EFFECTS OF MICROWAVE COMBINED WITH ULTRASONIC OR ULTRAVIOLET PROCESSING ON THE PHYSIOCHEMICAL AND ALLERGENIC PROPERTIES OF SOYMILK

BY

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ABSTRACT

Human beings have consumed soybean as an excellent food source for thousands of years due to its rich protein, fatty acids, minerals, and fibers. However, soybean was recognized as one of the big eight allergens resulting in a series of allergic symptoms and it even could lead to death. With the increasing demand for soybean products, the challenges caused by soybean allergy need to be resolved urgently. The main allergens present in soybeans, including Gly m Bd 28k, Gly m Bd 30k, Gly m Bd 60k (Gly m 5), and the structures of soybean allergens may be altered by thermal or non-thermal treatments. Some novel food processing techniques such as ultrasound treatment and pulsed ultraviolet technology have shown potential application in reducing soybean allergenicity.

In this study, the effects of microwave treatment combined with ultrasonic or ultraviolet processing on the allergenicity, trypsin inhibitor (TI) content, antioxidant capability, microstructures, secondary structures, in vitro protein digestibility of soymilk have been investigated. For microwave combined ultrasonic treatment, the results indicated that there was an increase in total soluble protein content and total antioxidant capacity compared to the untreated samples. Meanwhile, the decrease of trypsin inhibitor activities with the longer treatment was observed, and it is also related to higher values of in-vitro protein digestibility. Larger aggregations of soy protein were observed by Scanning Electronic Microscopy (SEM). Secondary structural changes with the increasing time, specifically showing the upward trend of β-sheets and the downward trend of α-helix. Finally, the Enzyme-linked Immunosorbent Assay (ELISA) test shows that a 16 min of ultrasound treatment significantly inhibited the IgE binding capacity of Gly m 5. For microwave combined ultraviolet treatment, the

percentage of reduced allergenicity was positively related to the increase of ultraviolet irradiation time. Meanwhile, the total soluble protein content increased by 31.1% after 60 min ultraviolet treatment, and improving the antioxidant capacity and decreasing trypsin inhibitor activity of soymilk. Also, in the In-Vitro Protein Digestibility (IVPD) of soymilk, there was no significant change compared to the control sample. Soymilk protein denaturation and aggregation may induce an increase of β-turns and α-helixes structures and a decrease of β-sheet and unordered structures in treated samples. Significantly, more microstructural holes were observed in treated samples with increased treated time.

Résumé

Les êtres humains ont consommé le soja comme excellente source de nourriture pendant des milliers d'années en raison de sa richesse en protéines, en acides gras, en minéraux et en fibres. Cependant, le soja a été reconnu comme l'un des huit grands allergènes, entraînant une série de symptômes allergiques et pouvant même entraîner la mort. Avec la demande croissante de produits à base de soja, les défis causés par l'allergie au soja doivent être résolus de toute urgence. Les principaux allergènes présents dans le soja, notamment Gly m Bd 28k, Gly m Bd 30k, Gly m Bd 60k (Gly m 5), et les structures des allergènes du soja peuvent être modifiés par des traitements thermiques ou non thermiques. Certaines nouvelles techniques de transformation des aliments telles que le traitement par ultrasons et la technologie des ultraviolets pulsés ont montré une application potentielle dans la réduction de l'allergénicité du soja.

Dans cette étude, les effets du traitement par micro-ondes combiné à un traitement par ultrasons ou ultraviolets sur l'allergénicité, la teneur en inhibiteur de la trypsine (TI), la capacité antioxydante, les microstructures, les structures secondaires, la digestibilité des protéines in vitro du lait de soja ont été étudiés. Pour le traitement par ultrasons combiné par micro-ondes, les résultats ont indiqué qu'il y avait une augmentation de la teneur totale en protéines solubles et de la capacité antioxydante totale par rapport aux échantillons non traités. Pendant ce temps, la diminution des activités des inhibiteurs de la trypsine avec le traitement plus long a été observée et elle est également liée à une digestibilité plus élevée des protéines in vitro. Des agrégations plus importantes de protéines de soja ont été observées par microscopie électronique à balayage (SEM). Changement structurel secondaire avec le temps croissant, montrant spécifiquement la tendance à la hausse des feuillets β et la tendance

à la baisse de l'hélice α. Enfin, le test ELISA (Enzyme-linked Immunosorbent Assay) montre qu'un traitement par ultrasons de 16 min a inhibé de manière significative la capacité de liaison aux IgE de Gly m 5. Pour le traitement ultraviolet combiné par micro-ondes, le pourcentage d'allergénicité réduite était positivement lié à l'augmentation des ultraviolets. temps d'irradiation. Pendant ce temps, la teneur totale en protéines solubles a augmenté de 31,1% après 60 minutes de traitement aux ultraviolets, améliorant ainsi la capacité antioxydante et diminuant l'activité inhibitrice de la trypsine du lait de soja. Mais pour la digestibilité in vitro des protéines (IVPD) du lait de soja, aucun changement significatif par rapport à l'échantillon témoin n'a été observé. La dénaturation et l'agrégation des protéines du lait de soja peuvent induire une augmentation des structures des spires β et des hélices α et la diminution des structures en feuillet β et non ordonnées dans les échantillons traités. Des trous microstructuraux plus nombreux ont été observés dans les échantillons traités avec un temps de traitement plus long.

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Thesis Format

This thesis is submitted in the format of papers suitable for journal publication. This thesis format has been approved by the Faculty of Graduate and Postdoctoral Studies, McGill University, and follows the conditions outlined in the Guidelines: Concerning Thesis Preparation, which are as follows:

“As an alternative to the traditional thesis format, the dissertation can consist of a collection of papers of which the student is an author or co-author. These papers must have a cohesive, unitary character making them a report of a single program of research. The structure for the manuscriptbased thesis must conform to the following:

1. Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together a s an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis).

2. The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.

3. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts.

The thesis must include the following

(a) A table of contents;

(b) An abstract in English and French;

(c) An introduction which clearly states the rational and objectives of the research;

(d) A comprehensive review of the literature (in addition to that covered in the introduction to each paper);

(e) A final conclusion and summary;

4. As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.

5. In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers".

Contribution of Authors

The following are the manuscripts prepared for publication:

1. Zhaoyi He, Jin Wang and Vijaya Raghavan. 2021. “Soybean Allergy: Characteristics, Mechanisms, Detection and Its Reduction Through Novel Food Processing Techniques” (Under Review)

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3. Zhaoyi He, Xin Dong and Vijaya Raghavan. 2021. “Effects of Microwave combined Ultraviolet Processing on the Physiochemical and Allergenic Properties of Soymilk (In Progress)

The work reported here was performed by Zhaoyi He and supervised by Prof. Vijaya Raghavan of the Department of Bioresource Engineering, Macdonald Campus of McGill University. The entire research work was carried out at the Post harvest Technology laboratory, Macdonald Campus of McGill University, Montreal. Prof. Raghavan has provided scientific advice and is directly associated with reviewing the manuscript. Dr. Jin Wang contributed in reviewing the manuscript. Mr. Yvan Gariépy facilitated and instructed me in the lab procedure.

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CHAPTER I

INTRODUCTION

Soybean (*Glycine max*) is rich in nutrients that the human body needs. Studies reported that soy protein in foods could decrease cholesterol levels and reduce the risk of cardiovascular disease (Gagnon, Poysa, Cober, & Gleddie, 2010). Soybean protein is a complete plant-based protein containing abundant of bioactive factors, including soy isoflavones, soy saponins, soy oligosaccharides, etc. These bioactive factors have the functions of anti-oxidation, angiotensin-converting enzyme inhibition, and blood pressure reduction (Cucu et al., 2013). In addition, soybean is the only vegetable food that contains all eight essential amino acids, and the physiological value of soy protein can reach 100 (Asif & Acharya, 2013).

Soymilk, the water extract of soybean, is usually produced by grinding the soaked soybean with water, and it also has very high nutritional value. As an inexpensive and convenient source of high quality proteins, soymilk is one of the most important traditional beverages that are consumed widely in Asian countries, including China, Japan, Korea, Singapore, and Thailand. However, soybean was recognized as one of the major allergic food items belonging to the "big eight." Studies reported that approximately 0.3% - 0.4% of children are allergic to soybean worldwide (Savage, Kaeding, Matsui, & Wood, 2010). The presence of natural anti-nutrients (such as trypsin inhibitor (TI), lectins, phytic acid) and other allergic proteins in soymilk limit its consumption. Various studies have shown that the soymilk allergenicity could be reduced by using different processing methods.

Thermal processing is one of the most common food processing methods to eliminate or reduce the allergic proteins present in food. Heat treatment can cause damage in the spatial conformation of the protein, which in turn could result in reduction of the allergenicity of the

food.

Ultrasonic treatment is also used to reduce the allergenicity of soymilk. The sound wave provides enough energy to modify the related molecules (Shriver & Yang, 2011). It may cause conformational changes, secondary structure damage, other intra/inter molecular interactions, and the recombination of disulfide bonds (Stanic-Vucinic et al., 2012) of proteins and lead to alteration of allergenicity of soymilk.

In the past few decades, ultraviolet light technology has obtained great attention from researchers due to its advantages in microbial decontamination of food products. Also, ultraviolet rays could provide a low temperature, strong antimicrobial effect, and small impact on food quality (Koutchma, 2008). Side-chain oxidation, protein cross-linking, and aggregation may occur due to the absorption of light by protein chromophores. The formation of insoluble proteins and backbone fragmentation may reduce food allergenicity (Ekezie, Cheng, & Sun, 2018).

In soymilk most of the anti-nutritional factors and some allergic proteins can be reduced through heat treatment, and it also improves the digestibility of soy protein (Jiang, Cai, & Xu, 2013). However, excessive heating will destroy amino acids to a large extent and reduce the overall nutritional value of soymilk. Studies have shown that microwave and ultrasonic treatment can effectively remove anti-nutritional factors in soymilk (Vanga, Wang, & Raghavan, 2020b), but there are very few experiments of these two treatment combinations. Besides, using ultrasonic treatment alone has little effect on soybean protein (H. Hu, Cheung, Pan, & Li-Chan, 2015). Ultraviolet light is usually used as a sterilization technology, and there is no experimental work in the area of allergenicity of soymilk after ultraviolet light

treatment. In this thesis, the soymilk is subjected to microwave and ultrasonic combined treatment and ultraviolet treatment to remove its undesirable substances and to detect its improved properties.

1.1 Hypothesis

After the soymilk is thermally treated and then ultrasonics are added, the combination of these two treatment methods is expected to have a greater change in the structure of its protein molecules, which can effectively remove allergenicity of proteins and anti-nutrients. And it would have a certain effect on other physical and chemical properties such as protein digestibility. As a non-thermal treatment, ultraviolet rays can not only sterilize but may also affect its nutrients and protein structural changes. In this project, the impacts of microwave combined ultrasound processing and ultraviolet light processing are discussed on the trypsin inhibitor activity, antioxidant capability, secondary structures, microstructures, in vitro protein digestibility, and allergenicity of soymilk samples.

1.2 Research objectives

The study's overall objective is to investigate the modification of soybean allergens under microwave combined ultrasonic treatment and ultraviolet treatment and simultaneously attain physicochemical properties such as trypsin inhibitor activity and total antioxidant capability of soymilk. Additionally, protein structures and in vitro protein digestibility of soymilk samples are to be investigated.

