; Shen & Tang, 2012).

When the temperature was controlled, the PSH of the MF-treated samples showed an increased trend compared with the untreated SPI (1277.40 ± 31.89 if/g × µM). Surface hydrophobicity increases with the number of passes from 1307.54 ± 15.24 (if/g × µM) to 1490.45 ± 27.45 (if/g × µM) and 1986.29 ± 34.26 (if/g × µM) for 1, 3, and 5 passes, respectively. However, a significant difference ($p < 0.05$) was observed for the 3 and 5 cycles samples when they were compared with the non-treated sample (Fig. 5-7).

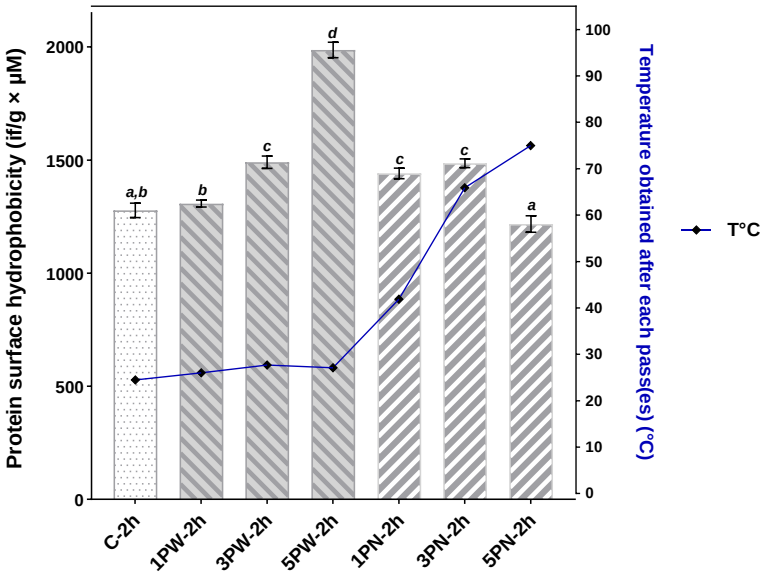


Figure 5-7. The influence of microfluidization (137 MPa) on protein surface hydrophobicity at the natural pH of treated and non-treated SPI. The data refers to the mean of triplicate measurements. Values in the column with different letters (a-d) represent significant differences ($p < 0.05$). The blue line is represented by the temperature achieved after each number of passes of microfluidization for both conditions.

These findings indicated that certain hydrophobic structural sections of treated SPI, such as hydrophobic amino acid residues, could become exposed after microfluidization due to the intense impact of high shear forces, high pressure, and cavitation. This exposure led to the dissociation of insoluble protein aggregates and protein unfolding (Fig. 5-8) (He et al., 2021; Fang et al., 2020).

Another factor that may cause the increase in PSH can be related to the peptide chain expansion or the dissociation of the protein subunits or β-conglycinin denaturation (Z. Wang, Li, Jiang, et al., 2014).

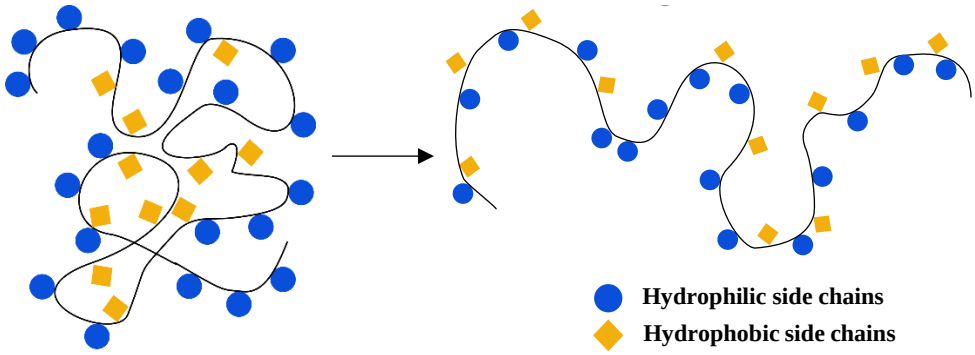


Figure 5-8. Unfolding of protein structures and exposure of hydrophobic amino acids residues after microfluidization.

Furthermore, previous studies reported similar results, where PSH increased when samples were subjected to high-shearing treatment. For example, after exposing the peanut protein isolate to 210 MPa, the PSH increased by 2.2 times more than the control (Gong et al., 2019).

Moreover, for pea albumin aggregates, it was found at 130 MPa, pH 7, that PSH increased and reached the maximum at this pressure (2.11 × 106 a.u) (Djemaoune et al., 2019). An increase in PSH was also observed for soybean protein isolate at 120 MPa - preheated and unheated samples (Shen & Tang, 2012).

On the other hand, compared with the non-MF sample (1277.40 ± 31.89 if/g × μM), an increase for the treated samples was observed for 1 (1441.02 ± 22.87 if/g × μM) and 3 passes (1485.96 ± 19.30 (if/g × μM) and a decrease for 5 passes (1217.28 ± 36.13 if/g × μM), if the temperature was not controlled.

However, between 1 and 3 cycles, no significant difference was observed. The PSH of 5 cycles shows a statistical difference compared to 1 and 3 passes but remains similar to the control. This reduction may be attributed to the intrinsic heating formed naturally during the microfluidization treatment (Z. Wang, Li, Jiang, et al., 2014). Also, the same authors reported that time, protein concentration, or temperature can influence the PSH.

To summarize, the order of increased surface hydrophobicity is as follows: 5PN-2h <C-2h < 1PW-2h < 1PN-2h < 3PN-2h < 3PW-2h < 5PW-2h.

All the results show that microfluidization treatment impacts the tertiary structure of soybean proteins for specific conditions. These modifications could improve the functional properties, such as foaming or emulsifying.

5.3.5. Effect of temperature control and number of passes on soybean protein structures in microfluidization: a comparative analysis

This section presents an analysis to individually compare the effect of temperature control for 1, 3, and 5 passes, highlighting the differences in protein structures between passes with and without temperature control. Fig. 5-9 provides an overview of the comparative analysis. Results showed that all the samples presented a similar profile in the primary structure for the powder state. When comparing 1PW and 1PN, the difference in secondary structure was observed for almost all structural arrangements except random coil and A2.

Additionally, for 3 and 5 passes, with and without temperature control, microfluidization influenced all secondary structure elements. The second group of microfluidized samples is related to their solution state. Analysis reveals no variation in the secondary structure between a single pass with and without temperature control. With 3 passes, differences were observed only in the β-sheet, β-turn, and random coil structures. Under more intense conditions with 5 passes, heating and multiple passes impact only the intermolecular interactions within β-sheets (A1).

Regarding surface hydrophobicity it was observed a difference between samples subjected to 1 and 5 passes. The results indicate that the internal heating produced by shearing and the number of cycles are essential to consider when selecting optimal parameters for specific protein structure modification.

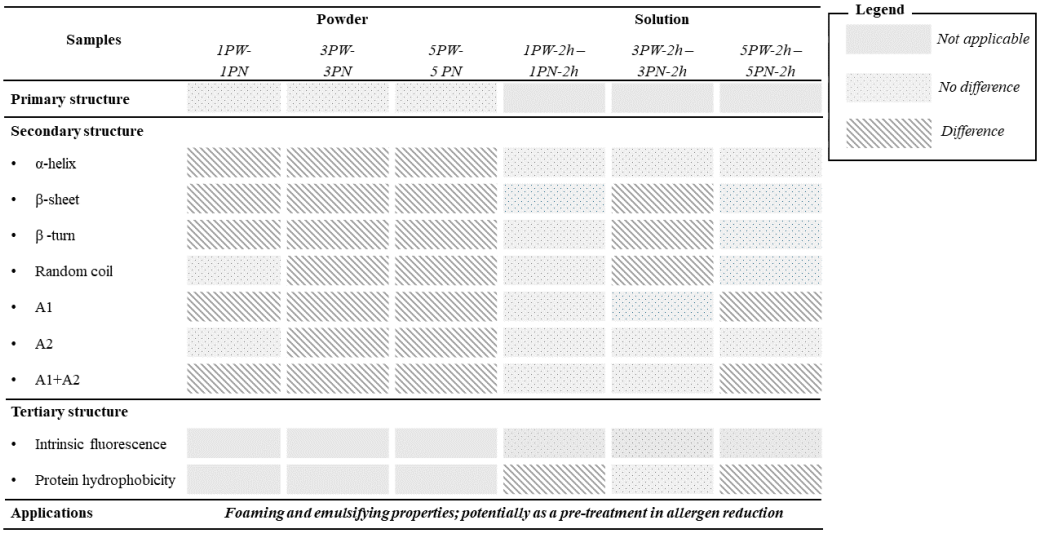


Figure 5-9. Summary of the effect of microfluidization on the soybean protein structures and potential applications.

Furthermore, based on particular requirements, microfluidization can serve as a method to enhance foaming and emulsifying characteristics and potentially serve as a preliminary treatment for reducing allergens.

5.4. Conclusions

The first part of the results investigated the impact of high shear force, cavitation, and high pressure produced by microfluidization on the primary, secondary, and tertiary structures of soybean protein isolate by comparing different conditions, including the temperature and number of passes. Regarding the primary structure, the SDS-PAGE analysis under reducing and denaturation conditions showed that the protein bands remained unchanged when exposed to microfluidization treatment.

On the other hand, microfluidization treatment impacted the secondary structure of the protein in a powder form, as measured by FTIR. The high shear force can influence the protein digestibility and flexibility for the powders, leading to protein unfolding. Moreover, at a higher number of passes (> 3), the resulting mechanical forces seem to cause protein rearrangement and aggregation. Furthermore, no differences were observed for secondary structural features at 3 and 5 passes for both studied thermal conditions (temperature controlled or not), meaning that after a certain point no further impact is observed, and the system reaches equilibrium, with results remaining consistent.

More interestingly, it is for the secondary structure of the proteins in the solution, where the structure changes completely when the powders are reconstituted in water, with a significant difference only in α-helix, β-sheet and β-turn. Lyophilization could explain this difference in results compared with the powders because this process also subjects protein and peptide molecules to unique stressors, causing potential modifications in conformation.

Moreover, microfluidization could also contribute to this effect by decreasing the particle size and opening more sites, therefore encouraging ice crystal formation before freeze drying. Regarding the tertiary structure, microfluidization impactes the SPIs. Microfluidization increases the surface hydrophobicity, and some hydrophobic amino acid residues become exposed due to the intense effect of high shear forces, high pressure, and cavitation. This could improve the functional properties of proteins, such as foaming or emulsifying. However, the most significant difference was observed for 5PW.

Among all the conditions tested, the samples where the temperature is controlled seem the most suitable. Having this overview of the structure of soybean, the conclusion obtained is used to design the experimental plan for the next chapter.

6

Effect of high shear, enzymatic hydrolysis, and their combination on soy allergens

Description of Chapter 6

In the previous chapter, we demonstrated the effect of microfluidization on the structure of soybean proteins. As a result, this chapter investigates its impact on allergenicity alone and in combination with enzymatic treatment.

This work is an original contribution adapted from Kerezsi, A. D., Figula, A., Jacquet, N., Francis F. & Blecker, C. (2024), ″Challenges in mitigation of soybean proteins allergenicity using combined techniques: high shearing and enzymatic hydrolysis.″ This paper is submitted for publication.

Chapter 6. Effect of microfluidization, enzymatic hydrolysis, and their combination on soy allergens

6.1. Introduction

The previous chapter showed the effect of microfluidization on the structure of soybean proteins. As a result, this chapter will explore its impact on allergenicity alone and in combination with enzymatic treatment.

In this way, previous studies have demonstrated the efficacy of enzymatic treatments in decreasing the allergenic properties of various allergenic foods like soy proteins (Meinlschmidt et al., 2016; Wang et al., 2014; Meinlschmidt et al., 2015), milk proteins (Liang et al., 2021), lentil proteins (Cabanillas et al., 2010), and peanut proteins (Cabanillas et al., 2010). For example, different enzymes (Alcalase® 2,4L FG, Flavourzyme® 1000 L, Corolase® 7089, Corolase® 2TS, Papain (0.05%), Pepsin) were evaluated on two main allergens of soybean, β-conglycinin and glycinin. Alcalase, Pepsin, and Papain demonstrated the highest efficacy in degrading the major allergens, achieving proteolytic activities of 100%, 100%, and 95.9% (Meinlschmidt et al., 2015). Also, Xing et al., 2019 showed a 32.2% reduction in the allergenicity of soy milk following a 4-hour enzymatic cross-linking process using microbial transglutaminase (MTGase, 3.0 U/g). This decrease might be due to the formation of aggregates, masking the antigenic epitopes.

However, the impact of microfluidization alone and combining it with enzymatic hydrolysis on soybean allergenicity has still not been explored. Some researchers describe this combination in order to improve the emulsifying properties of the hydrolysates obtained.

After exposing the SPI to microfluidization treatment (120 MPa), the resulting dispersion was further proteolyzed using pancreatin at pH 7.0 and 50°C. They observed improved accessibility of specific subunits (α subunit of β-conglycinin, acidic and basic glycinin subunits) and increased protein solubility (39.4% to 85.1%). Regarding the surface hydrophobicity, the control of microfluidized samples exhibited a higher value (1323.5) compared with the control of the non-treated sample (1099.8), indicating that the initial buried hydrophobic groups were exposed after microfluidization (L. Chen et al., 2016).

This combination was also investigated on other allergens, such as milk. For example, Zhong et al., 2014 combined the application of microfluidization and enzymatic treatment on β-lactoglobulin. They demonstrated that the microfluidization treatment performed before an enzymatic treatment with trypsin increased the hydrolysis rate of β-lactoglobulin compared to non-treated proteins, thus generating an increased reduction in allergenicity. This is explained by the fact that microfluidization results in the exposure of cleavage sites for the enzyme, therefore promoting hydrolysis of the protein.

For the experiments detailed in the following sections, we chose samples with controlled temperature. The allergenicity assessment for these samples was done by using three well-known methods: SDS-PAGE, Western Blot, and indirect enzyme-linked immunosorbent assay (ELISA).

6.2. Material and methods

6.2.1. Materials

Soybean protein isolate was obtained from SEAH International (France). The protein content is 90.0% wet weight (WW) (determined using the Dumas method (N×6.25) (Serrano et al., 2013)). Flavourzyme (proteases from *Aspergillus oryzae*, P6110, activity ≥ 500 LAPU/g) was purchased from Sigma-Aldrich. Flavourzyme is a blend of endoprotease and exopeptidase with an activity of 1 leucine aminopeptidase unit (LAPU) per gram. One LAPU unit corresponds to the amount of enzyme that hydrolyzes 1 mmol of L-leucine-p-nitroanilide per minute (technical data from the supplier).The human sera used in this study were obtained from PlasmaLab International (Everett, WA, United States), with a specific IgE level of 15.4 kU/l (PL 28282) and 6.48 kU/l (PL 28940) from patients with soybean allergy. Soybean-specific IgE and the concentration of IgE are confirmed with Immunocap. Following the manufacturer's guidelines, a positive outcome was considered when the sIgE value was ≥ 0.35 IU/ml.