CHAPTER II

LITERATURE REVIEW

Soybean Allergy: Characteristics, Mechanisms, Detection and Its Reduction through Novel Food Processing Techniques

2.1 Abstract

Human beings have consumed soybean as an excellent food source for thousands of years due to its rich protein, fatty acids, minerals, and fibers. However, soybean was recognized as one of the big eight allergens resulting in a series of allergic symptoms and it even could lead to death. With the increasing demand for soybean products, the challenges caused by soybean allergy need to be resolved urgently. This review described the pathogenesis and clinical characteristics of soybean allergy and its advantages and disadvantages related to four different diagnostic methods. The main allergens present in soybeans, including Gly m Bd 28k, Gly m Bd 30k, Gly m Bd 60k, were reviewed. Further, three different types of mechanisms causing soybean allergy (Type I, III, and IV) were reviewed. The advantages and disadvantages of various food processing methods in reducing soybean's allergen were evaluated. Some novel food processing techniques such as ultrasound and breeding technology have shown potential application in reducing soybean allergenicity. Also, some suggestions regarding the management and treatment of food allergies were addressed in this review. Hopefully, some technologies resulting from medical and food science research can be combined to solve food allergy challenges in future applications.

Keywords

Soybean allergy; Skin prick test; Food processing techniques; Protein structure; Immunotherapy

2.2 Introduction

Soybean (*Glycine max*) is one of the members of the legume family, which is rich in proteins (36.5 g/100g), total lipid (20 g/100g), minerals (e.g., calcium, 277 mg), vitamin (e.g., Vc, 6 mg), fibers (6 g/100g), and fatty acids (2.9 g) (Table 2.1). Studies reported that soybean protein content is 4-5 times higher than that of cereals and twice that of eggs, meat, and fish (Friedman & Brandon, 2001). The Food and Drug Administration (FDA) of the United States of America suggests that adding soy protein to foods can lower cholesterol levels and reduce cardiovascular disease risk (Gagnon et al., 2010). Soybean protein is a complete plant-based protein containing abundant bioactive peptides. These bioactive peptides have the functions of anti-oxidation, angiotensin-converting enzyme inhibition, and blood pressure reduction (Cucu et al., 2013). In addition, soybean is the only vegetable food that contains all eight essential amino acids, and the physiological value of soy protein can reach 100 (Asif & Acharya, 2013).

Soybean has great potential marketing value. In 2017, the Food and Agriculture Organization (FAO) of the United Nations reported that the total worldwide soybean production was 352,643,548 tons, and the output has increased by about 27% compared to five years ago. Furthermore, soybean has obtained significant attention from the food industry due to its unique processing properties, such as emulsification and foaming characteristics. Soybean was considered as an excellent source to produce various products such as soybean oil, soybean flour, soy milk, tofu, soy flakes, or fermented soy products (e.g., miso and soy sauce) for our daily consumption (Tamari, Shoyu). As a meat alternative, it shows an increasing trend and has become popular among vegetarians.

Table 2.1. Nutrients in 100 grams (g) Soybeans, mature cooked, boiled, without salt (Source: USDA Nutrient Database, 2018).

Nutrients	Ingredients	Concentration (100 g soybean)
Proximates	Energy	721kJ
	Water	62.55g
	Carbohydrate	8.36g
	Fiber	6g
	Sugar	3g
	Protein	18.21g
Minerals	Calcium	102mg
	Magnesium	86mg
	Iron	5.14mg
	Potassium	515mg
	Phosphors	245mg
	Zinc	1.15mg
Vitamins	Vitamin B-6	0.234mg
	Vitamin A, IU	9IU
	Vitamin K (phylloquinone)	19.2µg
Lipids	Fatty acids, total saturated	1.297g
	Fatty acids, total monounsaturated	1.981g
	Fatty acids, total polyunsaturated	5.064g

However, soybean was recognized as one of the major allergenic food items among the "big eight," which can trigger various allergic reactions ranging from mild symptoms to life-threatening symptoms (Ogawa, Samoto, & Takahashi, 2000; Jin Wang, Vanga, McCusker, & Raghavan, 2019). Studies reported that approximately 0.3% - 0.4% of children are allergic to soybean worldwide (Savage, Kaeding, Matsui, & Wood, 2010). A survey containing 1,397 volunteers aged from 20 to 44 was conducted, and the results found that about 2% of the total population was allergic to soybean (Ballmer-Weber & Vieths, 2008). Besides, soybean may also have cross-allergic reactions with other allergens due to their sharing of similar epitopes in the proteins. Gly m 4 is a major soybean allergen which is also a homologous protein of

Bet V1 present in birch pollen. Thus, those individuals with a history of birch pollen allergy may get induced allergic reactions when exposed to soybean or related product (Calamelli et al., 2019; Mittag et al., 2004). Furthermore, one study has reported that 43% of infants with cow's milk allergy showed cross-reactivity with soybean (Ahn et al., 2003). Also, the incidence rate is increasing year by year due to the increase of exposure to soybean or relevant products. Thus, solving this global challenge is urgent, using some novel desensitization techniques.

Soybean desensitization techniques commonly can be subdivided into physical, chemical, and biological methods. The physical method includes heating, high-pressure treatment, ultrasound, cold plasma, etc. Physical methods generally do not involve the primary structures of protein. Chemical methods mainly include glycosylation and chemical reagent treatment. Chemical reagent treatment for food products may leave harmful chemical residues in the product. Chung reduced the allergenicity of peanut butter by adding tannic acid but it reduced its nutritients and made it indigestible (S.-Y. Chung & Reed, 2012). The main methods to reduce the allergenicity of soybean protein by biological methods are microbial fermentation, enzyme treatment, plant breeding, and genetic modification. Although the genetic modification method can remove endogenous genes of soybean allergens, its safety remains widely controversial.

This review attempts to describe the pathogenesis, and clinical characteristics of soybean allergy and the advantages and disadvantages of the different diagnostic methods suggested. The main allergens present in soybeans, including Gly m bd 28k, Gly m bd 30k, Gly m bd 60k, were reviewed. Finally, we also discuss the advantages and disadvantages of different

processing methods to reduce soybean allergenicity.

2.3 Pathogenesis, clinical features, diagnosis, and epidemiology of soybean allergy

2.3.1 Pathogenesis and clinical features

Hypersensitivity is also called allergy, which develops when the antigen is recognized by the immune system, and then sensitized lymphocytes or specific antibodies are produced under the interaction of certain conditions (Jin Wang, Vanga et al., 2019). When the patient is exposed to the antigen again, hypersensitivity reactions will occur, and four types of allergy were found (Table 2.2. & Figure 2.1.). Soybean can mainly cause allergic reactions in the human body, including Type I, III, and IV.

Table 2.2. Mechanism of the four types of hypersensitivity (Marc & Olson, 2009; Sampson & Burks, 1996)

Types	Participating ingredients	Effector cells	Primary mediator	Secondary mediator	Major diseases
Type I Immediate immune	IgE	Mast cell, basophils	Vasoactive amine	leukotrienes, Cytokine, Prostaglandin D2, Platelet activating factor	Hives, Redness, Angioedema
Type II Cytotoxic hypersensitiv -ities	IgG, IgM	Fragment crystallizable receptors+cells (phagocyte, Natural killer cells)	Complement, membrane attack complex	Lysosomal enzyme, Perforin	Hemolysis, Autoimmune hemolytic anemia
Type III Immune complex	IgG, IgM	Fragment crystallizable receptors + cells, complement	Antigen-antibo dy complex	Lysosomal enzyme	Systemic lupus erythematosus, Serum sickness, Arthritis
Type IV Delayed-type	Th1,Th2, CTL	Mφ, Eosinophil	IFN-γ, IL-4, IL-5, IL-12, eotaxin	eotaxin, Cytokine, Inflammatory factor	Contact dermatitis, Rash, Tuberculosis

For Type I, the soybean antigen protein enters the intestinal immune system and produces a

large amount of immunoglobulin E (IgE) (Stenton, Vliagoftis, & Befus, 1998). The fragment crystallizable of IgE binds to the fragment crystallizable receptor of mast cells in the tissue to sensitize the body. When the body is exposed to the sensitizing protein again, the sensitizing protein will bind to IgE, which can cause a release of active media such as cell degranulation, histamine, serum toxin, prostaglandin, and enzyme. Eventually, the protein in the plasma leaks into the intestinal lumen, intestinal mucosal edema, goblet cell mucous exudation, and intestine, where absorption of liquid and electrolyte occurs, leading to severe local inflammation in the intestinal mucosa and submucosa (Marc & Olson, 2009; Sun, Li, Li, Dong, & Wang, 2008).

In comparison with other allergic reactions, Type III hypersensitivities need a longer time (from several hours up to weeks) to develop. As shown in Figure 2.1, after soy antigen passes through the human body's intestinal wall and enters the circulation, it can stimulate lymph nodes throughout the body to produce more IgG, forming an IgG-antigen complex (Ellsworth et al., 2008). Antigen-antibody complexes are deposited in the intestinal wall tissues, which activate the complement system to release allergic toxins and vascular permeability enhancers; or adhere to platelets and promote the release of reactive amines leading to inflammation. Research has suggested that Type III reactions may contribute to systemic lupus erythematosus (SLE), serum sickness, and farmer lung (Brostoff, Scadding, Male, & Roitt, 1991; Coico & Sunshine, 2009).

Type IV hypersensitivities are referred to as delayed-type hypersensitivities, mediated by specific T lymphocytes. Antigen protein activates T-lymphocytes through a specific and intricate reaction with T cells, causing a late-type allergic reaction, causing proliferation of

intestinal epithelial lymphocytes in animals, and greatly accelerating the mitotic rate of crypt cells, leading to changes in the structure of intestinal villi (Gell & Benacerraf, 1961).