6.2.2. Microfluidization (high shearing treatment)

A protein solution of SPI at 5% w/v was prepared by dissolving the SPI in distilled water under pH 7.0. The solution was stirred for 4 hours at room temperature (25°C) and then overnight at 4°C to achieve complete hydration. The SPI solution was then treated with the Microfluidizer Processor M-110EH (Microfluidics International Corporation) equipped with two Z-chambers, the first with a 200 µm micro-channel (H210Z) and the second of a 100 µm micro-channel (H10Z ceramic). The applied pressure was 137 MPa. Different numbers of passes through the interaction chamber were tested (1, 3, and 5 passes). Microfluidization was performed by controlling the temperature using a heat exchanger, and the temperature was set at 10°C (SC5000 Recirculating cooler, JULABO GmbH). The SPI solutions reached 26°C after one pass, then increased to 27.7°C after three passes, and it ended with 27.1°C passing 5 times in the microfluidizer. The untreated sample was used as a control. After treatment, microfluidized SPI solutions were frozen immediately before freeze drying or used for enzymatic treatment. Freeze-drying was done for 96 h (Christ Gamma 2-16 LSC 144 Plus), and then samples were ground and stored at 15°C before analysis.

The samples passed in the microfluidization system are represented by M1, M3, and M5, where 1, 3, and 5 represent the number of cycles used. On the other hand, M0 is the reference, which is a non-microfluidized sample.

6.2.3. Enzymatic hydrolysis of soybean protein isolate

Enzymatic hydrolysis was performed either on treated or non-treated SPIs. As described above, a 5 % w/v protein solution was prepared for non-treated SPI. Samples already treated with microfluidization were directly used as a substrate for hydrolysis.

Before selecting the optime parameters, preliminary tests were conducted (data not shown) to evaluate various enzyme concentrations and hydrolysis time. In this way, papain and Flavourzyme were used for hydrolyzation. Four enzyme concentrations were tested for papain: 2.5, 5, 10, and 20 U/g of SPI, and three for Flavourzyme: 12, 16, and 32 LAPU/g. Four different hydrolysis times were used for all ratios: 5, 15, 30, and 60 minutes (data not shown). These hydrolysis conditions were chosen based on the results of previous studies from the literature (Meinlschmidt et al., 2017; Meinlschmidt et al., 2016; Panda et al., 2015; Meinlschmidt et al., 2015; Cabanillas et al., 2010). As a result, Flavourzyme (12 U/g) was chosen for the following experiments presented in this article.

In this way, the enzymatic hydrolysis with Flavourzyme (12 U/g) occurred at 50°C for 15 minutes, under a pH of 7.0. In addition, the hydrolysis was stopped at 95°C for 10 minutes, and the samples were finally frozen before freeze-drying. A non-microfluidized SPI with the enzyme addition is named E. In this case, the control C is represented by the SPI without enzyme addition and treated under the same conditions (enzymatic hydrolysis for 15 minutes after being stopped at 95°C for 10 minutes).

6.2.4. Combined techniques: high shearing and enzymatic hydrolysis

The two treatments were combined by first applying microfluidization to SPI, followed by hydrolysis with Flavourzyme. Both treatments are described in detail in sections 6.2.2 and 6.2.3. Fig. 6-1 provides a schematic overview of the experimental plan conducted in this study.

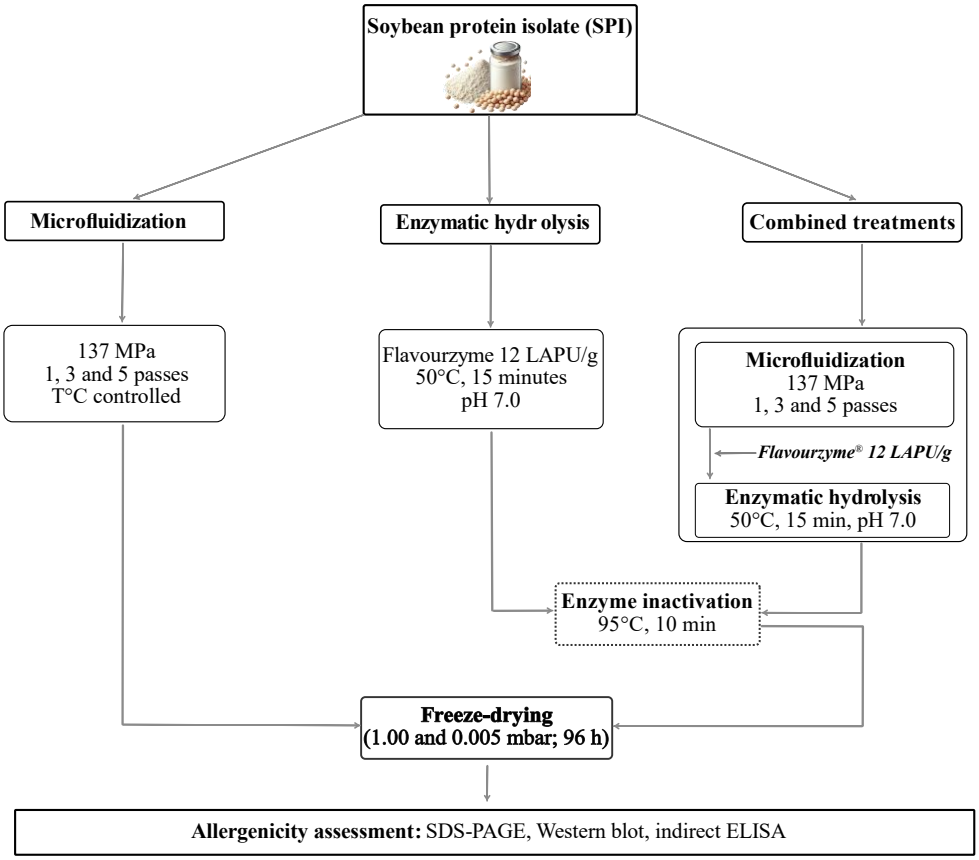


Figure 6-1. Roadmap of the experimental plan.

6.2.5. SDS-PAGE under reducing conditions and native polyacrylamide gel electrophoresis (Native-PAGE)

The impact of the treatments described above was analyzed using the SDS-PAGE method (Laemmli, 1970) under reducing conditions on the primary structure of proteins. In this way, 5% stacking gel and 12% or 15% separating gel (1 mm thick) were used (Blancher & Jones, 2001)The soybean proteins were solubilized in a Tris-SDS buffer containing 10 mM Tris HCl (pH=7.4), 4% (w/v) SDS, and a protease inhibitor.

Quantification of proteins was performed using the DC Protein assay kit for microplates from Bio-Rad. Then, the samples were heated at 70°C for 10 minutes. Subsequently, 20 µL of the solution, containing 20 µg of proteins, was loaded into each well of the gel. For the ladder was used 5 µL. SDS-PAGE under reducing conditions was carried out at room temperature, initially at a constant voltage of 60 V for 30 minutes, followed by 1 hour and 30 minutes at 150 V. Two molecular weight markers were used: PageRuler™ Prestained Protein Ladder, 10 to 180 kDa (26616),

Thermo Scientific™ and Precision Plus Protein™ Unstained Protein Standards, 10–250 kDa, Strep-tagged recombinant (1610363), Bio-Rad.

After electrophoresis, the gels were stained overnight in a Silver Blue solution. They were used for SDS-PAGE or Western Blot. Gel analysis was conducted using the ChemiDoc MP imaging system and the Image Lab 6.1 Software provided by Bio-Rad Laboratories.

Native-PAGE was performed following the same protocol, but without adding SDS, β-mercaptoethanol, and heating the samples (Arndt et al., 2012). Treated and non-treated samples were solubilized in pure water (pH 9), and the insoluble parts were removed by centrifugation at 10.000 x g, 20 min at 20°C.

6.2.6. Western Blot

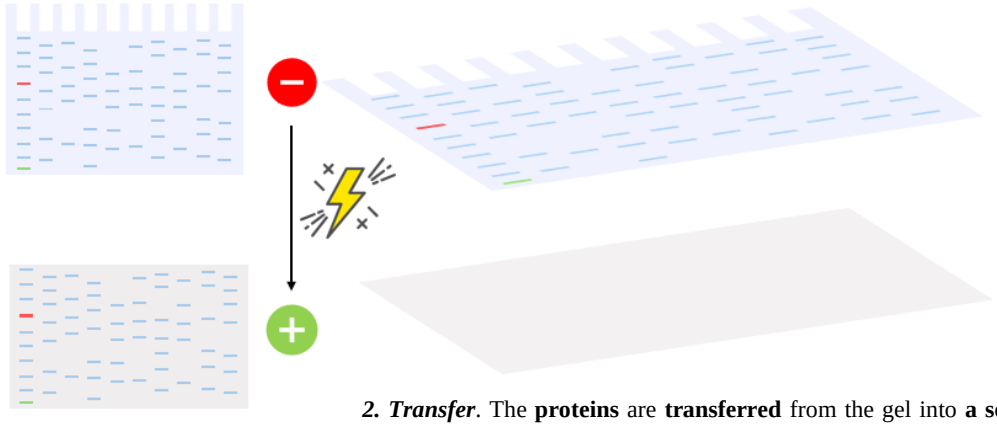
In order to perform the Western Blot analysis, the initial step involves the preparation of the gel (described above). Prestained gels were soaked in transfer buffer (methanol, Trizma base, glycine, and SDS). A Western Blot sandwich was assembled by placing two Whatman papers, the PVDF membrane (Immun-Blot PVDF Membrane, Bio-Rad Laboratories), the gel, and another Whatman paper, avoiding bubble formation. Protein transfer was done using the Trans-blot Turbo Transfer System (Bio-Rad Laboratories) at 1.3 A – 25 V constant for 7 minutes.

The transfer was confirmed with Ponceau S Staining solution (Thermo Fisher Scientific). The membranes were washed 3 times for 1 min in distilled water, stained with Ponceau S for 15 min at room temperature under agitation, and washed between 30 and 90 seconds with distilled water to achieve the desired staining. The blots were finally destained with a 0.1% NaOH solution for 2 min and rinsed in distilled water before blocking. The membranes were blocked with 5% Bovine Serum Albumin (BSA) in Tween TBS buffer (TBST) (25 mM Tris, 250 mM NaCl, 10 mM KCl, 0.1 % Tween 20, pH = 7,4) during 1 h at room temperature under gentle agitation and then washed 3 times for 5 min with TBST under agitation. The blocked membranes were incubated with 1/20 dilution of the human sera (15.4 kU/l and 6.48 kU/l) in TBST supplemented with 5% BSA for 2h at room temperature and washed 6 times for 8 min in TBST to remove unbound and non-specifically bound antibodies.

The secondary antibody (Goat Polyclonal anti-Human IgE Antibody Alkaline Phosphatase (AP) conjugated, Bethyl Laboratories) was added at 1/1000 in TBST + 5% BSA and incubated for 2h at room temperature. The membranes were once again washed 6 times for 8 min in TBST. Detection was performed using an AP substrate solution (0.1 M Tris pH 9.5, 0.1 M NaCl, 50 mM MgCl2·6H2O, 2% NBT/BCIP Stock Solution, Roche - Millipore Sigma). Revelation was stopped with distilled water when the desired staining was achieved. The blots were read after using the ChemiDoc MP imaging system and analyzed using the Image Lab 6.1 Software (Bio-Rad Laboratories). Figure 6-2 presents a schematic overview of the Western Blot method, briefly describing each step involved.

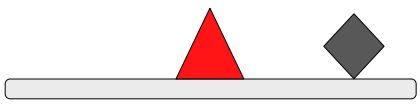
Western Blot method

1. Separation by size. **SDS-coated** proteins, **negatively charged**, migrate towards the anode in an electric field, **separating** based on their **size**.

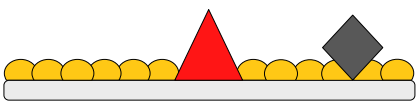


2. Transfer. The **proteins** are **transferred** from the gel into **a solid support,** using **electrical current.**

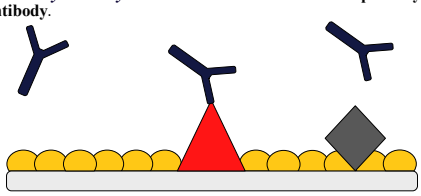
3. Successful Transfer. Proteins are **immobilized** on the membrane and **accessible** for **antibody binding.**



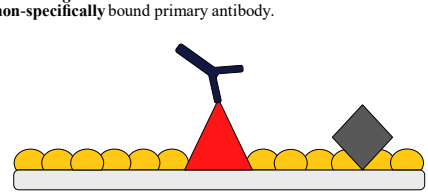
4. Blocking Membrane. The membrane is incubated with a **blocking** solution to **occupy all protein-binding sites on the membrane.**



5. Primary Antibody. The membrane is **incubated** with **primary antibody**.



6. Washing. The membrane is **washed** to remove **unbound** and **non-specifically** bound primary antibody.



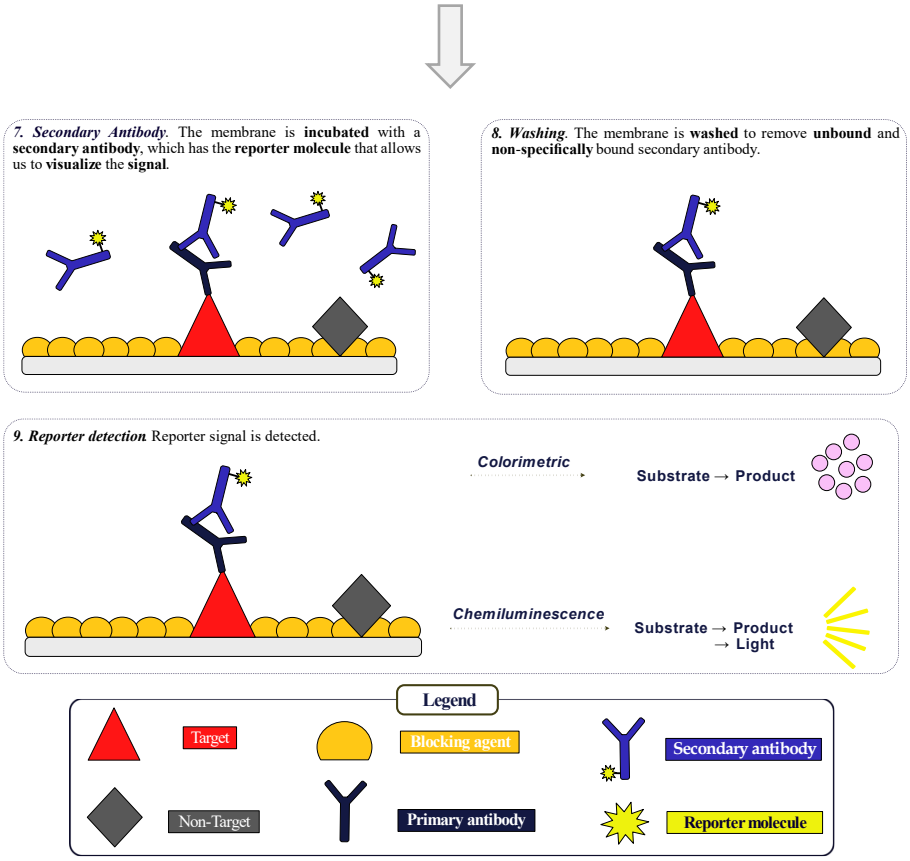


Figure 6-2. Schematic representation of Western Blot with colorimetric detection.