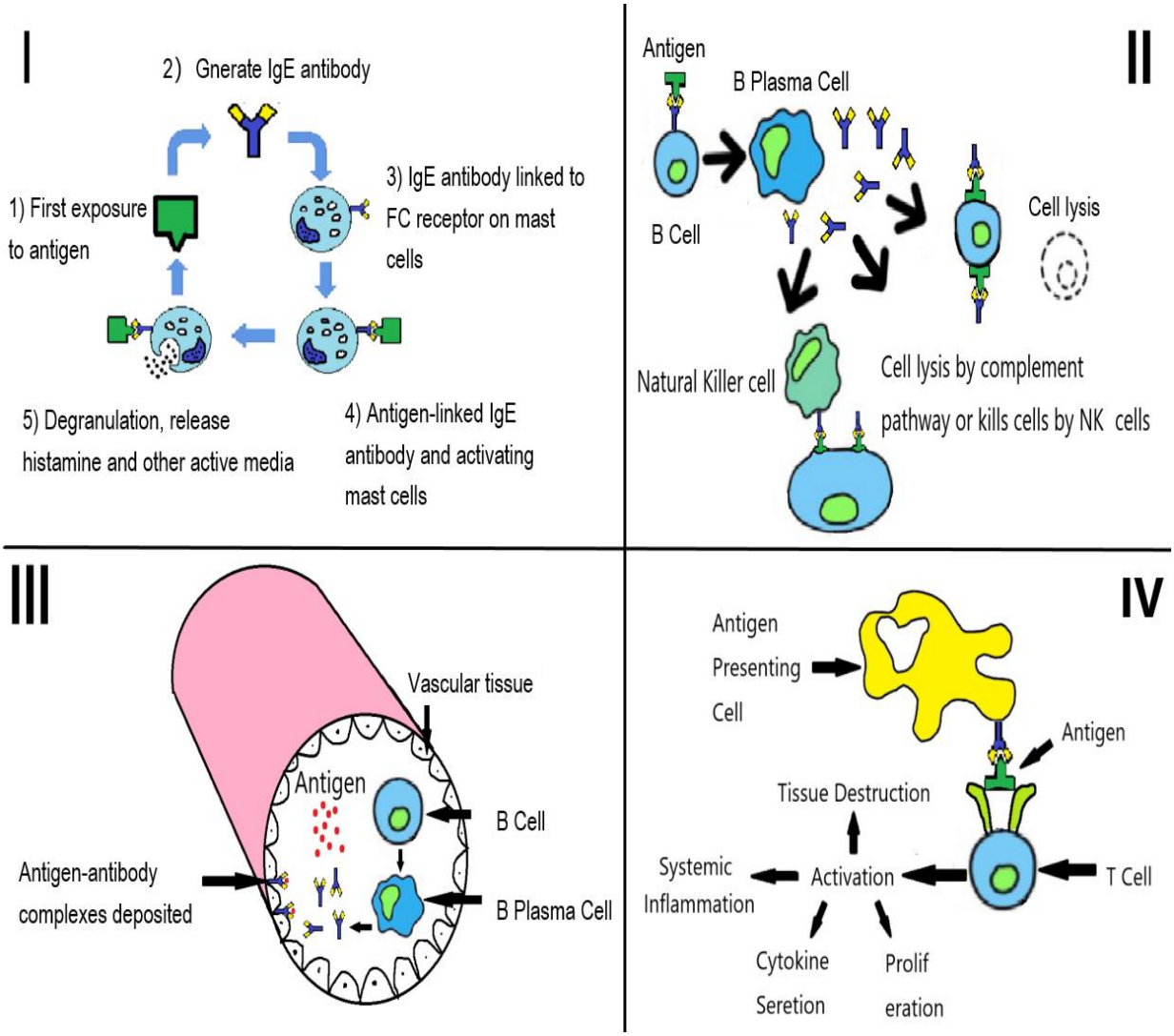

Figure 2.1. Four different types of food allergies and their mechanisms

2.3.2 Diagnosis and detection

2.3.2.1 Skin prick test (SPT)

Skin prick test is one of the most common methods which is used to identify the allergies and the relevant allergic food sources in the hospital. In an SPT, safe doses of allergens are introduced into a patient's skin. If there is a skin reaction, it means that the patient is reacting to the allergen, and the size and color of skin lesions can indicate the severity of the allergy;

normally, a wheal with a mean diameter of 3 mm or greater, would be considered as a positive SPT (Bock, Buckley, Holst, & May, 1977). According to Guidelines for the Diagnosis and Management of Food Allergy in the United States (Panel, 2010), SPT effectively detects the presence of sIgE. It is valuable in identifying the food(s) responsible for IgE-mediated food allergy, but it has low positive predictive value for making an initial diagnosis of food allergy; some patients have sIgE without clinical food allergy so that it may cause over-diagnosis.

2.3.2.2 Allergen-specific serum IgE (sIgE)

sIgE test is an *in vitro* immunodiagnostic test applied to detect the concentration of sIgE in the serum and identify foods that potentially provoke IgE-mediated food-induced allergic reactions. sIgE levels were originally measured using the radioallergosorbent test (RAST), but this test has been replaced by more sensitive fluorescence enzyme-labeled assays (Panel, 2010). The ImmunoCAP system is a new assay for sIgE (detecting sIgE down to 0.1 kU/litre). It has been identified by the Food and Drug Administration (FDA). Similarly, Enzyme-linked Immunosorbent Assay (ELISA) is another method used to determine the levels of sIgE. IgE in the patient's blood is bound to the antigen, and then enzyme-conjugated secondary antibodies are added. The complex reacts fluorescently with the developing agent. The higher the response value, the more specific IgE is present in the specimen. To evaluate the test results, the responses of the patient samples are transformed into concentrations with the use of a calibration curve. These IgE-based diagnostic methods have a high sensitivity (60% to 71%) and specificity (98% to 99%) (Jin Wang, Vanga et al., 2019).

2.3.2.3 Component-resolved diagnostics (CRD)

Component-resolved diagnostics (CRD) is a diagnostic test. The sIgE antibodies respond against the individual allergenic molecules when detected by using purified native or recombinant allergens. CRD can discriminate genuine sensitization from sensitization due to cross-reactivity (Calamelli et al., 2019). For example, Gly m 4 in soybean and Ara h 8 in peanut were identified as homologous proteins of Bet v1. A patient with birch pollen allergy might also be allergic to peanuts or soybean because of cross-reactivity (Mittag et al., 2004; Oriel & Wang, 2019). If CRD leads to a definitive diagnosis, oral targeted food in-take should be avoided in some patients.

2.3.2.4 Oral food challenge (OFC)

Oral food challenge (OFC) is the most definitive method for diagnosing of food allergies. There are three types of OFC: Double-Blind, Placebo-Controlled Food Challenge (DBPCFC), Single-Blind Food Challenge, and Open-Food Challenge. The DBPCFC is the gold standard (Oriel & Wang, 2019). The patient will receive increasing doses of the suspected food allergen and a harmless placebo. Food allergens and placebo are taken separately, at intervals of hours or days. Because allergens and placebo look similar, neither the patient nor his doctor will know which one is received. This procedure term is "double-blind." However, DBPCFC is time-consuming and expensive (Panel, 2010). The detection speed of single-blind and open food challenges are faster than DBPCFC, so these two methods are more used in clinical settings. Single-blind food challenges is that the patient does not know what food is being eaten, while open food challenges is that both the patient and the doctor

know what food is being eaten. Both the subjective factors of the patient and the doctor may affect the diagnosis. Overall, OFC is a good way to diagnose food allergy, but any form of OFC can expose patients to potentially severe allergic reactions.

2.3.3 Global epidemiology of soybean allergy

The soybean allergy has been documented since the 1980s (Ballmer-Weber & Vieths, 2008). Soybeans are widely grown in Asia and the USA. According to statistics from FAO in recent years, soybean production and consumption in the world have been rising. Moreover, due to the development of food processing technology, soy proteins, and their processed products are widely used in the food industry. Allergic cases caused by it are increasing. From 1991 to 1992, Björnsson et al. (1996) surveyed 1,397 unselected adults aged 20-44. They used the RAST method to test the candidates and found that about 2% of people were allergic to soybeans. In addition, in France, Rance et al. (1999) studied 544 pediatric cases in 703 patients with food allergies confirmed by a food challenge, and they found that 1.2% of patients were allergic to soybean. Generally, soybean allergy occurs primarily among young children with atopic dermatitis (Savage et al., 2010). But soybean allergies could also induce serious consequences. Fatal cases of anaphylaxis after ingestion of soybean have been reported (Foucard & Malmheden Yman, 1999). Also, there is a high degree of concern about the allergenicity of genetically modified soybeans, but overall information about soybean allergies is scarce, epidemiologic data on soybean allergy in adults and children is lacking (Ballmer-Weber & Vieths, 2008; Besler, Helm, & Ogawa, 2000).

2.4 Types of soybean allergens

Soybean allergens are proteins present in soybean that can trigger allergic reactions in humans. The World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee has identified eight key allergenic proteins present in soybean. According to the allergen database of the UNIVERSITY of NEBRASKA-LINCOLN, soy allergy proteins can be subdivided into 43 types (http://www.allergenonline.org/). As shown in Table 2.3, the major antigen proteins in soybean were listed, and protein structures of the main soybean allergen are also presented in Figure 2.2.

Table 2.3. Main allergenic proteins of soybean and their relative characteristics.

Biochemical name	Group	MW (kDa)	Types	References
β-conglycinin α-subunit	Gly m Bd 60K	67	Storage protein	(Maruyama et al., 2003)
Hydrophobic seed protein	Gly m 1	8	Structural protein	(Gijzen, Kuflu, & Moy, 2006)
Hull Protein	Gly m 2	8	Structural protein	(Codina et al., 2002)
Profilin	Gly m 3	14	Structural protein	(Mittag et al., 2004)
SAM22	Gly m 4	17	Defense protein	(Kleine-Tebbe et al., 2002)
7S Storage Globulin	Gly m Bd 28K	26	Storage protein	(Liu, Teng, Wang, & Wang, 2013; P. Xiang, Haas, Zeece, Markwell, & Sarath, 2004)
Cysteine protease (P34)	Gly m Bd 30K	32	Storage protein	(Ogawa et al., 2000)
Trypsin inhibitor	Gly m TI	21.5	Defense protein	(Kunitz, 1947)
Glycinin	Glycinin G1, G2, G3, G4, G5	320-360	Storage protein	(Rozenfeld, Docena, FOSSATI, & CA, 2002; P. Xiang, Beardslee, Zeece, Markwell, & Sarath, 2002)

2.4.1 β-conglycinin α-subunit

As shown in Table 2.3, β-conglycinin is one of the most allergenic proteins, which accounts for 30% of total globulin in soybean seed (Maruyama et al., 2003; Shibasaki, Suzuki, Tajima, Nemoto, & Kuroume, 1980). β-Conglycinin is composed of three subunits, namely, α′ (76 kDa), α (72 kDa), and β (52kDa) (Thanh & Shibasaki, 1977). These three subunits have high homology, but only α-subunit of β-conglycinin could cause allergic reactions (Harada, Barker, & Goldberg, 1989). β-conglycinin α-subunit m RNA with a total length of 1955 nt, with poly (A) tail, encoding 605 amino acids, including a 22 amino acid signal peptide at the N-terminus, the precursor peptide after removing the signal peptide is 583 amino acids. Finally, 40 amino acids were cut from the N-terminus during processing to form a mature α-subunit of 543 amino acids.

2.4.2 Cysteine protease (P34, Gly m Bd 30K)

P34 (Gly m Bd 30K) is a kind of soybean storage protein that belongs to the 7S component. Studies have reported that P34 is the most active allergenicity protein in soybeans, which could be frequently recognized by IgE antibodies in the serum of patients with atopic dermatitis (Ogawa et al., 2000). The mature P34 vacuolar protein is composed of 257 amino acid residues, which is obtained by removing a portion of the 122 amino acid residues at the N-terminus from the precursor protein with a molecular weight of about 47,000 during maturation in the vacuole (Kalinski, Weisemann, Matthews, & Herman, 1990). It also has 30% homology with Der p 1 (a dust mite allergen) (L'Hocine & Boye, 2007). The gene structure belongs to the papain superfamily, but it is likely not to have protease activity

because its active center is missing a cysteine. Mature P34 protein has at least ten epitopes. Different IgE epitopes have significant differences in serum binding capacity for different allergic patients. Five major epitopes have been identified in this protein (Helm et al., 1998).