6.2.7. Indirect enzyme-linked immunosorbent assay (ELISA)

After protein solubilization in 10 mM Tris buffer pH 9.5, samples were diluted to a protein concentration of 2.5 µg/ml in carbonate buffer (14 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). The wells of a MaxiSorp Clear Flat-Bottom Immuno Nonsterile 96-Well Plates (Nunc, Thermo Fischer Scientific) were coated with 100 µL of these solutions for 18h at 4°C. Between each step, the plate was washed 5 times with 250 µL of TBST (25 mM Tris, 250 mM NaCl, 10 mM KCl, 0.05% Tween 20, pH = 7,4).

Wells were blocked with 100 µL of TBST supplemented with 2% BSA for 1h at 37°C in a moist atmosphere (to avoid drying the wells). The sera used (15.4 kU/l and 6.48 kU/l) were diluted 1/10 in TBST + 2% BSA, and after 5 washes, the plates were incubated with 100 µL of diluted serum in each well for 1h30 at 37 °C in a moist atmosphere. After 5 washes, 100 µL of a 1/1000 dilution of Goat Polyclonal anti-Human IgE Antibody Alkaline Phosphatase (AP) conjugated (Bethyl Laboratories) were added to the plates and incubated for 1h at 37°C. Finally, after 5 new washes, 100 µL of the revelation solution (1 mM $MgCl_2 \cdot 6H_2O$, 9.7% diethanolamine, 1 tab of

4-nitrophenyl phosphate disodium salt per 5 ml (Agdia) were added to the plates. These were placed in the dark for 2h before reading the absorbance at 450 nm with the iMark microplate absorbance reader (Bio-Rad Laboratories).

A standard curve was also prepared following the same protocol by diluting untreated SPI control at concentrations of 0.5, 0.75, 1.0, 2.5, 3.5, 5.0, 10, and 20 µg/ml to verify the analysis response linearity. Two negative controls were used: wells coated only with carbonate coating buffer, all the following steps being realized in the same way as described above, and wells coated only with carbonate coating buffer and blocked but incubated with TBST + 2% BSA without antibodies in the following steps. Each sample was analyzed in duplicate. Reduction in immunoreactivity of protein samples was determined using the following equation:

$$\frac{ABS_{sample}-ABS_{blank}}{ABS_{SPI}} x\ 100$$

- ABS_{sample}: the absorbance of each treated sample
- ABS_{blank}: the absorbance of blank
- ABS_{SPI}: the absorbance of the non-treated sample, a native SPI

The following Figure (6-3) presents a schematic overview of the indirect ELISA method, briefly describing the main steps involved.

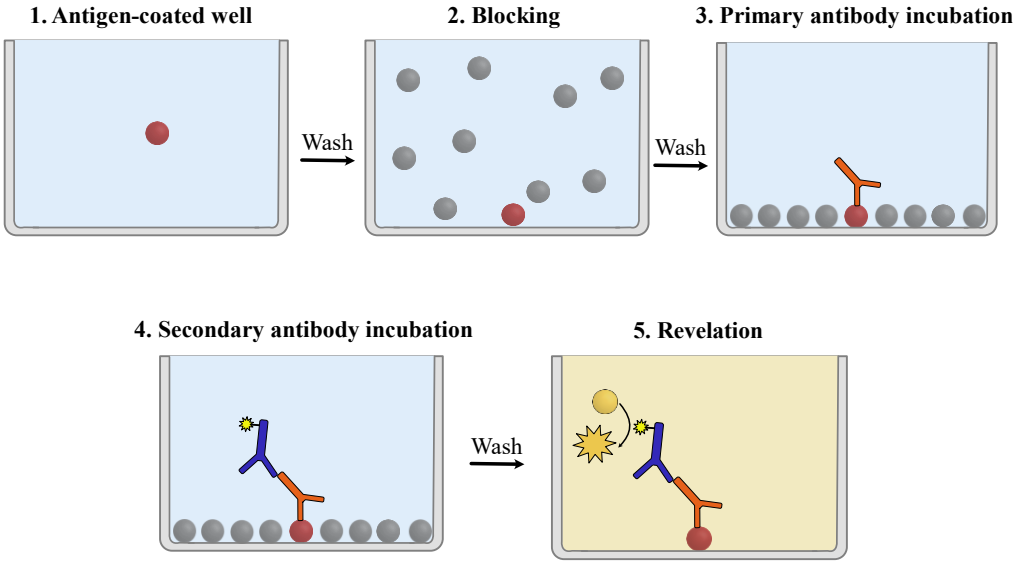


Figure 6-3. Schematic representation of indirect ELISA, including the main steps.

6.2.8. Statistical analysis

Statistical analysis was performed with RStudio (2023.06.0 +421). All data were analyzed using one-way analysis of variance (ANOVA), and differences between means were evaluated using Tukey's HSD test with a p-value < 0.05 for significant differences.

6.3. Results and discussion

In this part, the outcomes are divided into three main categories: the impact of microfluidization (6.3.1), enzymatic treatment (6.3.2), and their combination (6.3.3) on soybean allergens. Each category will discuss allergenicity assessment for SDS-PAGE, Western Blot, and indirect ELISA.

6.3.1. Effect of microfluidization on soybean allergens

It is well known that during microfluidization, two phenomena can occur: particle disruption and/or protein aggregation formation (Gong et al., 2019). The intense energy input of microfluidization causes significant particle disruption within the interaction chamber, creating new interfaces as particles break apart (Jafari et al., 2007). However, these newly formed particles are thermodynamically unstable. Under high pressure, velocity, vibrations, cavitation, and shear forces, collisions between particles can lead to re-aggregation. The results from the following sections demonstrate these phenomena.

6.3.1.1. SDS-PAGE under reducing and denaturation conditions

Fig. 6-4 (a) shows the SDS-PAGE profile obtained for the untreated and treated samples. Under reducing conditions, the SDS-PAGE showed a molecular weight ranging from 15 to 150 kDa.

The three prominent bands correspond to β-conglycinin subunits (Gly m 5), considered one of soybean's main allergens. These are α', α, and β (Wiederstein et al., 2023) with a molecular weight of ~76 kDa, ~72-74 kDa, and ~48-50 kDa, respectively. Another significant allergen, glycinin (Gly m 6) is presented in the gel. Its acidic and basic chains have ~33-40 kDa and ~20-22 kDa (Q. Li et al., 2023; L'Hocine & Boye, 2007). Gly m Bd 30 K was also detected, and it is a highly allergenic storage protein with the potential to induce atopic dermatitis (Mulalapele & Xi, 2021).

Under reducing conditions, the band intensities of the microfluidized samples at 137 MPa did not change with the increasing number of cycles (1, 3, and 5). These results suggest that microfluidization does not impact the primary structure of soy proteins, which is consistent with what has been observed in the literature (Yang et al., 2023; Gong et al., 2019; Chen et al., 2019).

For example, a study conducted on Ara h 2 under different pressures of 60, 90, 120, 150, and 180 MPa showed that microfluidization treatment did not affect the primary structure of the peanut allergen (C. qiu Hu et al., 2011). Another study examined the effects of ultrasonication (24 kHz, 100 W) over 5–60 minutes on faba bean isolates. Electrophoresis showed no significant changes in the primary structure of the native

and sonicated proteins (Badjona et al., 2024). The band intensities of the α, α', and β subunits in lecithin-β-conglycinin complexes also remained unchanged across heating temperatures (40°C, 60°C, 80°C) in the SDS-PAGE analysis (H. Yang et al., 2023). Moreover, both untreated and treated SPI samples showed similar protein profiles, containing β-conglycinin and glycinin, indicating that neither ultrasonic nor hydrodynamic cavitation affected the molecular weights of SPI (Ren et al., 2020). To conclude, the linear epitopes, derived from short contiguous segments of eight to ten amino acids (X. Li et al., 2015), may not be directly influenced by the pressure and the number of cycles applied in this study.

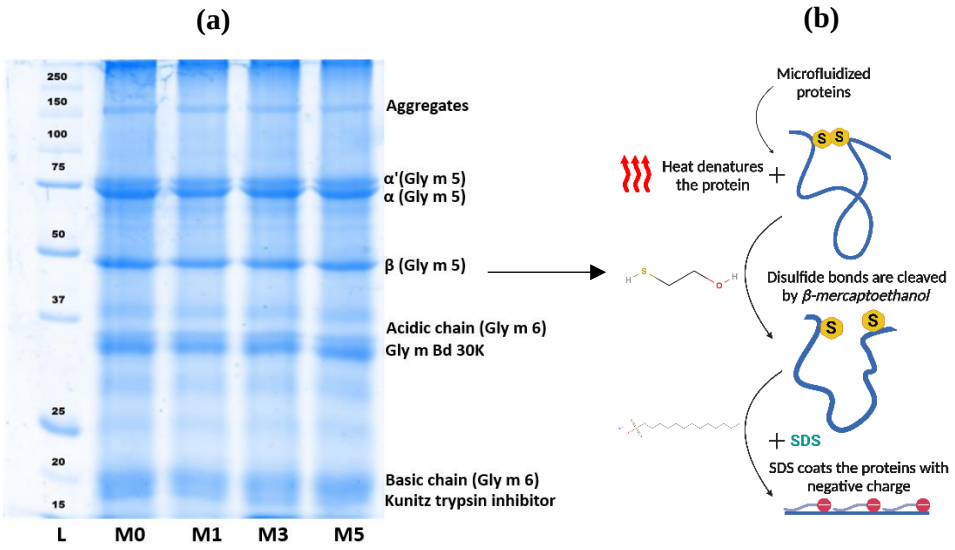


Figure 6-4. **(a)** SDS-PAGE (12 % separating gel) of untreated and microfluidized SPIs. Lane 1- unstained marker (kDa). Lane 2 - untreated native SPI (M0). Lanes 3, 4, and 5 - microfluidized samples 1, 3, and 5 pass(es) (M1, M3, M5); **(b)** Proteins under reducing and denaturing conditions become linear, allowing them to be separated by MW.

6.3.1.2. Native-PAGE

Native PAGE patterns allow the detection of high-shearing effects on the modifications of soybean allergens without the presence of SDS, reducing agent and sample heating, thereby eliminating interference produced by these. Native electrophoresis provides information based on their native charge and size without denaturing the proteins (H. Zhang et al., 2010).

Fig. 6-5 illustrates the Native-PAGE protein profile of soybean allergens, highlighting the outcomes under non-reducing conditions produced by the untreated and treated samples. In this case, it can be observed that there are no significant differences between the native SPI and the microfluidized ones. The only slight difference is that the sample passed 3 times in the system, where the band's intensity is less than the other treatment conditions containing the allergen with a molecular weight of ~70 kDa (β-conglycinin).

Additionally, it is unexpected that the intensity of the bands is less compared with the reducing conditions. However, other researchers have already observed this phenomenon (Shen & Tang, 2012), who used the same technique, microfluidization, but additionally preheated the samples at 75, 85, and 95°C. Another interesting aspect is related to the aggregates, which did not enter the separating gel. This could be due to proteins in the form of aggregates through inter-subunit disulfide bonds (Shen & Tang, 2012).

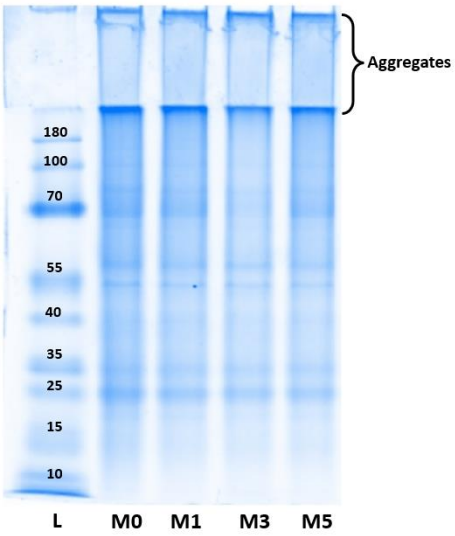


Figure 6-5. Native-PAGE of untreated and microfluidized SPIs. Lane 1- prestained marker (kDa). Lane 2 - untreated native SPI (M0). Lanes 3, 4, and 5 - microfluidized samples 1, 3, and 5 pass(es) (M1, M3, M5).

6.3.1.3. Western Blot using human serum

The effect of microfluidization treatment on the Western Blot of SPIs is illustrated in Fig. 6-6. Results showed no significant decrease in IgE binding or band intensity between the untreated (M0) and the treated SPIs (M1, M3, M5). As mentioned, allergens are stable at processing, and soybean allergens investigated in our work may have a higher thermostability (Cuadrado et al., 2011).

In addition, the absence of detectable changes in the Western Blot may also be attributed to the lack of significant effect of microfluidization on the antigenic properties of the proteins. While microfluidization can induce structural changes in proteins (Sethi et al., 2022; Guo et al., 2020). These changes may be insufficient to alter the accessibility of epitopes recognized by the antibodies used in the Western Blotting assay.

However, it is also crucial to consider that proteins are separated under reducing and denaturing conditions during SDS-PAGE prior to Western Blotting. It is assumed that conformational epitopes present in the samples are denatured during the preparation

of the SDS-PAGE, thus becoming unrecognized by the antibodies (Zhou et al., 2007). However, this hypothesis is still incomplete or contradictory in the literature.

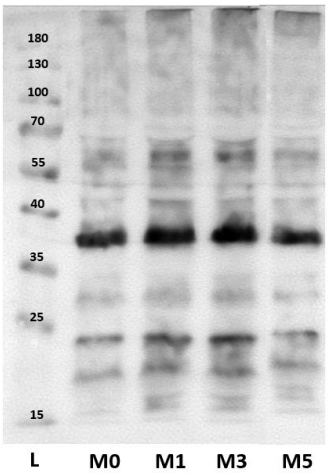


Figure 6-6. Western Blot (12 % separating gel) (IgE immunoblot of individual serum 15.4 kU/l). Lane 1- prestained marker (kDa). Lane 2 - untreated native SPI. Lane 3, 4, and 5 - microfluidized samples 1, 3, and 5 pass(es).

6.3.1.4. IgE-binding of soybean proteins using indirect ELISA

The ELISA method was instrumental in quantifying the percentage of the allergenic response for each treatment studied. Thus, the results for the soybean-allergic patient are shown in Fig. 6-7 (a) with an IgE level of 15.4 kU/l. The microfluidization performed at 3 passes shows a level of immunoreactivity significantly different from the untreated SPI. Its level is considerably higher and reached 123.54% of initial immunoreactivity.

For 1 and 5 cycles, residual immunoreactivity levels ranged from 89.81% to 97.82% and were non-significantly different from the native SPI level. This increase in immunoreactivity for some treatment parameters might be attributed to structural modifications resulting from high-shearing treatment, leading to exposure of previously masked epitopes. These modifications can, for example, include protein unfolding or disaggregation, creating more new interfaces available (Ozturk & Turasan, 2021; H. Chen et al., 2019; Zhong et al., 2011). The exposed epitopes thus become accessible to antibodies, which can then bind to them, increasing immunoreactivity (H. Chen et al., 2019). Similar results were observed for serum 6.48 kU/l as shown in Fig. 6-7 (b). The immunoreactivity for M1E decreased to 87.4%, but this reduction was not significantly different from the native SPI ($p > 0.05$). On the other hand, an increase in immunoreactivity was noted for both M3E (129.9%) and M5E (112.5%).

Similar findings have been reported in the literature, where microfluidization was applied to β-lactoglobulin for 3 passes at different pressures. The antigenicity of β-

lactoglobulin was higher at 0.1 MPA (13.41 µg mL-1) and 80 MPa (12.27 µg mL-1), but it decreased at 160 MPa (7.19 µg mL-1) (H. Chen et al., 2019). The changes resulting from microfluidization have subsequently been associated with increased protein digestibility, ultimately reducing allergenicity (H. Chen et al., 2019; Zhong et al., 2014).

Different studies described the connection between increased digestibility and reduced allergenicity, notably in the case of the application of high-pressure or microfluidization treatment to food proteins (H. Chen et al., 2019; Rahaman et al., 2016; Zhong et al., 2014). However, other researchers have demonstrated no strict relationship between digestibility and allergenicity (Bøgh & Madsen, 2016). However, considering some results are contradictory, a closer inspection is needed to fully elucidate the correlation between digestibility and allergenicity.

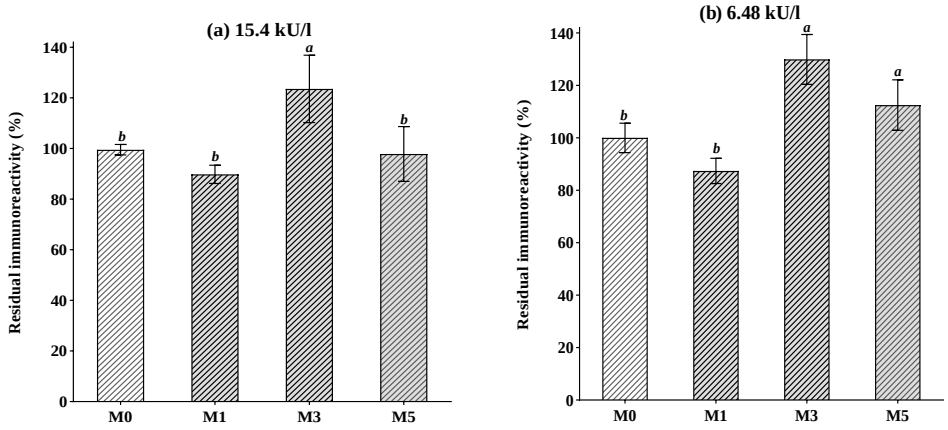


Figure 6-7. **(a)** Indirect ELISA using 15.4 kU/l and **(b)** 6.48 kU/l. The residual immunoreactivity (%) for untreated native SPI (M0) and MF samples 1, 3, and 5 pass(es) (M1, M3, M5).

To illustrate these results, Fig. 6-8 presents how microfluidization treatment under the applied conditions increased soybean allergens. The figure shows proteins in their natural state, where some epitopes may be hidden. However, when exposed to pressure and shear forces, the proteins unfold or disaggregate, revealing these epitopes. This exposure triggers an immune response as IgE antibodies bind to the exposed epitopes, activating mast cells. Consequently, the immune system reacts more strongly due to recognizing these newly exposed protein regions. The presented epitopes are found in the α-helix structure, but this serves only as an illustrative example.

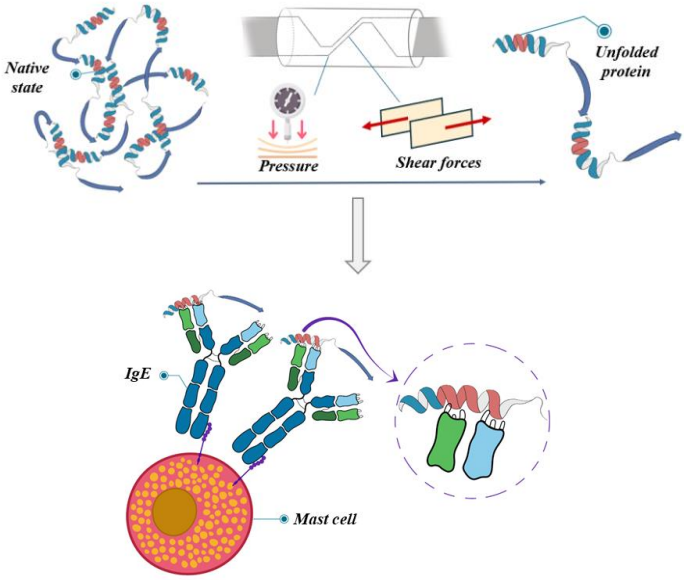


Figure 6-8. Effect of pressure and shear forces on protein unfolding and epitope recognition.

6.3.2. Effect of enzymatic treatment on soybean allergens

Due to enzymatic hydrolysis, conformational epitopes undergo a rapid collapse, while linear epitopes are cleaved, and their persistence depends on the specific enzyme employed and the degree of hydrolysis (Rahaman et al., 2016). The selected enzyme for this study, Flavourzyme, constitutes a blend of endoprotease and exopeptidase activities. This mixture selectively liberates N-terminal amino acid residues, thereby facilitating the degradation of bitter peptides (Meinlschmidt, Schweiggert-Weisz, et al., 2016). Reducing bitterness is essential for a hypoallergenic product to be acceptable to consumers; hence, this type of enzyme was chosen.

6.3.2.1. SDS-PAGE under reducing and denaturation conditions

The SDS-PAGE profiles of native SPI, control, and sample exposed to enzymatic hydrolysis are shown in Fig. 6-9. The control (C) is represented by the SPI without enzyme addition and treated under the same conditions (enzymatic hydrolysis for 15 minutes after it was stopped at 95°C for 10 minutes). Upon examination of the sample subjected to enzymatic hydrolysis (E), Flavourzyme showed considerable changes in the SDS- PAGE profile. It can be observed that the intensity of the band is very low compared with native SPI and control.

In this way, Flavourzyme seems highly efficient for the chosen hydrolysis parameters and helps to detect the modifications visibly in the SDS-PAGE profile. As an illustration (Fig. 6-13 (b)), the adjusted volume (intensity) of the hydrolyzed sample (lane 4) was reduced by 65.63% compared with the control (lane 3).

This parameter is the sum of all the intensities in the volume, with the background subtracted, which was done using the Image Lab Software 6.1 from Bio-Rad. In addition, the bands still presented in the gel can be associated with peptides resulting from the hydrolysis of higher molecular weight proteins (Panda et al., 2015).

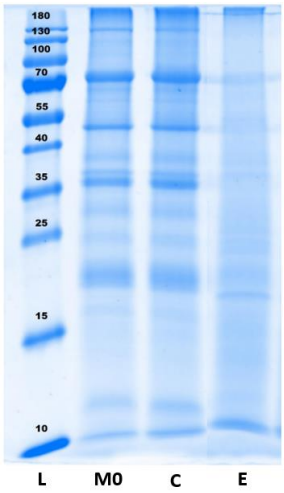


Figure 6-9. SDS-PAGE (15 % separating gel) of untreated and hydrolyzed SPI. Lane 1- prestained marker (kDa). Lane 2 - untreated native SPI (M0). Lane 3 – control, represented by the SPI without enzyme addition and treated under the same conditions (C). Lane 4 - hydrolyzed SPI (E).

6.3.2.2. Western Blot using human serum

The effect of enzymatic hydrolysis on the IgE-binding of soybean proteins is shown in Fig. 6-10. Multiple bands were detected for the control (C) and native SPI (M0), which corresponds mainly to α, α', and β subunits of β-conglycinin (Gly m5) and acidic and basic chains subunits of glycinin (Gly m6). This means the antibody was strongly recognized when the untreated samples were incubated with the individual serum (15 kU/l) from the patient allergic to soybean. Moreover, no bands were detected when the soybean was exposed to the enzymatic treatment with Flavourzyme (lane 4). This result is consistent with the literature (Cabanillas et al., 2010; Lee et al., 2007). Moreover, the presumed big allergens Gly m5 and Gly m6 were not recognized anymore by the antibody. However, a slight interaction between the antibody-antigen can still be detected on the membrane (lane 4), confirmed further by the ELISA technique.

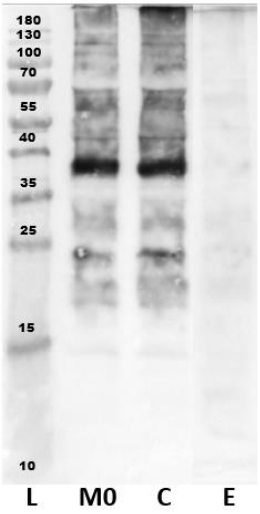


Figure 6-10. Western Blot (15 % separating gel) (IgE immunoblot of individual serum 15.4 kU/l). Lane 1- prestained marker (kDa). Lane 2 - untreated native SPI (M0). Lane 3 - control (C). Lane 4 - hydrolyzed SPI (E).

6.3.2.3. IgE-binding of soybean proteins using indirect ELISA

The ELISA test was conducted to evaluate the decrease in the antigenic activity of protein hydrolysates. This assessment was carried out using the same human sera with concentrations of 15.4 kU/l and 6.48 kU/l. As can be observed in Fig. 6-11 (a), between native SPI and control, there is no significant difference ($p > 0.05$). However, despite no significant difference, the IgE binding increased for the control and reached 105.10%. This observation was also applied when the treated samples were incubated with the second serum (Fig. 6-11 (b)), where the value reached 102.3%. This can be due to the thermal treatment, which can lead to the exposure or generation of new epitopes (Pi et al., 2021; Kranthi Vanga et al., 2017; Rahaman et al., 2016; Shriver & Yang, 2011).

Regarding the protein structure, the formation of new intra- and/or intermolecular covalent or non-covalent bonds and the rearrangement of disulfide bonds can also be observed (Rahaman et al., 2016; Shriver & Yang, 2011). The heat treatment applied in this case for the control is without enzyme addition but treated under the same conditions (enzymatic hydrolysis for 15 minutes at 50°C after being stopped at 95°C for 10 minutes).

On the other hand, compared with the native SPI and control, the sample treated with enzymatic hydrolysis (E) declined at 46.84% (15 kU/l) and 36.80% (6.48kU/l) of the allergenicity degree and a significant difference ($p < 0.05$) between them can be observed. For example, a group of researchers studied the effect of enzymatic hydrolysis on lentil allergenicity. In this case, ELISA indicated complete destruction of antigenic epitopes after Flavourzyme hydrolysis (degree of hydrolysis = 56%). On the other hand, immunoblotting revealed that two sera still detected IgE-binding

proteins (12, 14, and 45–48 kDa) after 300 minutes, while two other sera faintly recognized 18 kDa protein and putative Len c 1 in the same sample (Cabanillas, Pedrosa, Rodríguez, et al., 2010). In addition, previous studies have shown that soy proteins are generally resistant to proteolysis, as noted by different researchers (Govindaraju & Srinivas, 2007; Henn & Netto, 1998) .This resistance is mainly due to the compact quaternary and tertiary structures of globular soy proteins, which protect many peptide bonds from enzymatic attack (L. Chen et al., 2016). The effectiveness of proteolysis in breaking down these proteins is influenced by factors such as the specificity of the protease used, the degree of hydrolysis (DH), and the characteristics of the substrate (Tavano, 2013).

In this way, most of the residual immunoreactivity seems to come from the peptides derived from enzymatic hydrolysis (Panda et al., 2015). These were not further degraded with the enzyme/substrate ratio chosen.

Another essential aspect is the hydrolysis time. An excessive amount of time can improve the residual immunoreactivity, but it also may form bitter peptides, thereby impacting the flavor profile of proteins and influencing their suitability for food applications. The bitterness comes from the low molecular weight peptides, mainly composed of hydrophobic amino acid residues (Cruz-Casas et al., 2021; Zhao et al., 2024).

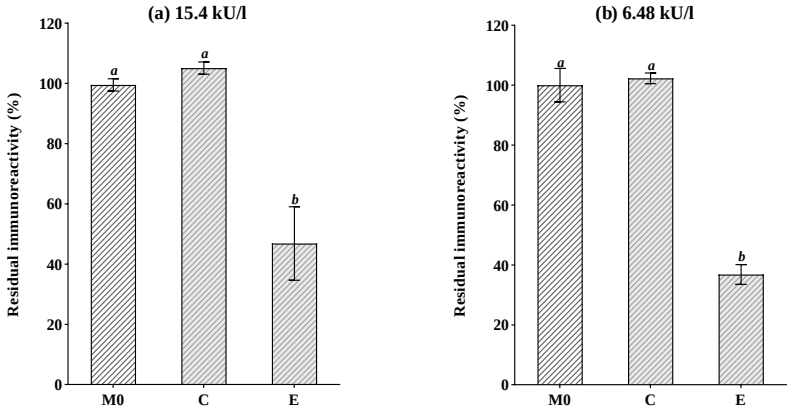


Figure 6-11. **(a)** Indirect ELISA using 15.4 kU/l and **(b)** 6.48 kU/l. The residual immunoreactivity for untreated native SPI (M0), control (C), and hydrolyzed SPI (E).

To illustrate these results, Fig. 6-12 (see below) presents how enzymatic hydrolysis under the applied conditions efficiently reduced soybean allergens. In this way, the soybean protein, in its native state, has some epitopes that may be hidden within its folded structure. Following enzymatic hydrolysis, the protein breaks down into smaller peptides. Some epitopes may remain unchanged, others may be shortened, and some could be destroyed. This reduction in epitope size and conformational changes make these peptides less detectable by the immune system.