2.4.3 7S Storage Globulin (Gly m Bd 28K)

Gly m Bd 28K was initially isolated from soybean as a glycoprotein and was later found to be an unknown Asn glycoprotein with a mass of 26 kDa (Tsuji et al., 1997). It was identified that the Asn-N linked glycan moiety having the same sugar composition as Gly m Bd 30K was located in the Asn20 residue of Gly m Bd 28K. The N-terminal amino acid sequence analysis gave a result of FHDDEGGDKKSPKSLFMSDSTRVFK (Ogawa et al., 2000). Gly m Bd 28K is another important allergen in the 7S component and is recognized by the serum of approximately 25% of patients with soybean allergy (Ogawa et al., 1991). Gly m Bd 28K cDNA is 1567 bp in length with a polyadenylated tail. It has an open reading frame of 1419bp and a 3 'untranslated region of 148bp. One of the open reading frames encodes a protein precursor consisting of 473 amino acids (Tsuji et al., 2001).

2.4.4 Glycinin acidic peptides and other soybean allergenic proteins

The overall sensitizing capacity of glycinin is lower than that of the 7S component. The allergenicity is derived from the constituent subunits. Studies have shown that the acid peptides A1a, A1b, A2, A3, and A4 of 11S globulin are all allergic proteins (Djurtoft, Pedersen, Aabin, & Barkholt, 1991; Zeece, 1999). Also, soybean trypsin inhibitors, Gly m 1, Gly m 2, Gly m 3, and Gly m 4 (SAM22), are several soy allergens that have been studied a lot. The overall content of these allergic proteins is relatively low, accounting for only 15%

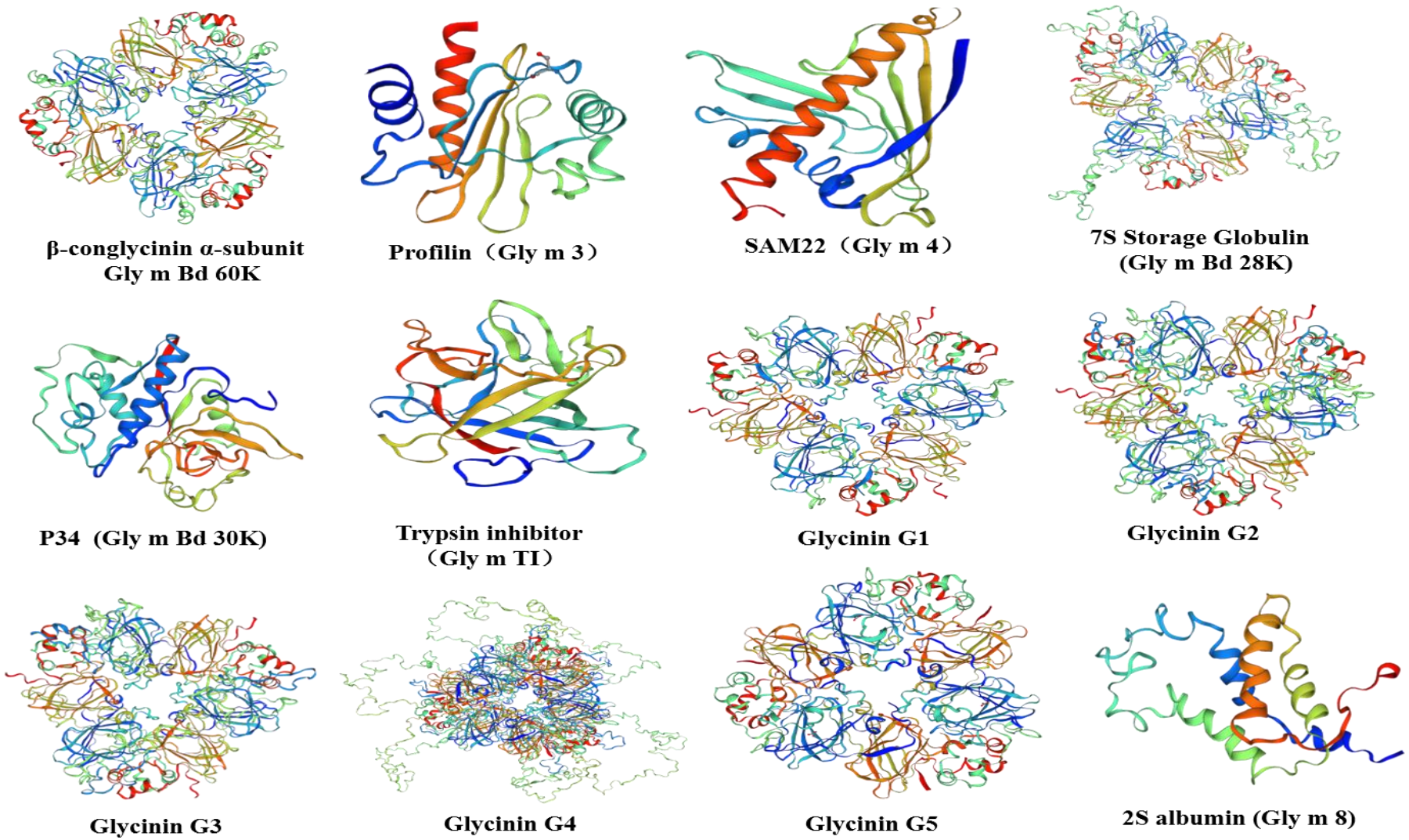

Figure 2.2. Protein structure of soybean allergens obtained from protein data bank.

-20% of the total protein of soybean, and the thermal stability and antigenicity are relatively weak. Hence it can easily remove the allergenicity of these proteins (Lui, White, & Litster, 2007; Müller et al., 1998).

2.5 Effect of processing techniques on the reduction of soybean allergens

Although avoiding consuming foods containing soybean allergens is the best way to prevent allergic reactions suggested by the doctor (Asensio, González, García, & Martín, 2008), soybean as fillers, emulsifiers, and protein fillers are now widely used in the food industry. Meanwhile, many food products have not been clearly marked as containing soy ingredients, so it is challenging to avoid soy allergens' ingestion entirely. Therefore, it is very necessary to reduce the content of active allergens in soybeans or relevant products using different food processing techniques. In this study, we reviewed and compared three different techniques that may be used to reduce the allergenicity of soybean or related products (Table 2.4 & Table 2.5).

2.5.1 Physical method

2.5.1.1 Thermal processing

Heat processing methods can be divided into moist heating, dry heating, and dielectric heating (RF and Microwave). Thermal processing is one of the most common food processing methods used to inhibit bacteria and enzymes' activity through different temperatures and times (Verma, Kumar, Das, & Dwivedi, 2012). For allergic proteins present in food, heat treatment can cause damage in the spatial conformation of the protein, which in turn could result in a reduction in the allergenicity of the food. Lakemond et al. (2000) found

that processing under high temperatures induced significant changes in the spatial conformation and three-dimensional structure of glycinin, which led to a decrease in the allergenicity or removed allergenicity of glycinin. Similalry, Cabanillas et al. (2018) boiled soybeans for 15 min, 30 min, and 60 min at 100 ℃ (Table 2.4). The results showed that soybeans boiled for 15 min and 30 min rarely affected the IgE-binding capacity of soybean proteins. In comparison, the IgE-binding capacity of soybean proteins was significantly inhibited when boiled for 60 min (Cabanillas et al., 2018). Shibasaki et al. (1980) studied the effect of heat treatment on the binding capacity of IgE-specific antibodies in different components (2S, 7S, and 11S-globulins) of soybeans. However, when fractions were heated to 80 ℃, the ability of 2S-fraction to combine with IgE was enhanced, and the allergenic potencies were increased, but allergenic potencies of other fractions were reduced by 40%-75% (Shibasaki et al., 1980). In an EAST experiment, only nine of 15 soybean allergic patients had detectable specific serum IgE against microwave heated (70 w, 25 min) soybean protein (Vieths, Zagon, Fischer, Dehne, & Boegl, 1995).

Although many studies have reported that thermal processing could destroy the spatial structure of proteins, resulting in a reduction in the allergenicity of soybeans. Davis et al. (2001) found that the Maillard reaction in the glycosylation product generated during the thermal processing might cause the formation of some new antigens or allergen epitopes. Similarly, Friedman et al. (2001) confirmed this conclusion. They found that high-pressure heat treatment significantly enhanced the IgE binding ability of Gly m Bd 30K protein (a major soybean allergen). Besides, Boxtel et al. (2008) found that heat treatment did not affect the binding capacity of glycinin to IgE. Therefore, it is difficult to effectively reduce the

allergenicity of soybean protein by heat treatment alone due to the complexity of the allergen epitope structure of soybean, and some novel food processing techniques are needed in the future.

2.5.1.2 Non-thermal processing

In contrast to heat-based processing methods, non-thermal treatments are beneficial to maintain the sensory attributes and nutritional properties of food while changing the allergenicity. In recent years, many studies have been performed to develop non-thermal processing techniques, which could be potentially used as an alternative method to reduce the allergenicity of soybean. These food processing techniques include high-pressure treatment, cold plasma treatment, pulsed ultraviolet, and ultrasonic processing.

High-pressure treatment

High-pressure treatment can provide pressure over 100 MPa with specific ranges of temperatures, which could be used to inhibit the activity of microorganisms and modify the enzymes, proteins, starch, and other biological macromolecules in food (H. W. Huang, Hsu, Yang, & Wang, 2014). Studies found that high-pressure treatment can effectively maintain the natural taste, flavor, and nutritional value of the food and may produce some new textural characteristics (H. W. Huang et al., 2014). Recently, studies have reported that high-pressure processing could break down the non-covalent bonds in the protein molecule and cause significant changes in its structures (e.g., secondary structure).

As shown in Table 2.4, Tang et al. (2009) treated glycinin with high-pressure treatment at 300-500 MPa for 10 min. The results found that glycinin was dissociated into subunits, and

the conformation of these subunits also changed after processing at 300 MPa for 10 min. At the same time, the number of sulfur-containing groups, hydrophobic regions, and amino acids showed a significant increase. After treatment at 400 MPa for 10 min, glycinin was denatured entirely. The secondary structure, including α-helix and β-sheet of glycinin, were destroyed and transformed into random coils when a further increase of the pressure up to 500 MPa. Peñas et al. (2006) found that 7S globulin (β-conglycinin) and 11S globulin in soybean were denatured and inactivated under high-pressure treatment at 300-400 MPa.

Further, they also found that high-pressure processing at 200-300 MPa could cause a significant reduction in the allergenicity of Gly m 1 in soybean. Similalrly, Li et al. (2012) processed soybean protein isolates with high pressure at 200 - 300 MPa for 5-15 min, and the results found that the content of hydrophobic bonds and free sulfhydryl groups of soybean protein isolates increased significantly compared with the untreated samples during treatments. The allergenicity of soybean protein decreased by 48.6% when treated at 300 MPa for 15 min compared with the control group. This reduction in the allergenicity of soybean proteins was strongly associated with the protein structural changes during processing. Also, in this study, they found the interaction between the hydrophobic bond and the thiol group, which gradually decreased, and the average length of the soybean protein isolate spiral increased significantly (Li, Zhu, Zhou, & Peng, 2012). The possible reason is that the soy protein allergen epitope is closely related to the α-helix and β-sheet secondary structures, and these interactions and secondary structural changes provide direct evidence for reducing soybean allergenicity.

In summary, high-pressure treatment shows potential applications in reducing the allergenicity of soybeans through the secondary structure effect (e.g., α-helix and β-sheet) of proteins under different pressures. However, the effect of high-pressure treatment in reducing food allergenicity is very limited. Thus, to provide safe food products for individuals with soybean allergy history, the relevant parameters need to be improved, and some novel processing methods are still in need.