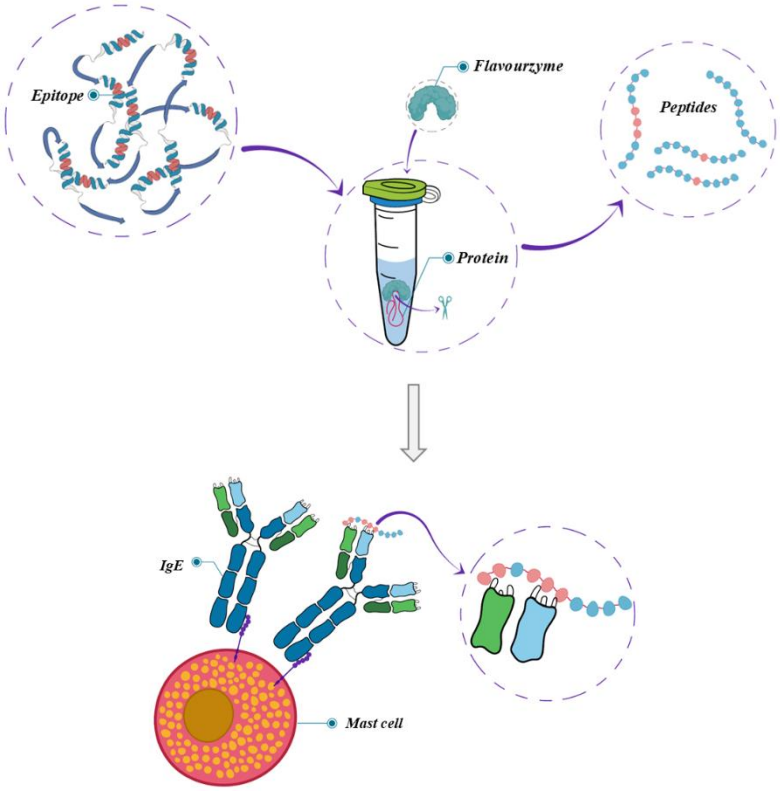


Figure 6-12. Impact of enzymatic hydrolysis on protein structure and epitope recognition.

6.3.3. Effect of combined microfluidization and enzymatic treatments on soybean allergens

Microfluidization can serve as a pretreatment along with enzymatic hydrolysis to decrease allergens. This process enhances protein accessibility to enzymes and improves hydrolysis efficiency (L. Zhang et al., 2021; Guo et al., 2020; H. Chen et al., 2019). Interestingly, during the processing of the commercial SPI, the protein is more susceptible to forming aggregates, which can further result in burying the cleavage sites (L. Chen et al., 2016). Microfluidization has been shown to not only modify the structure of globular proteins but also break down insoluble, heat-induced protein aggregates into smaller and soluble ones. This process exposes internal groups previously hidden inside the folded protein structure (Shen & Tang, 2012). By unfolding the proteins, microfluidization exposes hydrophobic and hydrophilic regions, thereby increasing the available area for enzymatic action and facilitating enzyme binding and subsequent hydrolysis (L. Chen et al., 2016). In this way, a pre-treatment can be necessary to modify the structural characteristics of soybean proteins to enhance the accessibility of the enzyme and better reduce allergens.

6.3.3.1. SDS-PAGE under reducing and denaturation conditions

SDS-PAGE profiles of SPI treated with microfluidization and hydrolyzed with Flavourzyme are shown in Fig. 6-13 (a). The protein profile indicates that hydrolyzed SPI (E) and SPI hydrolyzed after microfluidization (M1E, M3E, and M5E) have similar protein primary structures. This suggests that microfluidization does not significantly improve SPI hydrolysis by Flavourzyme, possibly due to the lack of microfluidization efficiency or the high accessibility of SPI proteins to enzymes.

However, in the literature, microfluidization has been proven effective in enhancing enzymatic treatments on various substrates such as peach pomace, milk, rice, or soy proteins (L. Zhang et al., 2021; Chen et al., 2019; L. Chen et al., 2016; Xu et al., 2015; Zhong et al., 2011).

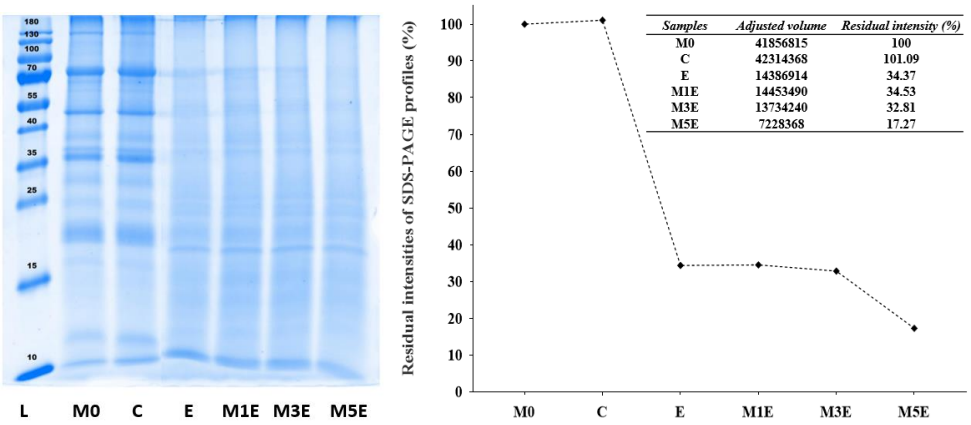


Figure 6-13. (a) SDS-PAGE (15 % separating gel) of untreated and hydrolyzed SPI. Lane 1- prestained marker (kDa). Lane 2 - untreated native SPI (M0). Lane 3 – control (C). Lane 4 - hydrolyzed SPI (E); Lane 5, 6, 7 – combined treatments (M1E, M3E, M5E); **(b)** Percentage of the residual intensity (%) for the untreated, control, hydrolyzed SPI and combined treatments.

Moreover, an interesting aspect concerning the protein profile is the intensity of the bands, as previously discussed in Section 6.3.2.1. Thus, as shown in (Fig. 6-13 (b)), a noticeable decrease and differences in intensity are noticed when comparing the treated samples (microfluidization + enzymatic) with the native SPI.

For instance, after passing through the microfluidization system once with enzymatic treatment (M1E), the lane intensity decreased by 65.47%. Notably, the intensity reduction continues with more passes; in M3E, the intensity reduced by 67.19%. This trend persists even when the sample is passed through the microfluidization system five times, resulting in an 82.73% reduction.

Furthermore, upon examining the control, there was a 1.09% increase in the intensity of the bands. This could be attributed to the thermal treatment (Pi et al., 2021; Kranthi Vanga et al., 2017; Rahaman et al., 2016; Shriver & Yang, 2011). The

intensity parameter represents the total intensity within the volume after background subtraction, which was performed using Bio-Rad's Image Lab Software 6.1. The untreated SPI was used as a reference. The intensity values of C, E, M1E, M3E, and M5E were normalized by dividing each by the value of M0 and then multiplied by 100 to obtain the corresponding percentages.

6.3.3.2. Western Blot using human serum

The Western Blot analysis enhances the SDS-PAGE findings, offering more insights into the combing treatments. Untreated native soybean and the control exhibit pronounced signals (Fig. 6-14), indicating that the patient reacts with mostly allergenic proteins, especially Gly m5 and Gly m6.

The lanes corresponding to the combined treatments (M1E, M3E, and M5E) show decreased signal intensity. However, when comparing the enzymatic treatment alone to the combined treatment, the pattern of remaining allergens appears to be consistent.

The intensity of M1E and M3E are more pronounced. Additionally, the faint bands in the blot could be attributed to the increased immunoreactivity of specific proteins, which may be due to the exposure of new epitopes or the generation of peptides from the breakdown of larger proteins that retain some immunoreactivity (Panda et al., 2015).

Despite the general reduction in immunoreactivity observed for the hydrolyzed proteins, the application of microfluidization before the enzymatic treatment does not have an additional effect on the allergenicity of soybean proteins.

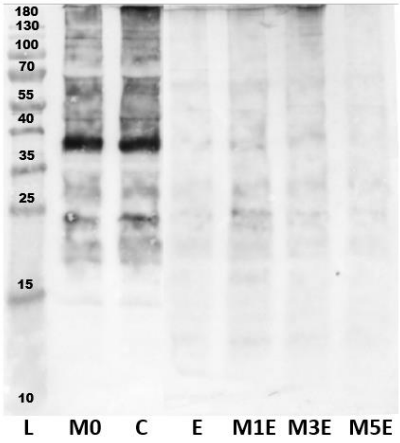


Figure 6-14. Western Blot (15 % separating gel) (IgE immunoblot of individual serum 15.4 kU/l). Lane 1- prestained marker (kDa). Lane 2 - untreated SPI (M0). Lane 3 – control (C). Lane 4 - hydrolyzed SPI (E); Lane 5, 6, 7 – combined treatments (M1E, M3E, M5E).

6.3.3.3. IgE-binding of soybean proteins using indirect ELISA

Fig. 6-15 (a) and (b) illustrate the results of ELISA assessing the immunoreactivity of soybean allergens by combined microfluidization (MF) and enzymatic hydrolysis. The data indicate that the sample treated with one pass of microfluidization and enzymatic hydrolysis (M1E) exhibits a residual immunoreactivity of 45.87%, which is close to the sample treated with enzymatic hydrolysis alone (46.84%). After three treatment passes at 137 MPa, the residual immunoreactivity is reduced to 35.92%. A further increase to five passes yields a residual immunoreactivity of 39.93%, which is not statistically different from the three-pass treatment, suggesting a limit to the treatment's efficacy. These results were obtained for the concentration of 15.4 kU/l.

In Fig. 6-15 (b), a similar trend is observed using the second serum (6.48 kU/l). Enzymatic hydrolysis alone results in a residual immunoreactivity of 36.80%. For the combined treatments, M1E starts at 44.10%, while M3E and M5E yield 36.80% and 40.30%, respectively.

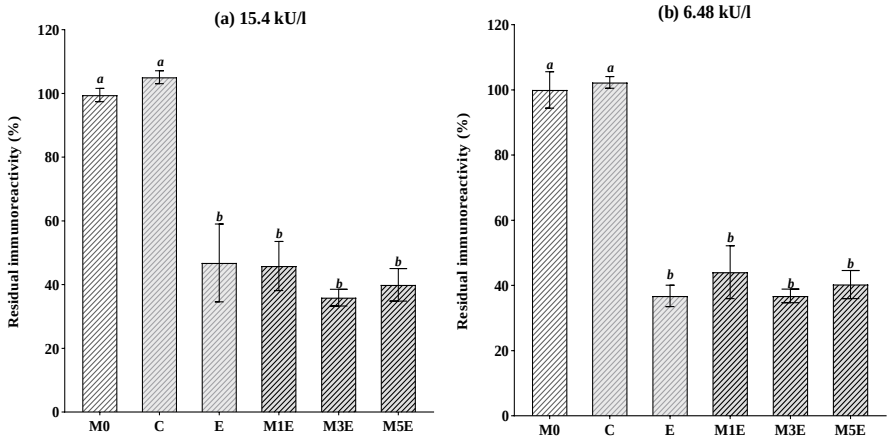


Figure 6-15. **(a)** Indirect ELISA using 15.4 kU/l and **(b)** 6.48 kU/l. The residual immunoreactivity for untreated native SPI (M0), control (C), hydrolyzed SPI (E), and combined treatments (M1E, M3E, M5E).

Despite the lack of significant differences among the combined treatments, all samples subjected to microfluidization and enzymatic hydrolysis (M1E, M3E, and M5E) demonstrate a reduction in immunoreactivity relative to the untreated control (M0) and the control sample (C).

This reduction implies that microfluidization, potentially in synergy with enzymatic hydrolysis, effectively lowers the allergenic potential of soybean proteins, as reflected by the decreased residual immunoreactivity observed. Inactivating enzymes through heat treatment in the hydrolysates may also contribute to diminished antigenicity (Peñas, Snel, et al., 2006).

The combination of microfluidization's mechanical action and enzymatic biochemical action theoretically enhances allergen reduction. As illustrated in Fig. 6-

16, this process leads to more efficient hydrolysis. The unfolded proteins allow enzymes to bind more effectively, breaking peptide bonds and partially degrading some epitopes, which will be less recognized by the immune system. However, further investigations are required for their combination.

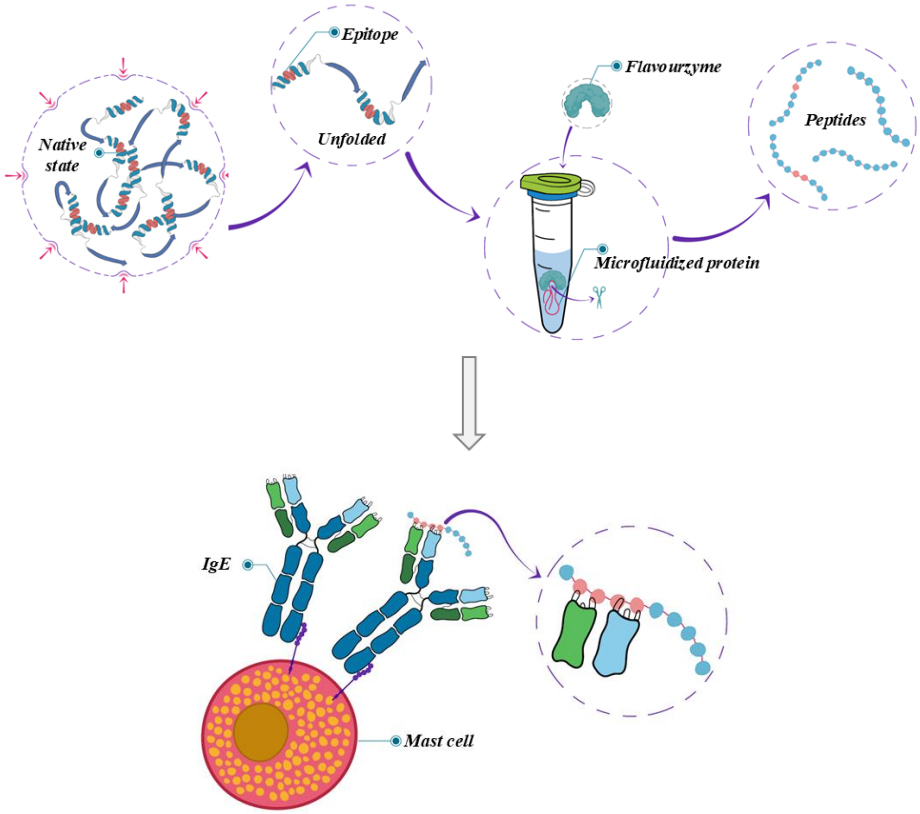


Figure 6-16. Combined effects of microfluidization and enzymatic hydrolysis on soy protein structure and epitope recognition.