Cold plasma

In the past decade, cold plasma technology has attracted researchers' attention due to its advantages in microbial decontamination of food products. Also, cold plasma could provide a low temperature, short processing time, high power efficiency, strong antimicrobial effect, and minimal impact on food quality (Bourke, Ziuzina, Boehm, Cullen, & Keener, 2018). Ultraviolet photons, ions, electrons, free radicals, molecules, motivated atoms etc., are generally referred to as ionized gases in the fourth state of matter and are composed of a range of reactive substances (Dasan et al., 2017). Many studies found that cold plasma could induce the reactions between proteins resulting in their conformational structural changes and leading to a reduction in food allergenicity (Ekezie et al., 2018). Meinlschmidt et al. (2016) used cold plasma technology to treat soybean allergen, β-conglycinin, and glycinin. After cold plasma treatment, the results found that the corresponding protein band strength decreased, and then a new protein band of 50 kDa was formed. In addition, it was found that the disappearance of protein bands in SDS-PAGE was caused by insoluble aggregates formed after treatment. Finally, in the analysis of ELISA, it was found that the immunoreactivity of soybean protein samples decreased by 89-100%.

Also, Segat et al. (2016) unfolded whey protein molecules and changed their 3D structures by using direct Cold Plasma treatment (Segat, Misra, Cullen, & Innocente, 2016). These studies illustrate that cold plasma treatment shows a potential application in reducing the allergenicity of food. The related mechanisms are still not clear, and thus further studies are needed in the future.

Pulsed ultraviolet (PUV)

Pulsed ultraviolet is a type of pulsed excitation technology equipped with a unique inert gas tube that is used to excite and process ultraviolet rays in a pulse form. In recent years, pulsed ultraviolet light has been applied to ionize molecules due to the formation of high energy within a short time (Meinlschmidt et al., 2016). Molecular vibrations and rotations are driven by visible and infrared light. PUV light has photothermal, photophysical, and photochemical properties, which may cause protein aggregation, loss of conformational epitopes, and thus change the allergen structure and reduce IgE-binding ability. Yang et al. (2010) treated the soybean protein extract with PUV light and found that glycinin (11 S, 14 ~ 34 kDa) and β-conglycinin (7S, 50 kDa) were significantly reduced as analyzed by SDS-PAGE. Also, the results observed that protein molecules with a molecular weight of 150 to 250 kDa were crosslinked or polymerized during the cold plasma, which may contribute to the reduction in the allergenicity of food proteins (W. W. Yang et al., 2010). However, long-time PUV light treatment could increase temperature, moisture loss, and weight loss due to the formation of photothermal effect during processing (S. Y. Chung, Yang, & Krishnamurthy, 2008; Krishnamurthy, Irudayaraj, Demirci, & Yang, 2008). Therefore, PUV showed a potential application in modifying the allergic soybean proteins, while some functional characteristics

such as digestibility and nutrients content may be affected negatively during processing. Further studies are needed in the future.

Ultrasonic processing

The frequency of mechanical waves used in ultrasonic processing varies from 20 kHz to 100 kHz (Feng, Barbosa-Cánovas, & Weiss, 2011). The sound wave will spread through the medium, resulting in a series of compression and rarefaction. The interference between bubbles and the critical size of bubbles leads to fracture, which results in an increase in the temperature and pressure in the medium. When bubbles explode, the temperature and pressure in the local area may be as high as 5000 K and 1000 atm, which provides enough energy to modify the related molecules (Shriver & Yang, 2011). For protein molecules, hydrogen interactions between polar and nonpolar groups can contribute to a variety of changes in the natural structure, such as conformational changes, secondary structure damage, other intra/inter molecular interactions, and the recombination of disulfide bonds (Stanic-Vucinic et al., 2012). At the same time, the high shear stress and velocity gradient will lead to the generation of micro streams, and the intense agitation of micro streams may destroy the van der Waals interaction and hydrogen bond in peptides, leading to protein denaturation. Therefore, ultrasound treatment showed potential effects on the characteristics of soybean allergens (Soria & Villamiel, 2010; Tian, Wan, Wang, & Kang, 2004).

Yang et al. (2015) treated soybean seeds with ultrasound at different power levels (0-300 W) and then germinated them for 5 days. The results found that ultrasound processing disrupted or eliminated the epitopes in the proteins, which led to a significant reduction in the

allergenicity of soybeans. Specifically, the allergenicity of soybeans treated with ultrasound at 300 W was reduced by 51% compared to the untreated samples (H. Yang, Gao, Yang, & Chen, 2015). In addition, Choudhary et al. (2013) treated soybeans with a high-intensity ultrasonic processor at a frequency of 37 kHz for 10 minutes. The results reported that high-intensity ultrasound processing at 37 kHz caused significant changes in the secondary structure of soybean proteins, which resulted in a 24% reduction in the sensitization of soybean proteins (R Choudhary, Gautam, Perez-Alvarado, & Kinsel, 2013).

At present, even if there is very limited literature regarding the effects of ultrasound on soybean allergens, ultrasonic processing, a non-thermal processing method, might have a potential application in modifying the allergic proteins present in soybeans, and further research is needed in the future.

2.5.2 Chemical method

Glycosylation

Glycosylation is a chemical reaction in which a carbohydrate is covalently bonded to an α or β-amino group on a protein molecule to form a glycosylated protein. Recently, studies have reported that glycosylation, as a chemical method, could be used to reduce the allergenicity of soybeans. Van de Lagemaat et al. (2007) used fructose or oligofructose (FOS) powder and liquid systems to saccharify soy protein isolate (SPI) by Maillard reaction. The results of SDS-PAGE showed that the protein band of 7S and 11S fragments present in soybeans was affected during the reaction of glycosylation. Further, an ELISA test was used to confirm these changes and the results found that the allergenicity of soy protein isolate was reduced

by 90% compared with untreated samples (van de Lagemaat, Silván, Moreno, Olano, & Del Castillo, 2007). Simialrly, Babiker et al. (1998) obtained a soybean protein-galactomannan complex by Maillard reaction. The results also found that one of the major soybean allergens, a 34-kDa protein that is easily recognized by IgE antibodies in the serum of patients with a soybean allergy history, was removed during the reaction (Babiker et al., 1998). Wilson et al. (2005) also reported that galactomannan prepared with guar gum masked the recognition structure of Gly m Bd 30 K antibody, thereby successfully eliminating the sensitization of Gly m Bd 30 K. And the latest research reported glycation reaction with lactose could also remove the antigenicity and allergenicity of glycinin, the antigenicity of 11S after glycosylation was reduced by approximately 30% compared to raw 11S (Bu, Zhang, & Chen, 2015). These reductions in the allergenicity of soybeans during the glycosylation reactions could be attributed to the blocking of Ig-E binding epitopes of proteins by the added compounds. The second pass is the chemical solution, such as glutaraldehyde solution, copper sulfate, sodium sulfite, and ethanol, which can change the molecular structure of trypsin inhibitors and destroy disulfide bonds and achieve the purpose of passivating antigens. However, Ogawa et al. (1995) performed a western blotting experiment on the hydrolyzed β-conglycinin peptide and found that the peptide that binds to IgE is not a glycopeptide. They believe that glycosylation is not a necessary condition for binding to IgE. Calabozo et al. (2002) used dot blot detection to find that IgE can also react with deglycosylated peptides or proteins. Therefore, the role of sugar chains in sensitization is not certain. At present, there are different conclusions and opinions on glycosylation in soybean sensitization. The reasons for the disagreement are mainly due to two aspects. One is that

there are many diagnostic methods for determining allergic reactions; the other one is that the type of sera (animals or humans) used in the experiments are numerous. Therefore, the role of glycosyl groups and their structures in sensitization needs further investigation.

2.5.3 Biological method: fermentation and enzymatic hydrolysis

Fermentation is one of the traditional food preserving methods that could be used to extend the shelf life of food products. During processing, the big food molecules like proteins could be decomposed into smaller molecule peptides and amino acids, which increased the protein content and digestibility and altered the structure of their epitopes, thereby reducing the allergenicity of food products and improving the nutritional quality of soybeans (Shekib, 1994). As shown in Table 2.5, Hong et al. (2004) posted soybeans in a bed-packed solid fermentor with Aspergillus oryzae GB-107 for 48 hours, and then its trypsin inhibitor and nutrient content of soybeans were measured. The results showed that fermentation increased the protein content in soybeans, eliminated trypsin inhibitors, and reduced the size of peptides (Hong, Lee, & Kim, 2004). In Japanese soy sauce, after soybeans are fermented by moromi, the protein was completely degraded into peptides and amino acids by microbial proteolytic enzymes (such as koji), and their allergenicity was in an undetectable range. At the same time, it was found that polysaccharides derived from soybean cell walls still exist in soy sauce after fermentation treatment. These polysaccharides have effective anti-allergic activity *in vitro* and in vivo and are effective interventions for patients with allergic rhinitis (Kobayashi, 2005). Yamanishi et al. (1995) soaked soybeans in water overnight, then autoclaved at 120 ℃, 0.2 kgf /cm^2 for 20 minutes, and inoculated 0.8 mg of dry Bacillus natto powder per 1 g of soybeans. The fermentation process was performed at 40 ℃ with sufficient moisture. The

results demonstrated that the sensitization of Gly mBd 30 K in soybean was significantly reduced during the fermentation of Bacillus natto. During the fermentation process, the microorganisms and the internal environment of the fermentation are not very stable, the fermentation cycle is long, and unknown anti-nutritional components may appear in the fermentation process; therefore, the use of microbial fermentation cannot completely remove the antigen in soybean.

Enzymatic hydrolysis is another similar method that can be used to reduce the allergenicity of soybeans by destroying the spatial structure of proteins or breaking down the chemical bonds of soybean proteins. Tsumura et al. (1999) found an alkaline protease of Proleather FG-F, it can effectively hydrolyze Gly m Bd 30K, Gly m Bd 28K sensitized protein 7S globulin α subunit when the hydrolysis degree reaches 25%. In addition, Lee et al. (2007) hydrolyzed soybean 11S globulin with pepsin and chymotrypsin, and the results showed that the sensitization of the 11S enzymatic fragment was reduced up to 100%. However, some studies found that partial hydrolysis does not reduce or remove food allergies in a certain way because cross-contamination may occur during processing, and new allergens appear (Wigotzki, Schubert, Steinhart, & Paschke, 2000). Cabanillas et al. (2010) hydrolyzed lentils with protein and found that proteolysis destroyed an important Ig E binding epitope in *in-vitro* experiments, but protein allergens were still detected in the patient's serum indicating that enzymatic hydrolysis can destroy some protein allergen but not completely removed. In general, in the enzymatic hydrolysis process, due to the different enzymes and the enzymatic hydrolysis process, the enzymatic hydrolysis conditions are not easy to control, and there are also differences in the components and antigenicity in the final product obtained.