6.4. Conclusions

Microfluidization alone did not affect the primary structure of soy proteins across different passes (1, 3, and 5) under both reducing and non-reducing conditions. The major allergens (Gly m5, Gly m6, Gly m Bd 30K, Kunitz trypsin inhibitor) were present in all samples and strongly recognized by the patient with a clinical allergy to soybean. Moreover, microfluidization alone, at 3 and 5 passes, increased immunoreactivity (123.54% at 15.4 kU/L for 3 passes, and 129.9% and 112.5% at 6.48 kU/L for 3 and 5 passes, respectively) compared to untreated SPI. This increase might be attributed to structural modifications resulting from high-shearing treatment, leading to exposure of previously masked epitopes due to the disruption of particles

and the formation of new available sites. These modifications can include protein unfolding or disaggregation. Moreover, enzymatic treatment with Flavourzyme alone significantly ($p < 0.05$) reduced soybean allergens by 53.16 % and 63.2 %, although some residual peptides remained visible on the gel and blots. Hydrolysis duration is important as excessive time can enhance residual immunoreactivity but potentially generate bitter peptides affecting flavor profiles. Combining microfluidization with enzymatic treatment should be confirmed because of the high variability of the enzymatic treatment alone.

7

General discussion

Chapter 7. General discussion

7.1. Characterization of the soy protein structure

This study has comprehensively examined soybean allergenicity and the relation between protein structure and the reduction of allergens. The research has integrated various analytical techniques with a dual objective: (1) to analyze the impact of microfluidization on soybean structure and (2) to explore the relationship between the structural changes and the presence of allergens. Given that proteins are the primary triggers for allergic reactions, we assumed that modifying soybean protein structure through microfluidization would also affect their allergenic properties.

The initial phase of the experimental work highlighted structural differences in proteins between the microfluidized samples. When applying microfluidization, it is important to account for various processing conditions. A key challenge is to delimit the specific effects of pressure, shear, and temperature on the proteins, as these come from the microfluidization process.

This treatment allows for either temperature control or not during the process. With temperature control, only shear forces and pressure affect the process, avoiding thermal effects. Without control, the temperature rises with each pass, meaning the process is influenced by shear, pressure, and heat generated from the high shear and pressure in the chamber.

It is also interesting to note that beyond a certain point, no further impact is observed, and the system reaches equilibrium, with results remaining consistent. For most secondary structure elements, one to three passes of microfluidization are sufficient. This is economically advantageous as it reduces scaling challenges, such as increased energy consumption, production volume, and other cost-related factors. This observation was applied to the samples in powder form.

Additionally, we aimed to investigate whether the structure of these microfluidized samples remained unchanged when reconstituted in water. Surprisingly, the protein behavior changed upon reconstitution in water, impacting only few structural elements. This finding highlights the significance of the protein state when selecting conditions for a specific application, such as foaming or emulsifying, to achieve the desired final product. Shear and pressure from microfluidization mainly affect α-helix, β-sheet, and β-turn, and controlling the temperature did not change the overall trends. In most cases, after 3 passes, we do not have a significant additional impact, indicating that the system has reached a saturation point in terms of structural modifications. On the other hand, for the samples where the temperature was not controlled, the shear, pressure, and temperature affect mainly α-helix and A1 if we compared with a non-treated SPI. Changes in these conditions were observed for a higher number of passes (>3), meaning that temperature plays an important role.

If intending to use a protein as a solution, considering lyophilization and its effects is crucial. Microfluidization enhances molecular availability by reducing particle size significantly (from 253.98 µm to 0.22 µm for approximately 90% of particles D(90) - Appendix 5). During the freezing process, more sites are exposed, leading to increased ice crystal formation and additional stress on the molecules. The mechanical forces applied during microfluidization seem to drive protein rearrangement and aggregation, forming new interactions and bonds.

In our study, when discussing the tertiary structure of treated and untreated soybean protein isolate, the number of passes and the consistent temperature were critical factors (for the temperature-controlled samples). In this case, we observed that surface hydrophobicity increases with the number of passes, the maximum reached by 5 passes. However, the results were inconsistent for samples without temperature control, with an increase of surface hydrophobicity after 1 and 3 passes, followed by a decrease at 5 passes. Moreover, there was no statistical difference between 1 and 3 passes. This suggests that the internal heating caused by the lack of temperature control results in aggregate formation, which subsequently hides hydrophobic amino acid residues. As expected, microfluidization impacts the tertiary structure of soybean protein, which aligns with existing literature.

The significance of the number of passes becomes evident upon analyzing the secondary and tertiary structures. However, this importance does not extend to the primary structure of proteins. Our findings indicate that whether under reducing conditions or in their native state, the soybean protein profile remained consistent across all treated samples. It is well-established that allergens exhibit stability during processing, suggesting that the soybean allergens studied in our research may possess increased thermostability. This aspect adds further interest to explore our second objective.

7.2. Characterization of the soy allergens

The initial characterization of the soybean protein structure helps in designing the experimental plan for the second objective, which is the detection of soybean allergens. Our comprehensive literature review, presented in Chapter 3, revealed that various techniques have been employed to reduce the allergenicity of soybeans. Interestingly, we identified a gap in the literature where microfluidization as a technique for reducing soy allergenicity has not been explored. Given that microfluidization has been shown to impact the structural properties of proteins, we hypothesized that it may also affect the allergenicity of soy proteins. After completing the secondary structure experiments, we selected 1, 3, and 5 passes (temperature-controlled) for different reasons: (i) consistent results, (ii) presumed increased flexibility, as evidenced by lower α-helix content compared to the control, and (iii) variations in tertiary structures among the different passes (1, 3, 5). Three methods were employed to assess the allergenicity of the treated samples: SDS-PAGE,

Western Blot, and indirect ELISA. The Western Blot method required several optimization steps to determine the appropriate parameters. Details of these optimizations can be found in Appendices 1, 2, and 3.

The general objective of the thesis is to decrease soybean allergenicity by using physical treatments. However, our findings indicated that more than a single technique was needed to achieve this goal. Specifically, microfluidization for 1, 3, and 5 passes at 137 MPa (temperature-controlled) did not enhance soybean allergenicity. Although we focused on temperature-controlled conditions, we also examined samples without temperature control (these results were presented in Appendix 6 – Fig. A2). When Western Blot was conducted with Alkaline Phosphatase (serum 15.4 kU/l), we noted that even under increased internal shearing, allergens remained unchanged, with only a minimal decrease in band intensity for some allergens, which proved insignificant. We even attempted to perform electrophoresis without reducing conditions to observe their native charge (Appendix 6 – Fig. A1). It was shown that the protein profile was similar for all samples except for a protein at 100 kDa, which was less intense for the 5 passes without temperature control.

A noteworthy observation in our study involved comparing a commercial SPI (used in our research) with SPI produced at the laboratory scale from the local Belgian soybean, coming from two different farmers Nijs and Terryn (in Appendix 4 – Fig. A1 is presented the comparison between them, using SDS-PAGE method). Moreover, more information about the chemical composition of local Belgian soybean is found in the Appendix 4 – Fig. A2.

Regarding the soybean protein profile obtained by Native-PAGE, we found that some proteins obtained on a laboratory scale could not enter the gel, and there was a higher abundance of aggregates than the commercial SPI (Appendix 6 – Fig. A1). This phenomenon might be due to high temperature and/or prolonged heat treatment during SPI processing, which can induce protein aggregation through non-covalent or covalent forces (L. Zhang et al., 2024). However, in our case, alkaline extraction was conducted at room temperature (alkalinization at pH 9 and precipitation by acid at pH 4.5), with no heat treatment applied. The use of NaOH for alkalinization during protein extraction and HCl for protein precipitation might have resulted in salt formation (Karaca et al., 2011). This is an essential aspect because the presence of $CaCl_2$ has been shown to induce the formation of insoluble aggregates (Añón et al., 2001). In our study, the increased salt concentration from the protein extraction might be responsible for the formation of insoluble aggregates, further impacting the aggregates' ability to enter the gel. The protein profile differed between electrophoresis methods, whether under reducing conditions or not. For instance, a 40 kDa protein was more prominent in the native gel (Appendix 6 – Fig. A1), along with the abundance of aggregates, and two extra visible bands were found between 50-55 kDa and one at 25 kDa. This observation is interesting because SPI protein profiles might be influenced by soybean genotypes/cultivars and processing steps (L. Zhang et al., 2024). In summary, when aiming for a hypoallergenic product, it is evident that industrial-scale production is necessary, given the larger quantities involved. In this

context, the commercial SPI more closely aligns with the industrial standards for producing hypoallergenic products.

Previously, we discussed the application of microfluidization as a single physical treatment to reduce soybean allergens, exploring a range of conditions from gentle (1 pass) to more intense treatment (5 passes). However, our findings indicated that more than a single technique was needed, and combining it with other methods, such as enzymatic hydrolysis, was necessary. This approach is not new, as previous researchers have also combined different techniques to optimize allergen reduction. Studies have shown that combining fermentation or enzymes with other treatments can effectively reduce allergenicity levels, as no single treatment can currently eliminate 100% of all allergens.

Literature proved that enzymatic hydrolysis enhances the reduction of different allergens, and it was also confirmed in our case by Western Blot and ELISA analyses on soybean allergens. When microfluidization was applied alone, the antibody strongly identified all potential allergenic proteins. However, when enzymatic hydrolysis was applied, the big allergens Gly m5 and Gly m6 were no longer recognized by the antibody. Although a faint interaction between the antibody and antigen persisted on the membrane, as confirmed by ELISA, the significant reduction in allergen recognition was important. Interestingly, while both Western Blot and ELISA aim to detect specific proteins in a sample, ELISA can detect allergens at lower concentrations, even when they are not visibly detected on a Western Blot. This is because ELISA can detect proteins presented in smaller quantities, and it does not require the protein to be separated by molecular weight as in Western Blotting. This difference explains the reduced visibility of interactions on the Western Blot compared to the residual immunoreactivity detected by ELISA. The complementary nature of these techniques is advantageous, as Western Blot allows for visualizing antigen-antibody interactions, while ELISA excels at detecting low levels of allergens.

Upon examining the combined effect of microfluidization and enzymatic hydrolysis, we observed a better reduction, particularly for 3 and 5 passes, even if a significant difference was not identified between the treated and non-enzymatic control samples.

Moreover, reconsidering the structure of proteins, the outcomes from both objectives exhibit a correlation. In combination with enzymatic hydrolysis, proteins displayed increased flexibility (lower α-helix content) and enhanced susceptibility to hydrolysis compared to a rigid structure, which is less accessible to enzymes. A decrease in β-sheet and the increase in β-turns facilitated protein unfolding, exposing hydrophobic regions and making them more accessible to enzymes. The synergy between the two treatments proved beneficial. However, when considering microfluidization alone, the decrease in β-sheet content and the increase in β-turns gave an opposite result. The immunoreactivity level increased by 11.25%, 23.54% and 29.9%. This shift indicates that the exposed epitopes became more available for antibody binding, increasing immunoreactivity. Furthermore, the number of

aggregates observed in the SDS-PAGE profile of the treated samples adds an exciting dimension to the analysis.

Given that aggregates A1 and A2 signify intermolecular and intramolecular interactions between β-sheets, we observed a decrease in β-sheet content and an increase in the number of aggregates in powders. This interaction between β-sheets contributes to the aggregation phenomenon, which can be seen in gel electrophoresis. When protein samples were solubilized in Tris-SDS buffer containing protease inhibitors, maybe the proteins were protected and behaved similarly to their powder form. This connection aligns with the increased flexibility of proteins, which further supports enzymatic hydrolysis. Additionally, our study highlights that protein behavior varies in different environments.

To conclude, combinations using processing and enzymatic hydrolysis offer great potential in the industrial production of hypoallergenic and high-quality hydrolyzed proteins. This study evaluated the impact of using microfluidization alone and then in combination with enzymatic hydrolysis on reducing the allergenicity of soybean allergens. The study elucidated recent advances in mechanisms involved in processing, protein structure, and their correlation, highlighting the potential for developing hypoallergenic products in the industry. After performing this study, we can conclude that:

a) Although microfluidization impacts the secondary and tertiary structure of soy proteins, the applied treatment is insufficient for reducing soy allergens.
b) In the case of soy allergens and under the applied conditions, there was an increase in allergenicity because microfluidization reduces particle size, unfolds proteins, and exposes the allergenic epitopes.
c) By affecting the secondary and tertiary structure, under different conditions applied, microfluidization may serve as a pretreatment to improve the enzymic hydrolysis.

7.3. Limitations and future perspectives

In this study, we evaluated the allergenicity of soybean proteins following microfluidization, enzymatic hydrolysis, and their combination using methods such as SDS-PAGE, Western Blot, and indirect ELISA. While the results demonstrated that enzymatic hydrolysis proved to be most efficient by reducing the allergens by 53.16 % and 63.2 %, it is important to consider some limitations of this study, such as protein digestion.

Protein resistance to digestion is one candidate for determining allergenic potential (Clare Mills et al., 2009). Many allergens are characterized by their ability to resist degradation in the gastrointestinal tract, allowing intact or partially digested proteins to reach the intestinal mucosa, where absorption and sensitization can occur. For example, β-conglycinin of soybean was stable to the gastric model (simulated gastric fluid) for 60 min, compared with glycolate reductase from spinach leaf digested within 15 sec (Astwood et al., 1996). They concluded that allergens exhibited greater stability than nonallergenic proteins and suggested that digestive stability could serve as a

parameter to differentiate allergenic and nonallergenic proteins. On the other hand, some researchers extended the previous work described, and they found no clear correlation between digestibility and allergenicity (Fu et al., 2002). Digestibility is influenced by food processing, digestive conditions, and food matrix (Koidl et al., 2023). Although our study assessed the allergenicity of soybean proteins post-processing, future studies should incorporate *in vitro* digestion models for the powders obtained.

Another study limitation is that the treated powders were not evaluated in real systems. For instance, *in vivo* studies using a soybean-sensitized animal model would provide a more relevant allergenicity assessment. For example, a group of researchers investigated a three-week-old C3H/HeJ mice sensitized to cow's milk. The study found that specific IgE levels significantly increased, peaking at six weeks post-sensitization, which coincided with a systemic anaphylactic response upon intragastric challenge with cow's milk. The involvement of IgE in mediating cow's milk hypersensitivity was confirmed by reduced passive cutaneous anaphylaxis reactions following heat inactivation of immune sera. Additionally, the allergic response was likely mediated by TH2 cells, as indicated by elevated levels of IL-4 and IL-5 from the spleen cells of allergic mice (X. M. Li et al., 1999). These models should be useful for assessing the immunopathogenic mechanisms associated with food allergies. In addition, an ideal model for evaluating food allergens should effectively predict both strong and weak allergens, utilizing oral or skin sensitization routes. In addition to measuring IgE levels, recording functional or symptomatic responses is crucial to have the full spectrum of allergic reactions (Bøgh et al., 2016).