2.5.4 Breeding and genetically modified methods

Breeding is a traditional method for removing the allergenic protein from soybeans: Takahashi (1994) cultivated soybean lacking the α-subunit of β-conglycinin through mutation breeding. Also, researchers at Kyoto University in Japan used natural hybridization and γ-ray irradiation to induce low-sensitivity soybean varieties by inducing plant mutations as the preferred method, and clinical experiments showed that the taste of this low-sensitivity soybean did not change (Sathe, Kshirsagar, & Roux, 2005). In addition, L'Hocine et al. (2007) cultured a new variety of soybean, which excluded allergenic proteins such as P34, Gly m 2, Gly m 1.0101, Gly m 1,0102, and r Gly m 3 through breeding pathways. In 2013, Jeong et al. (2013) developed molecular markers and polyclonal antibodies to select soybean lines that express P34 at low levels efficiently. The selected soybean lines can continue to be crossed with other non-allergenic soybean varieties to breed low-allergenic soybean varieties. And in 2017, researchers used a marker-assisted method, selected low-P34 lines from a cross-population. Concentrations of the P34 protein, as identified with a polyclonal antibody, were reduced by 50–70% (Watanabe et al., 2017). Although plant breeding pathways are used to cultivate low-sensitized soybean varieties, the antigenic protein can be fundamentally removed. However, most antigen proteins are used for defense. Decreasing the content of antigen proteins may cause adverse effects on soybeans, such as yield and disease resistance (Clarke & Wiseman, 2000a, 2000b). Finally, whether the breeding method will bring some negative effects on nutrition and function or whether it will express new antigenic components also needs further investigation.

Genetic modified soybean protein is extensively used in processed foods. Genetic modification could eliminate the allergenicity of soybean allergens by eliminating endogenous genes. Herman et al. (2003) successfully removed major allergens Gly m Bd 30K in soybeans by applying the genetic modification method. However, the removal of allergens by genetic engineering is controversial, which may bring new potential allergens, and there are no clear standards to evaluate the safety of genetically modified foods. A widely publicized report on allergenicity has fueled speculation on the safety of genetically modified foods that genetically modified foods may be responsible for the rise in global allergies (Maghari & Ardekani, 2011). In 1966, methionine-rich albumin from Brazilian triangular walnut was introduced into soybeans to make up for the lack of this essential amino acid in soybeans. But Nord lee research found that allergen proteins were also introduced into the blood of human volunteers and recognized by the serum, triggering an allergic reaction (Nordlee, Taylor, Townsend, Thomas, & Bush, 1996).

Like breeding methods, transgenic technology may have certain effects on the physiological characteristics of soybean plants and the processing properties of soybean protein, such as disease resistance of soybean. Whether transgenic technology can be applied to desensitization in practice remains to be studied, and it will focus on attention in future food desensitization technology.

Table 2.4. Summary of recent studies regarding the effect of physical processing technologies on the allergenicity of soybean.

Type	Method	Treatment	Result	Reference
Physical methods	Moist heating	Boiled soybeans for 60min	Fewer IgE-binding proteins	(Cabanillas et al., 2018)
	Dry heating	Fractions of 2S,7S, and 11S-globulins of soybean, heated to 80 ℃	2S-fraction allergenicity enhanced, Other fractions were reduced by 40% -75%	(Shibasaki et al., 1980)
	Microwave heating	Microwave heated (70w, 25min)	9 of 15 soybean allergic patients had detectable specific serum IgE	(Vieths et al., 1995)
	High-pressure treatment	400Mpa, 10 min	Glycinin was denatured entirely	(Tang & Ma, 2009), (Jeong , 2005)
		500Mpa, 10 min	Secondary structure α-helix and β-sheet of glycinin were destroyed and transformed into random coils	
		200MPa - 400 MPa	7S globulin and 11S globulin were denatured and inactivated Gly m 1allergenicity was reduced	(Peñas, Préstamo, Polo, & Gomez, 2006; YAMANISHI, HUANG, TSUJI, BANDO, & OGAWA, 1995)
		300 MPa, 15 min	Soybean protein allergenicity decreased by 48.6%	(Li, Zhu, Zhou, & Peng, 2012)
	Cold plasma	Microwave-driven plasma torch (2.45 GHz, 12kW, air, 18slm, 10min),	Immunoreactivity of soybean protein samples decreased by 89-100%	(Meinlschmidt et al., 2016)
	Pulsed Ultraviolet	Xenon PUV system 13.2 cm from the light source 235.2J/cm2	11 S Glycinin (14 ~ 34 kDa) and β-conglycinin (7S, 50 kDa) were significantly reduced	(W. W. Yang et al., 2010)
	Ultrasonic treatment	300 W treated soybean seeds, then germinated for 5 days	Allergenicity was reduced by about 51.39%	(Yang, Gao, Yang, & Chen, 2015)
		37 kHz, 10min	Allergenicity was reduced by about 24%	(Choudhary, Gautam, Perez-Alvarado, & Kinsel, 2013)

Table 2.5. Summary of recent studies regarding the effect of chemical and biological processing methods on the allergenicity of soybean.

Type	Method	Treatment	Result	Reference
Chemical methods	Glycosylation	Used fructose or oligofructose (FOS) in powder and liquid systems to saccharify soy protein isolate (SPI)	Allergenicity was reduced by 90%	(van de Lagemaat, Silván, Moreno, Olano, & Del Castillo, 2007)
		Protein-galactomannan complex by Maillard reaction	Removed 34 kDa protein, Masked the recognition structure of Gly m Bd 30K	(Babiker et al., 1998) (Wilson, Blaschek, & De Mejia, 2005)
		Glycation reaction with lactose	Antigenicity of 11S reduced 30%	(Bu, Zhang, & Chen, 2015)
Biological methods	Fermentation	Fermented by Aspergillus oryzae GB-107 for 48 hours	Eliminated trypsin inhibitors	(Hong et al., 2004)
		Fermented by moromi	No longer allergenic	(Kobayashi, 2005)
		Autoclave (120 ℃, 0.2 kgf / cm2 for 20 minutes) inoculate 0.8 mg of dry Bacillus natto powder per 1 g of soybeans.	Gly mBd 30K in soybean was significantly reduced	(YAMANISHI et al., 1995)
	Enzymatic hydrolysis	Alkaline protease of Proleather FG-F (25% hydrolysis)	Hydrolyzed Gly m Bd 28K,30K,60K	(Tsumura, Kugimiya, Bando, Hiemori, & Ogawa, 1999)
		Hydrolyzed soybean 11S globulin with pepsin and chymotrypsin	Sensitization of the 11S enzymatic fragment was reduced	(Wigotzki, Schubert, Steinhart, & Paschke, 2000)
	Breeding	Gamma-rays mutation breeding	Lacking the Gly m Bd 60K	(Takahashi, Banba, Kikuchi, Ito, & Nakamura, 1994)
		Natural hybridization and γ-ray irradiation	Low-sensitivity soybean varieties was induced	(Sathe, Kshirsagar, & Roux, 2005)
		Marker-assisted method	Selected low-P34 lines P34 reduced by 50–70%	(Watanabe et al., 2017)
	Genetic Modified	Gene-silencing techniques	Eliminated P34	(Herman, Helm, Jung, & Kinney, 2003)

2.6 Management and treatment of food allergy

2.6.1 Dietary management

Currently, there are no effective medical methods that could be used to cure soybean allergy. One of the most effective treatments for people with a soy allergy history is to avoid ingestion of corresponding foods or related products. Further, learn about how to deal with allergic reactions that may occur after the accidental ingestion of soy or related products is also very necessary. Soybean is a versatile ingredient in food processing due to its rich protein content and inexpensive properties. Thus, avoiding the ingestion of soybean is difficult. Meanwhile, there are multiple allergenic proteins in soybean. The list of potential cross-reactive foods is significant. For those patients with soy allergies, it is difficult for them to know their dietary restrictions and a complete list of allergens related to the cross-reactivity of soy allergies. Even in developed countries, food ingredients in the food are unclear, and/or incorrect labeling is very common. Consumers who are allergic to soy should carefully read the detailed ingredient information of each product before purchasing them.

2.6.2 Emergency treatment

Food allergy could trigger a rapid multi-organ allergic reaction and even result in severe or fatal health conditions (Bird, Lack, & Perry, 2015). Thus, timely identification of signs and symptoms of an allergic reaction is essential for proper management. In 1987, the U.S. Food and Drug Administration (FDA) approved epinephrine which is available as a self-injectable medicine that is highly active and secure for the management of anaphylaxis. For patients with a severe allergy, intramuscular epinephrine is the first-line treatment in all instances of

anaphylaxis in both inpatient and outpatient situations. But many studies have shown that patients do not get enough adrenaline when they have a severe allergic reaction (Julie Wang & Sicherer, 2017). Part of the reason may be related to the fear of needles or adverse drug reactions. On the other hand, it may be that the patient lacks relevant knowledge, does not realize the importance of self-administration, and does not know how to use the adrenaline device (Shemesh et al., 2017). Fleming et al. (2015). studied 384 cases of severe allergic reactions due to food allergies and found that those who used adrenaline before the hospital needed fewer hospitalizations than those who used it after arriving at the hospital (17% VS 43 %, P <0.001).

2.7 Future immunotherapy

Currently, no immunotherapy can cure food allergies. The primary purpose of allergen immunotherapy is desensitization, a temporary increase in the response threshold that provides a degree of safety, the latter depending on continuous therapeutic exposure. Ideal treatment should allow patients to eat whatever they want without being affected by stimulants such as disease or exercise, that is, to achieve true, complete tolerance. At present, the hottest areas of immunotherapy are research on Oral immunotherapy (OIT), Epicutaneous immunotherapy (EPIT), and Sublingual immunotherapy (SLIT) routes.
The formation mechanism of oral tolerance is very complicated, and it is not clear so far. It is generally considered to be related to the regulation of immune response, which may be related to the loss or disability of immune cells and the activation of immune regulatory cells (Burks, Sampson, Plaut, Lack, & Akdis, 2018). But most clinical trials currently focus on the three most common allergens: peanuts, eggs, and milk, and get some positive results (Burks

et al., 2018). Although oral immunotherapy research on other food allergens is ongoing, this method is time-consuming and at risk of allergies. SLIT is another therapy for allergic reactions, which requires allergen extracts to be given sublingually every day during the treatment (sublingual 2 to 3 minutes after swallowing). SLIT therapy can desensitize to a certain extent, is well tolerated, and has mild adverse reactions, usually only oropharyngeal itching and tingling. However, it rarely achieves sustained unresponsiveness (Burks et al., 2018). EPIT therapy is a new therapy that is still in the research stage and needs further study. EPIT therapy involves applying a small patch of allergen patch to the back or upper arm and changing the patch every 24 hours for several years. The efficacy of this therapy is weaker than OIT and SLIT, but it is the safest and well-tolerated. Most patients will only have mild local skin irritation symptoms at the patch site (Burks et al., 2018). To sum up, these therapies pose a risk of allergies to patients. Therefore, there is still much work to be done, and more novel effective immunotherapy treatments need to be developed in the future.

2.8 Conclusions

Soybean is one of the eight major allergens, which could cause some cross-reactivity, with other related allergens, such as birch pollen, leading to an increasing trend all over the world. Soybean can cause a series of allergic symptoms, it even could lead to death. With the increasing demand for soybean products, the problem of allergenicity of soybean protein needs to be solved urgently. This review reported major soybean allergens in soybean such as Gly m Bd 28K, P34 (Gly m Bd 30K), and β-conglycinin α-subunit (Gly m Bd 60K). In the food industry, some physical or chemical treatments are often used to reduce the allergenicity of soybean by changing the protein structure or IgE binding site of allergens. However,

different processing methods have shown various effects on the modification of proteins and their functional characteristics, and currently, the effective processing methods are still very limited. Thus, more novel food processing techniques are still needed. Further, the processed soybean products should be evaluated using medical methods like skin prick test and blood test to detect their allergenicity. Finally, education on proper management of soybean allergy patients and the development of desensitization strategies can improve the physical condition and quality of life of patients with food allergy history.

CONNECTING TEXT

In the review reported in Chapter II, we have seen the characteristics, mechanisms, and detection methods of soybean allergy. And some novel processing techniques to reduce soybean allergenicity, including high-pressure processing, ultrasound, pulsed ultraviolet light, cold plasma, fermentation, enzymatic hydrolysis, and combination processing were described. In the next chapter, an experimental study on the effect of microwave (85℃ 10 min) combined with ultrasonic processing (0, 4, 8, 12, 16 min) on the trypsin inhibitors activity, antioxidant capability, secondary structures, microstructures, in vitro protein digestibility, and allergenicity of soymilk samples will be investigated.

CHAPTER III

Effects of Microwave combined with Ultrasonic Processing on the Physiochemical and Allergenic Properties of Soymilk

3.1 Abstract

Soymilk is one of the common beverages with high nutritional value derived from soybean. However, many individuals are allergic to soybean. This study investigates the influence of microwave combined ultrasonic treatment on the trypsin inhibitors content, antioxidant capability, secondary structures, microstructures, in vitro protein digestibility, and allergenicity of soymilk samples. Total soluble protein content and total antioxidant capacity increased gradually with the increase of time and reached the highest value at ultrasonic treatment for 16 min. Meanwhile, the decrease of trypsin inhibitor activities with the longer treatment was observed, and it was also related to higher values of in-vitro protein digestibility. Larger aggregations of soy protein were observed by SEM. Secondary structural change with the increasing time, specifically showing the upward trend of β-sheets and the downward trend of α-helix. Finally, the ELISA test shows that a 16 min of ultrasound treatment significantly inhibited the IgE binding capacity of Gly m 5. These results demonstrated that microwave combined ultrasonic treatment has the potential to promote the elimination process of soybean allergenicity and it would not bring about the adverse effects of long-term high temperature treatment of soybeans.

Keywords: Ultrasonic treatment, ELISA, Soymilk, Digestibility, Microstructure

3.2 Introduction

Soymilk is a traditional beverage in Asia. Due to its nutritional benefits and the increasing demand for plant-based milk substitutes combined with the challenges of lactose intolerance and dairy allergies, the popularity of soymilk in western countries is increasing (Vanga et al., 2020b). Many studies report that soymilk is a plant-based protein beverage and contains anti-nutritional factors, such as trypsin inhibitor (TI), which leads to lower digestibility and low absorption of nutrients (Nguyen, Bhandari, Cichero, & Prakash, 2015; Zhou, Han, Li, & He, 2017). Besides, soybean was recognized as one of the major allergic food items among the "big eight," about 15 types of soybean protein are recognized by the serum of soy-sensitive patients with atopic dermatitis, which could trigger various allergic reactions ranging from mild symptoms to life-threatening symptoms (Ogawa et al., 2000; Jin Wang, Vanga et al., 2019). Gly m 5 (Beta-conglycinin) is composed of several subunits leading to its classification as major allergic protein in soybean (Maruyama et al., 2018). It is 7S globulin which accounts for about 30% of the seed protein, and its molecular weight ranges from 50 kDa to 70 kDa (Holzhauser et al., 2009). Data showed 13 subjects had allergic reactions to Gly m 5 among a batch of 30 subjects (Holzhauser et al., 2009).

In recent years, microwave treatment as a novel processing technique has been applied to soy products. Microwave treatment can effectively reduce the activity of trypsin inhibitors and the allergenicity of soymilk (Shibasaki et al., 1980; Vanga et al., 2020b). While overheating will make soy proteins difficult to digest, a lower temperature will not decrease their allergenicity. Therefore, combined hybrid treatment may solve this problem. Ultrasonic treatment, as a new non-thermal processing treatment, is also used to treat soy products. This

treatment can improve the soy proteins solubility and digestibility (Jambrak, Lelas, Mason, Krešić, & Badanjak, 2009; Vanga et al., 2020b). In addition, studies have shown that ultrasound treatment could reduce the allergenicity of soy proteins to a certain extent (Amponsah & Nayak, 2016; R Choudhary et al., 2013). In this study, microwave treatment (85°C, 10 min) combined with ultrasound treatment (0, 4, 8, 12, 16 min) was performed. The reduction of soymilk allergenicity will be analyzed by the ELISA test. Further, physiochemical characteristics of soymilk such as trypsin inhibitor activity, secondary structure, microstructure, antioxidant capacity, in-vitro protein digestibility are to be investigated as well.

3.3 Material and Methods

3.3.1 Sample preparation

As shown in Figure 3.1, Soymilk was prepared as reported by Vanga (Vanga et al., 2020b). Raw soybeans (Agrocentre Belcan, Sainte-Marie, QC, Canada) were cleaned and were soaked for 24 h in water. After soaking, they were blended with water in a ratio of 1:9 (w/v) using Soyabella (Tribest, CA, USA). After filtering with 4-layer cheesecloth, soymilk was then stored at 4 ℃ until it was subjected to a plan for every part of the experimental study.

3.3.2 Microwave combined Ultrasonic treatment

Microwave processing was performed using the Mini Wave digestion system (SCP Science, QC Canada) with a frequency of 2450 MHz. All samples were first subjected to microwave treatment at 85°C for 10 minutes. Branson Sonifier 450 (Branson Ultrasonic Corp., Danbury, CT, USA) with a probe at a 20-kHz frequency, 400 W, was used to conduct the Ultrasonic processing. The sonication probe was immersed into the glass beaker that was filled with

soymilk samples. And the samples were placed in an ice bath and were treated by ultrasonic machine with a duty cycle of 50% for 0 min (US0), 4 min (US4), 8 min (US8), 12min (US12), and 16 min (US16), respectively (Figure 3.1). Samples were dried by using a freeze dryer (7420020, Labconco Corporation, Kansas City, USA) until it becomes powder for further analysis. All treatments and analyses were performed in triplicates.

3.3.3 Total soluble protein content

The soymilk sample of 50 mg was mixed with 7 ml distilled water with a 30 min residence time. The mixture was centrifuged at 4,000 x g for 10 min, and the supernatant was collected for analysis. The total soluble protein content of soymilk samples was analyzed by using the Pierce BCA protein assay kit. The samples and controls were tested according to the protocol suggested by the manufactures.

3.3.4 SDS-PAGE

The soymilk sample of 10 mg was mixed with 1 ml distilled water with 30 min residence time. The mixture was centrifuged at 4,000 x g for 10 min, and the supernatant was collected for further processing. 50 µL supernatant was mixed with the sample buffer (2.5µL β-mercaptoethanol, 47.5 µL 2x Laemmli sample buffer) and it was heated to 90℃ for 5 min. Then 10 µL of denatured protein samples and 5 µL Molecular weight marker (Precision Plus Protein™ Dual Color Standards) were loaded in each lane of the gel (4–20% Mini-PROTEAN® TGX Stain-Free™ Protein Gels, 10 well). Electrophoresis was performed in a vertical unit (Mini-PROTEAN® Tetra System, BIO-RAD, Philadelphia, PA, USA) at 150 V. Gels were either Coomassie-stained for 15 min and de-stained with a water solution containing 50% methanol and 10% acetic acid at 45℃ until the protein band was clear.

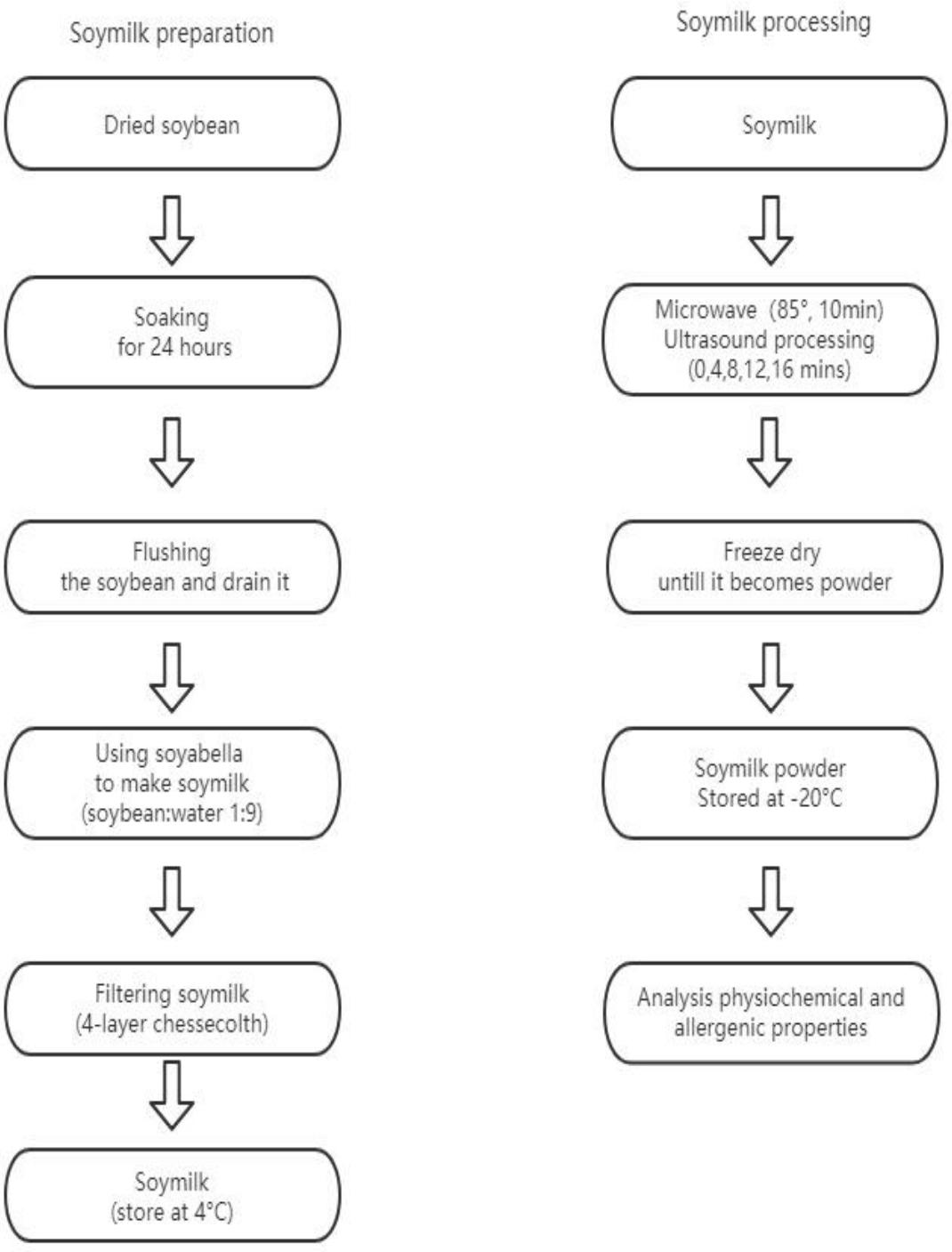

Figure 3.1 Soymilk preparation and processing

3.3.5 Allergenic protein content –Enzyme-linked immunosorbent assay (ELISA)

ELISA kit (Soybean Gly m 5 ELISA 2.0, Indoor Biotechnologies, USA) was utilized to quantify the Gly m 5 content in the soymilk samples. The ELISA kit consists of a microtiter

plate pre-coated with anti-Gly m 5 monoclonal antibody and reagents including Gly m 5 allergen standard (500 ng/mL), Biotinylated monoclonal antibody 1B9, Streptavidin-peroxidase, wash buffer (10x), assay buffer (10x), TMB developing substrate, and Stop Solution. All the steps were performed according to the protocol provided by the kit. The absorbance of the Gly m 5 was recorded at 450 nm, and the concentration of Gly m 5 was calculated based on the standard curve obtained by the standard.