Moreover, the current results show the impact of the mentioned treatments on soybean allergens using two concentrations of serum from patients allergic to soy. As a result, further research should investigate the effect of these treatments using a pooled serum (a mixture of different concentrations from many patients) to have more accurate response, especially because food allergies represent an individual response. Additionally, several other factors could be considered in future studies. For instance, comparing the effects of different protein concentrations (e.g., 1%, 3%, and 5%) on soy allergens could provide more insights. Previous studies have shown that the influence of high-pressure treatment (ranging from 200 to 600 MPa) on SPI properties is highly dependent on the initial protein concentration, with observed variations in solubility, gelling behavior, free SH content, emulsifying properties, and protein hydrophobicity (X. S. Wang et al., 2008). Another interesting area for exploration is the effect of different pressure levels, such as 0.1, 40, 80, 120, and 160 MPa, combined with lower protein concentrations. This could be compared with findings for β-lactoglobulin, where an increase in allergenicity was noted at 80 MPa, while the lowest allergenicity occurred at 120 and 160 MPa (J. Z. Zhong et al., 2012).

In conclusion, this section outlines several limitations of the study, including the absence of assessments for *in vitro* digestion and *in vivo* models. It also presents potential future directions, such as exploring various protein concentrations and processing parameters to understand better how high shearing affects allergenicity.

8

Conclusions and perspectives

Chapter 8. Conclusions and perspectives

8.1. Conclusions

Food allergies are a major global health concern, with efforts being made to address them. These allergies can be studied from two perspectives: clinical (related to medical aspects and patient care) and technological (methods and treatments used in the food industry to reduce allergens). When we look at the clinical aspects, managing food allergies involves a multifaceted approach to ensure safety and improve the quality of life for affected individuals.

Dietary management includes allergen avoidance and guidance from dietitians on safe food choices to maintain nutritional balance. Psychological support is crucial for people's emotional state, offering coping strategies and cognitive behavioral therapy through consultations with psychologists. A comprehensive treatment plan, including emergency medications like antihistamines and auto-injectors, and a written emergency protocol with healthcare contacts are essential for moderate to severe allergies. Immunomodulatory treatments such as Omalizumab and allergen-specific immunotherapy (e.g., oral, sublingual, subcutaneous, or epicutaneous) aim to modify immune responses and reduce allergic reactions over time.

Considering that all mentioned immunotherapies involve direct contact with allergens, these can present advantages and limitations.

Food challenges offer several advantages for individuals with food allergies. They can reduce anxiety related to food consumption, normalize eating habits outside the home, and enhance nutritional intake, improving overall quality of life. Additionally, by confirming tolerance to specific foods, food challenges can save medical and dietitian time, reduce family stress and expenses associated with specialized diets, and support healthy growth. This approach addresses immediate health concerns and contributes to allergy patients' long-term well-being and lifestyle normalization.

On the other hand, direct exposure to food allergens can trigger allergic reactions ranging from mild to severe. Additionally, this direct contact necessitates careful monitoring and multiple hospital appointments with a specialist. While these therapies can be effective, the risks associated with direct allergen exposure must be managed carefully. Moreover, these immunotherapies can be cost-effective. For instance, one person with peanut allergy using Palforzia might have a prescription cost of around 4700 euros per year. Additionally, the administration costs can be significant, with 12 visits to the hospital totaling around 4200 euros in one year (Paul J. Turner, London, UK, information obtained from EAACI congress, 2024). Therefore, managing food allergies is challenging not only for the individual affected but also from a financial perspective and other related aspects.

When examining food allergens from a technological point of view, we can see from this study that different efforts are made in this direction.

Very interestingly, humans have been processing food since ancient times to make it more palatable, last longer, and be safe to eat. In this way, when people started to cook food from the raw state, they did not think that cooking could impact the allergenicity of the food. At that moment, they did not realize that heat treatment, for example, produces significant alterations in protein structure, thereby reducing allergens or creating new ones. As previously mentioned, proteins are responsible for triggering allergic reactions.

Our research focused on the technological aspect of food allergens. It showed that different techniques were investigated to reduce food allergens, starting from thermal, pressure-based, fermentation, enzymatic, and wave-based treatments. However, we are interested in physical methods due to concerns about the negative effects of thermal processing on food quality.

After a comprehensive literature review, we observed that various physical treatments effectively reduce soy allergenicity, with some methods achieving over 70% reduction. High-level γ-irradiation, PUV, cold plasma, extrusion, and DIC show the greatest potential, while ultrasonication, microwave, HPH, and HHP have a more moderate to weak impact. These treatments typically induce changes in soy proteins, such as denaturation, epitope inactivation, structural alterations, aggregation, and cross-linking.

While these technologies offer promising alternatives to conventional methods for reducing food allergenicity, several challenges and limitations must be addressed. These include maintaining nutritional and sensory qualities, managing off-flavors, and preserving the bioaccessibility and digestibility of nutrients. Additionally, concerns about the safety and health impacts of technologies like PUV and γ-irradiation on workers and consumers must be addressed. From all the mentioned treatments, extrusion seems to be the most convenient technology to reduce soybean allergenicity.

While compiling the physical treatments applied to soybeans, it was noted that microfluidization was not included. Although the impact of microfluidization has been studied on peanut and milk allergens, it has not been investigated for soybeans.

Since microfluidization impacted the secondary and tertiary structure, it was hypothesized that it could reduce soy allergenicity. Understanding how microfluidization impacts soybean protein structure is essential because it will help design the second part of the experiments. Therefore, we explored how microfluidization affects the structure of soybean protein under various conditions (1, 3, and 5 passes, with and without temperature control, in both solution and powder forms).

While the primary protein structure remained stable, secondary and tertiary structures were notably modified. The treatment caused protein unfolding, disaggregation, or aggregation, especially with more passes and changed the secondary structure in powder and reconstituted state. More modifications were observed for the powders. Additionally, microfluidization increased surface hydrophobicity and exposed hydrophobic amino acids, potentially enhancing the protein's functional properties. Overall, microfluidization significantly impacts

protein structure, which could improve its applications in food processing. Among the samples tested, the ones where the temperature was controlled seem the most suitable.

In this way, these results helped us for the second part of the experiments, where we tested the allergenicity of soybean. The original objective of this thesis was to use only physical treatments, specifically microfluidization, to reduce soy allergens. After performing the allergenicity assessment, the protein profile was similar for all the conditions tested. Moreover, instead of lowering the allergens, we observed an opposite result: an increase due to the exposed hidden epitopes.

Observing that a single technique was insufficient to achieve the desired outcome, it became necessary to integrate microfluidization with another method. We selected enzymatic hydrolysis, a conventional technique, to complement microfluidization and enhance its effectiveness. On the other hand, enzymatic treatment significantly reduced allergens, though some residual peptides remained. Combining microfluidization and enzymatic hydrolysis seems the most effective in reducing allergenicity but further investigations are required. Microfluidization before hydrolysis did not notably enhance allergen reduction. The combination approach may offer a promising method for reducing soy protein allergenicity under different processing conditions.

After considering the two sides of food allergies, clinical and technological, we can conclude that at this moment, no technique/immunotherapy can entirely reduce or cure food allergies. However, investigating this technological aspect will help the following scientists to design and select the appropriate process conditions to improve the efficiency of allergen reduction in food products. Moreover, after obtaining a hypoallergenic product, it needs to be approved by the authorities (such as the FDA or EFSA) and tested *in vivo* and *in vitro* to ensure its safety and effectiveness. Indeed, communication and education are key from the patient's perspective because they need to be properly informed and educated before consuming food allergens.

8.2. Perspectives

This work has provided valuable insights into the soybean protein structure and allergenicity. However, some future research perspectives can be taken into account to complete better this study:

a) Protein characterization: using mass spectrometry to identify and quantify the proteins and peptides, providing detailed information on the allergenic proteins.

b) Optimizing microfluidization conditions: after observing that the number of passes did not influence the reduction of allergens, maybe at different pressures (low, middle, and high), soybean allergens react differently.

c) Oral food challenges: testing the obtained products in allergic individuals (ideally) under medical supervision to monitor any adverse reactions by controlled administration.

d) Skin Prick Tests: small amounts of hydrolyzed products are applied to the skin of allergic individuals to observe any localized reactions.

e) Combining microfluidization and enzymatic hydrolysis on other plant-based proteins, such as lupin, fava bean, or pea.

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Appendix

Appendix

Appendix 1. Optimization of the Western Blot method.

Step	*Test 1*	*Test 2*	*Test 3*	*Test 4*
Sample preparation	TPX BIO-RAD assay	TPX BIO-RAD assay	TPX BIO-RAD assay	TPX BIO-RAD assay
Gel electrophoresis	• 12% separating gel • 5% stacking gel • 6 µg of proteins	• 12% separating gel • 5% stacking gel • 20 µg of proteins	• 12% separating gel • 5% stacking gel • 20 µg of proteins	• 12% separating gel • 5% stacking gel • 20 µg of proteins
Membrane transfer	PVDF	PVDF	PVDF	PVDF
Blocking	3% BSA, 1h, RT	3% BSA, 1h, RT	3% BSA, 1h, RT	3% BSA, 1h, RT
Antibody detection	9 antibodies	3 antibodies	1 antibody (6,48 kU/l)	1 antibody (15,4 kU/l)
-primary antibody	1/10, overnight, 4°C	1/10, overnight, 4°C	1/10, overnight, 4°C	1/10, overnight, 4°C
-secondary antibody	HRP, 1/2000, 1h, RT	-HRP, 1/2000, 1h/4h/5h, RT -AP, 1/1000, 4h, RT	AP, 1/1000, 4h, RT	AP, 1/1000, 4h, RT
Detection	Chemiluminescent	Chemiluminescent Colorimetric	Colorimetric	Colorimetric

Appendix 2. Optimization of the Western Blot method.

Step	*Test 5*	*Test 6*	*Test 7*	*Test 8*
Sample preparation	TPX BIO-RAD assay	TPX BIO-RAD assay	TPX BIO-RAD assay	TPX BIO-RAD assay
Gel electrophoresis	• 12% separating gel • 5% stacking gel • 20 µg of proteins	• 12% separating gel • 5% stacking gel • 20 µg of proteins • Heating at 70°C, 10 min	• 12% separating gel • 5% stacking gel • 20 µg of proteins • Heating at 70°C, 10 min	• 12% separating gel • 5% stacking gel • 20 µg of proteins • Heating at 70°C, 10 min
Gel electrophoresis	PVDF	PVDF • transfer confirmation with Ponceau S	PVDF • transfer confirmation with Ponceau S	PVDF • transfer confirmation with Ponceau S
Membrane transfer	PVDF	PVDF • transfer confirmation with Ponceau S	PVDF • transfer confirmation with Ponceau S	PVDF • transfer confirmation with Ponceau S
Blocking	3% BSA, 1h, RT	Blocking Buffer Bio-Rad 5 min, RT -washing 3 times, 10 min	Blocking Buffer Bio-Rad 5 min, RT -washing 3 times, 10 min	Blocking Buffer Bio-Rad 5 min, RT -washing 3 times, 10 min
Antibody detection	1 antibody (6,48 kU/l)	1 antibody (6,48 kU/l)	1 antibody (15,4 kU/l)	1 antibody (15,4 kU/l)
-primary antibody	1/10, overnight, 4°C	1/20, 2h, RT -washing 6 times, 10 min	1/20, 2h, RT -washing 6 times, 10 min	1/20, 2h, RT -washing 6 times, 10 min
-secondary antibody	HRP, 1/2000, 5h, RT	AP, 1/1000, 2h, RT -washing 6 times, 10 min	AP, 1/1000, 2h, RT -washing 6 times, 10 min	HRP, 1/2000, 2h, RT -washing 6 times, 10 min
Detection	Chemiluminescent	Colorimetric	Colorimetric	Chemiluminescent

Tables in Appendices 2 and 3 were important in optimizing the Western Blot method. They provided valuable information on various parameters, including different antibodies, incubation times, washing times, detection methods, and whether the membrane was cut or not. After numerous tests, we selected two antibodies for further optimization to achieve the best possible blot.

Appendix 3. Background optimization for the Western blot method, using different blocking buffers and antibody dilutions.

1st Ab	*Blocking buffer*	*1st Ab dilution*	*1st Ab incubation*	*2nd Ab dilution*	*2nd Ab incubation*	*Detection method*	*Problem*
High (15.4 kU/l)	TBST + BSA 5%	1/10	Overnight	1/1000	2h	Colorimetry	Too high background
	TBST + BSA 5%	1/10	2h	1/1000	2h	Colorimetry	Too high background
	TBST + BSA 5%	1/100	2h	1/1000	2h	Colorimetry	Too low signal
	Bio-Rad	1/20	2h	1/2000	2h	Colorimetry	Almost no signal
	Bio-Rad	1/20	2h	1/1000	2h	Colorimetry	Weak signal/Revelation time too long
Mid (6.48 kU/l)	Bio-Rad	1/20	2h	1/2000	2h	Chemiluminescence	Low signal, background problem
	Bio-Rad	1/10	2h	1/2000	2h	Colorimetry	Weak signal/Revelation time too long

The following conditions were chosen for the experiments:

High antibody (15.4 kU/l)

- TBST + BSA 5% Blocking buffer
- 1st Ab 1/20 dilution, 2h incubation
- 2nd Ab 1/1000 dilution, 2h incubation (Alkaline Phosphatase-colorimetric)

Mid antibody (6.48 kU/l)

- TBST + BSA 5% Blocking buffer
- 1st Ab 1/20 dilution, 2h incubation
- 2nd Ab 1/1000 dilution, 2h incubation (Alkaline Phosphatase-colorimetric)

Appendix 4. Protein profile for SPIs obtained at the industrial and laboratory level.

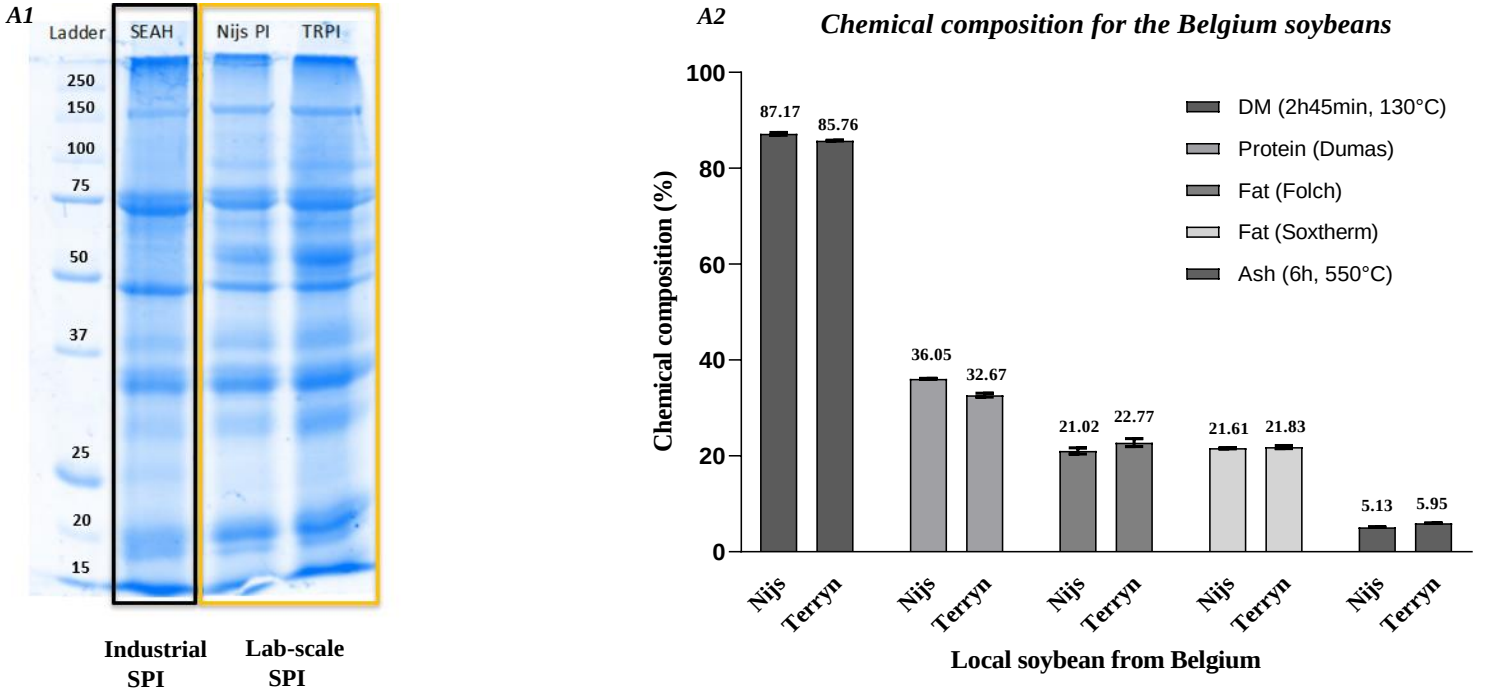
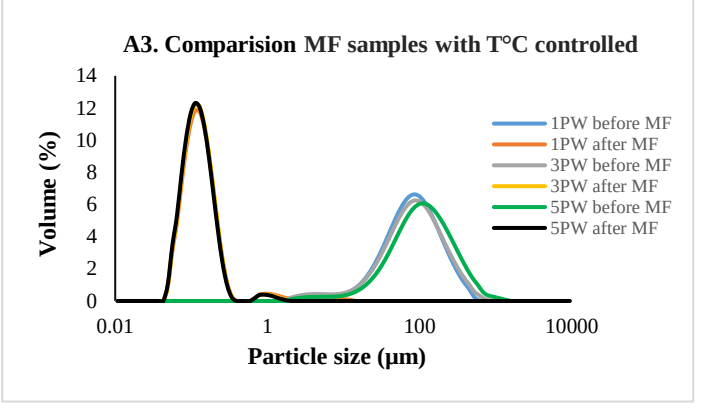
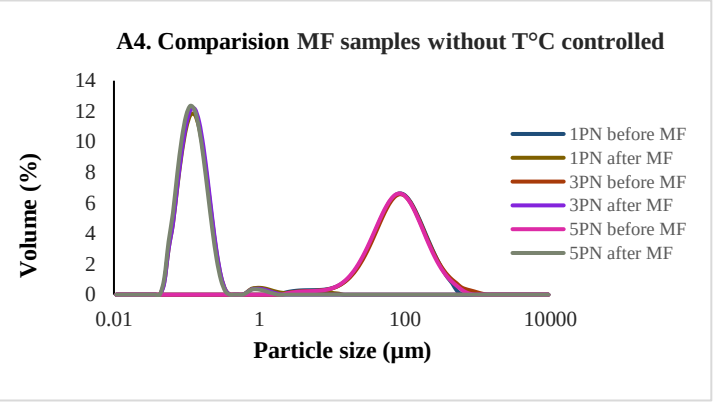


Fig. A1. SDS-PAGE profile under reducing and denaturation conditions 12% separating gel, 5% stacking gel. Lane 1– unstained marker (kDa). Lane 2 – commercial SPI from France, produced at industrial scale. Lanes 3 and 4 –SPIs coming from local farmers from Flanders (Nijs and Terryn) and obtained at lab-scale; **A2**. Comparison of the chemical composition of the Belgian soybeans (Nijs and Terryn). The protein, fat, and ash content is related to the dry matter.

Appendix 5. Particle size distribution before and after microfluidization treatment - with (A3) and without cooling system (A4). This is characterized by key parameters: D(10), D(50), D(90), span, and D[4, 3], measured by Malvern Mastersizer / Hydro 2000S, in the form of solution. Each parameter is based on 3 replicate measurements, and the values presented in the graph and table represent the mean of these replicates.





Samples	*Key parameters related to the distribution of particle sizes* MF samples with T°C controlled					Samples	*Key parameters related to the distribution of particle sizes* MF samples without T°C controlled				
	D10 (µm)	D50 (µm)	D90 (µm)	Span	D [4, 3] (µm)		D10 (µm)	D50 (µm)	D90 (µm)	Span	D [4, 3] (µm)
1PW before	23.26	83.97	259.48	2.81	118.79	1PN before	24.33	80.38	223.09	2.47	105.85
1PW after	0.07	0.12	0.23	1.28	0.26	1PN after	0.07	0.12	0.23	1.27	0.26
3PW before	21.85	82.91	248.33	2.73	114.16	3PN before	26.09	84.15	232.62	2.45	112.22
3PW after	0.07	0.12	0.21	1.18	0.15	3PN after	0.07	0.12	0.22	1.17	0.15
5PW before	29.64	106.17	330.29	2.83	151.94	5PN before	25.05	80.05	229.77	2.56	109.37
5PW after	0.07	0.12	0.21	1.17	0.14	5PN after	0.07	0.12	0.21	1.16	0.14

Appendix 6. Native-PAGE and Western Blot (under reducing and denaturation conditions) for the microfluidized samples (with and without temperature controlled) and SPIs from the local farmers from Belgium.

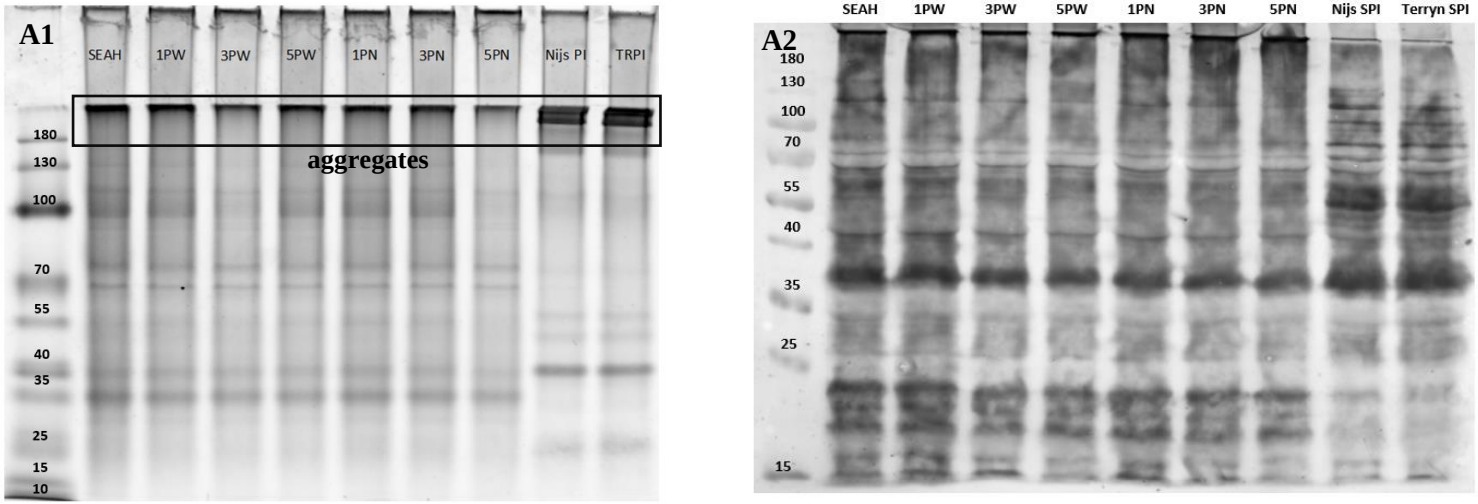


Fig. A1. Native-PAGE without SDS, β-mercaptoethanol and heating of the samples (samples are solubilized in water (pH 9) and the insoluble parts were removed by centrifugation (10.000 x g, 20 min at 20°C); **(A2)** Western Blot (under reducing and denaturation conditions: 12% separating gel, 5% stacking gel; blocking 5% BSA in TBST 1 h at RT, primary Ab 1/20 (15.4 kU/L) in TBST + 5% BSA 2h RT, secondary Ab 1/1000 (AP) in TBST + 5% BSA 2h RT); the abbreviations are the same for both figures; Lane 1 – prestained marker (kDa), Lane 2 – non-treated SPI. Lanes 3, 4, and 5 – microfluidized samples where the temperature was controlled. Lanes 6, 7, and 8 – the microfluidized samples without temperature control. Lane 9, 10 – Nijs, Terryn soybean proteins isolates were obtained at a laboratory scale without any treatment.

Appendix 7. Student contributions

Publications in international peer reviewed journals

1. **Kerezsi, A. D**., Jacquet, N., & Blecker, C. (2022), "Advances on physical treatments for soy allergens reduction - A review," *Trends in Food Science & Technology*, 122, 24–39. https://doi.org/10.1016/J.TIFS.2022.02.007.

2. **Kerezsi, A. D**., Lelia Pop, O., Othmeni, I., Figula, A., Francis, F., Karamoko, G., Karoui, R., & Blecker, C. (2024). "Impact of pilot-scale microfluidization on soybean protein structure in powder and solution," *Food Research International*, 188, 114466. https://doi.org/10.1016/J.FOODRES.2024.114466.

3. **Kerezsi, A. D**., Figula, A., Jacquet, Francis, F., N., & Blecker, C. (2024), "Challenges in mitigation of soybean proteins allergenicity using combined techniques: high shearing and enzymatic hydrolysis." Submitted for publication.

4. Nahimana, P., **Kerezsi, A.D**., Karamoko, G., Abdelmoumen, H., Blecker, C., Karoui, R.(2023), "Impact of defatting methods on the physicochemical and functional properties of white lupin protein isolates", *European Food Research and Technology Journal. 1*, 1–14. https://doi.org/10.1007/S00217-023-04305-X.

External collaborations for publications

5. Ciont, C., Suharoschi, R., **Kerezsi**, A. D., Vodnar, D. C., & Pop, O. L. (2024). "Lactobacillus plantarum: Food supplements and pharmaceutical uses". (pp. 75–114). *Nova Science Publishers, Inc.*

6. Ciont, C., Epuran, A., **Kerezsi**, A. D., Coldea, T. E., Mudura, E., Pasqualone, A., Zhao, H., Suharoschi, R., Vriesekoop, F., & Pop, O. L. (2022). "Beer Safety: New Challenges and Future Trends within Craft and Large-Scale Production". *Foods*, 11(17), 2693. https://doi.org/10.3390/FOODS11172693.

7. Pop, O. L., **Kerezsi**, A. D., & Ciont, C. (2022). « A Comprehensive Review of Moringa oleifera Bioactive Compounds—Cytotoxicity Evaluation and Their Encapsulation". *Foods*, 11(23), 3787. https://doi.org/10.3390/FOODS11233787.

8. Mihalca, V., **Kerezsi**, A. D., Weber, A., Gruber-traub, C., Schmucker, J., Vodnar, D. C., Dulf, F. V., Socaci, S. A., Fărcaș, A., Mureșan, C. I., Suharoschi, R., & Pop, O. L. (2021). "Protein-Based Films and Coatings for Food Industry Applications". *Polymers*, 13(5), 769. https://doi.org/10.3390/POLYM13050769.

Contributions at international meetings

1. **Kerezsi, A.D**., Figula, A., Jacquet, N., Francis, F., Blecker, C. "Exploring microfluidization and enzymatic techniques to mitigate soybean allergenicity". 31 May – 03 June 2024, EAACI Congress 2024, Valencia, Spain. Flash Talk.

2. **Kerezsi, A.D**., Figula, A., Jacquet, N., Francis, F., Blecker, C. "Exploring microfluidization and enzymatic techniques to mitigate soybean allergenicity". The second edition of JMA Task Force "Tell us about your project" (Thesis in 3 min), organized by EAACI, 21 May 2024. The oral presentation is available on their platform (https://hub.eaaci.org/education_webinars/2nd-edition-of-jma-task-force-tell-us-about-your-project/).

Scholarship to attend EAACI Congress, Valencia, Spain, 2024 for "Tell Us About Your Project" (Thesis in 3 min; one of the best 3 videos).

3. **Kerezsi, A.D**., Figula, A., Jacquet, N., Francis, F., Blecker, C. "Challenges in exploring microfluidization and enzymatic methods to mitigate soybean allergenicity". 28 April – 1 May 2024, Sustainable Protein Forum Co-located with the AOCS Annual Meeting & Expo, Montreal, Canada. Poster presentation.

4. **Kerezsi, A.D**., Jacquet, N., Lelia Pop, O., Othmeni, I., Figula, A., Francis, F., Karamoko, G., Karoui, R., Blecker, C. "Recent developments in soybean protein structure using high-shear treatment". 28 April – 1 May 2024, Sustainable Protein Forum Co-located with the AOCS Annual Meeting & Expo, Montreal, Canada. Poster presentation.

5. **Kerezsi, A.D**., Figula, A., Jacquet, N., Francis, F., Blecker, C. "Meet the unexpected increase of soybean allergens: how is it possible?". 24 November 2023, The SFMBBM (Structure and Function of Biological Macromolecules, Bioinformatics and Modelling), Liege, Belgium. Poster presentation.

6. **Kerezsi, A.D**., Figula, A., Jacquet, N., Francis, F., Blecker, C. "Impact of microfluidization, enzymatic treatment and their combination on soybean allergens". 27-29 September 2023, Food Allergy Forum - International Conference, Amsterdam, Netherlands. Poster presentation.

7**. Kerezsi, A.D**., Jacquet, N., Blecker, C. "Advances on physical treatments for soy allergens reduction". 08 July 2022, The National Symposium for Applied Biological Sciences (NSABS 2022), Antwerp, Belgium. Poster presentation.

Collaboration

2 weeks at Adrianor Centre Technique Agroalimentaire, Arras, France (5-9 December 2022; 12-16 June 2023) in collaboration with Mr. Romdhane KAROUI - to characterize the secondary and tertiary structure of soybean proteins.