3.3.6 Secondary structure

The secondary structures of soybean proteins were determined by Fourier transform infrared spectroscopy (FTIR). The sample was loaded on the diamond crystal of the FTIR spectrometer (Nicolet Magna 158,750 FTIR, Nicolet Instrument Corp., Madison, WI).

The 32 scan spectra at a resolution of 4 cm−1 were averaged in the mid-infrared region (1000–2000 cm−1), using OMNIC software (Version 8, Thermo Nicolet Co., Madison, WI) to analyze the spectra. The spectrum of an empty diamond was regarded as the background reference to avoid the air influence.

A curve-fitting procedure was performed using the Peakfit software to quantify the conformational changes of soy proteins in the amide I band (1700–1600 cm−1).

3.3.7 Analysis of total antioxidant capacity

Ferric Antioxidant Status Detection Kit (purchased from Thermo Fisher Scientific, Canada) was applied to the quantification and detection of Ferric Antioxidant Status (also referred as Ferric Reducing Antioxidant Power, FRAP) in serum, plasma, urine, teas, fruit juices, beer, cider, cell lysates, herbal and fruit extracts. Antioxidant capacity in the samples reacts with the FRAP color solution to generate a blue-colored product which is read at 560 nm. The kit

consists of 10 mM Ferrous Chloride Standard, Clear 96-well Half Area Plate, Assay Buffer Concentrate, FRAP Reagent A & B. The soymilk sample (50 mg) was mixed with 2.5 ml methanol for 30 min. The mixture was centrifuged at 4,000 x g for 10 min, and the supernatant was collected for further analysis. Then all the steps were performed according to the protocol provided by the kit.

3.3.8 Trypsin inhibitor activity (TIA)

The soymilk powder (250 mg) was mixed with 25 mL of 0.01 M NaOH for 3 h at room temperature with constant stirring to reach the final pH at 8.5 (Kakade, Rackis, McGhee, & Puski, 1974). The supernatant was diluted with distilled water so that 40–60% trypsin could be inhibited by two milliliter mixture. Two milliliters of trypsin solution (200 mL of 0.001 N HCl, 8 mg trypsin) was mixed with 1 mL of diluted soymilk supernatant in clean tubes and placed into the water bath at 37℃. 5 mL of preheated (37℃) benzyl-DL-arginine-para-nitroanilide solution (0.08 mg in 2 mL dimethyl sulfoxide which was diluted to 200 mL using preheated, 8.2 pH tris-buffer) was then added to the tubes and vortexed. Tris-buffer was made by Tris (hydroxymethyl) aminomethane (1.21 g) and 0.59 g of CaCL2.H20. Chemicals were dissolved in 180 ml of distilled water, and the pH was adjusted to 8.2 with 1N HCI (10-15 drops) and made up to 200 ml with distilled water. The reaction was stopped by using 1 mL of 30% acetic acid after a 10-min incubation. The samples were then centrifuged at 4000 x g for 10 min and the absorbance of the supernatant with a spectrophotometer at 410 nm was noted. The sample blank and trypsin standard from bovine pancreas were prepared and tested as described by Hamerstrand, Black, & Glover, 1981.

3.3.9 Microstructure

A Scanning Electron Microscope (SEM) (TM3000, Hitachi High-Technologies Corporation., Tokyo, Japan) was used to observe the microstructural alteration of each sample (Jin Wang, Wang, Ye, Vanga, & Raghavan, 2019). The freeze-dried soymilk samples were transferred to the measuring platform. The level of magnification was set at 100 ~500x, and the related figures were captured by using the software equipped with SEM.

3.3.10 Simulated vitro gastrointestinal digestibility

An IVPD was performed using pepsin and pancreatin as described by previous studies (Hejazi, Orsat, Azadi, & Kubow, 2016; Vilela, Lands, Chan, Azadi, & Kubow, 2006) with some modifications. In the extracting stage, 0.25 g of soymilk powder was mixed with 10 mL of phosphate buffer (0.01 M, pH 7.0). In the second-stage digestion, the pH of the remainder of the extract was adjusted to 1.5 using 1 M HCl. Then 100 μL of pepsin solution (10 mg pepsin/mL in 0.01 M HCl) was added. After 30-min water bath (37 ℃) incubation, 100 μL, 1.0 M NaOH solution was added to the sample to stop the digestion. A 1.0 M NaOH solution was used to adjust the pH to 7.8, and 300 μL of pancreatin solution (10 mg/mL in sodium phosphate buffer, pH 7.0) was added to start the third-stage digestion, and the mixture was incubated at 40 ℃ for 1 h. Then, 100 μL of Na_2CO_3 solution (150 mM) was added to stop the overall digestion stages.

The protein content of soymilk samples before and after the digestion was measured using the Pierce BCA Protein Assay kit.

3.3.11 Statistical analysis.

The analysis of variance (ANOVA) of the SPSS software (SPSS Inc., Ver.18, Chicago, IL,

USA) was used to analyze the experimental data. The Duncan multiple range test was applied to separate the means and to establish significance, which was accepted at $p \leq 0.05$.

3.4 Results and Discussion

3.4.1 Protein determination

Microwave combined ultrasonic treatment significantly influences the total soluble protein content of soymilk samples (Table 3.1). The results showed that the total protein content of soymilk samples increased after treatment ($P < 0.05$). Specifically, compared with the untreated samples, the total soluble protein content of the samples treated with US16 increased by 19.92 %, followed by US12 (17.34%), US8 (7.95%), and US4 (1.55%). Similarly, Similar to Jambrak, we used 20 – 40 kHz ultrasonic probes to treat soy protein isolates (SPI) and soy protein concentrate (SPC). The results showed that ultrasound treatment would increase the soluble protein content ($P < 0.05$), compared to untreated sample, protein solubility for 30 min 20 kHz probe treatment increased by about 78% (Jambrak et al., 2009). Hu's experiment also verified this view by using 20 kHz ultrasonication at varying power (200, 400, or 600 W) and time (15 or 30 min) to SPI dispersions ($P < 0.05$) (H. Hu, Wu et al., 2013). The main reason for the increase in protein solubility is that during the ultrasonic treatment, cavitation bubbles will cause the local temperature and pressure to rise around the collapsed bubbles, it leads to protein decomposition and the breaking of peptide bonds, making hydrophilic amino acid residues oriented toward water (Morel, Dehlon, Autran, Leygue, & Bar-L'Helgouac'h, 2000; Moulton & Wang, 1982).

3.4.2 Total antioxidant capacity (TAC)

As shown in Table 3.1, the influence of microwave combined ultrasonic treatment on the antioxidant capacity of soymilk samples are great. The TAC of soymilk samples increased with the processing time from 4 min to 16 min. The highest TAC of soymilk sample was US16 (35.90 μmol/g), followed by US12 (33.52 μmol/g), US8 (31.542 μmol/g), US4 (30.45 μmol/g), and US0 (26.851 μmol/g). These results showed that ultrasound processing could contribute to the increase of total antioxidant capacity with a longer time.

Phenolic is one of the antioxidant compounds in plants and foods of plant origin (Dai & Mumper, 2010). The increase of TAC in treated samples might be attributed to the release of phenolics from cell wall matrices and cell wall destruction due to cavitation effects and microwave heating during the treatment (Chiremba, Rooney, & Beta, 2012; Đurović et al., 2018). Also, ultrasonic treatment can add hydroxyl radicals to the aromatic ring of phenolic compounds. It has been proven that when a second hydroxyl group is added to the ortho or para position of these compounds, the antioxidant activity of phenolic compounds will increase (Tomadoni, Cassani, Viacava, Moreira, & Ponce, 2017). Many studies indicated that microwave and ultrasound treatment could increase the TAC of soy samples (Đurović et al., 2018; Zhao, Kim, & Eun, 2020).

3.4.3 Trypsin inhibitor activity (TIA)

In this study, the soymilk samples were treated with microwaves and ultrasound to evaluate the changes of the trypsin inhibitors. The results are shown in Table 3.1. A recent paper has pointed out in the article that after microwave treatment at 85℃ for 10 minutes, the activity of TI drops by nearly 60% compared with the untreated sample (Vanga, Wang, & Raghavan,

2020a). And this article points out that after microwave treatment followed by ultrasonic treatment, TI activity will be further reduced. As shown in Figure 3.1, The lowest TI activity of the soymilk sample was at US16 (40.49%), followed by US12 (43.93%), US8 (48.16%), US4 (51.31%), and US0 (54.99%).

Table 3.1. Changes of bioactive compounds in microwave combined ultrasonic-treated soymilk samples

Treatment	Total soluble protein (mg/g)	TAC (µMol/g)	Gly m 5 (µg/g)	TIA %
US0	118.05 ± 1.429 ^{c}	26.85 ± 0.51 ^{e}	99.38 ± 2.10 ^{a}	54.99 ± 0.13 ^{a}
US4	119.87 ± 0.67 ^{c}	30.45 ± 0.48 ^{d}	61.41 ± 5.17 ^{b}	51.31 ± 0.06 ^{b}
US8	127.44 ± 1.57 ^{b}	31.54 ± 0.16 ^{c}	52.53 ± 5.56 ^{c}	48.16 ± 0.39 ^{c}
US12	138.53 ± 0.48 ^{a}	33.53 ± 0.33 ^{b}	38.11 ± 3.76 ^{d}	43.93 ± 1.57 ^{d}
US16	141.58 ± 0.67 ^{a}	35.90 ± 0.72 ^{a}	33.46 ± 3.18 ^{d}	40.49 ± 2.31 ^{e}

Note: values with different letters in the same column are significantly different ($p < 0.05$) from each other.

Studies by Huang et al. (2008) and Vagadia et al. (2018) show that the destruction of disulfide bonds caused by ultrasound and the impact of high temperature on TI are the reasons for the decline in TI activities. Ultrasonic treatment for 20 minutes reduced TI activity by 55%, while microwave treatment could reduce TI activity only by 3%.

3.4.4 In-Vitro Protein Digestibility (IVPD)

As shown in Figure 3.2, the IVPD increased with the rise of processing time. The digestibility changed significantly. The IVPD reached to a maximum level 92.70% after 16-min ultrasound processing, followed by US12 (91.02%), US8 (89.99%), US4 (88.93%), and US0 (87.08%). The increase of digestibility could be explained by the decreasing TI activities in the sample or due to secondary structural changes and the sonication unfolding soy protein structure which leads to more exposed sites; thus making them available for enzymatic action (Khatkar, Kaur, & Khatkar, 2020). Studies have shown that both ultrasonic treatment and microwave treatment could improve the digestibility of soy protein (Vanga et al., 2020a), but a study reported that Long-time microwave treatment would reduce the digestibility of soy protein (Su and Chang, 2002).

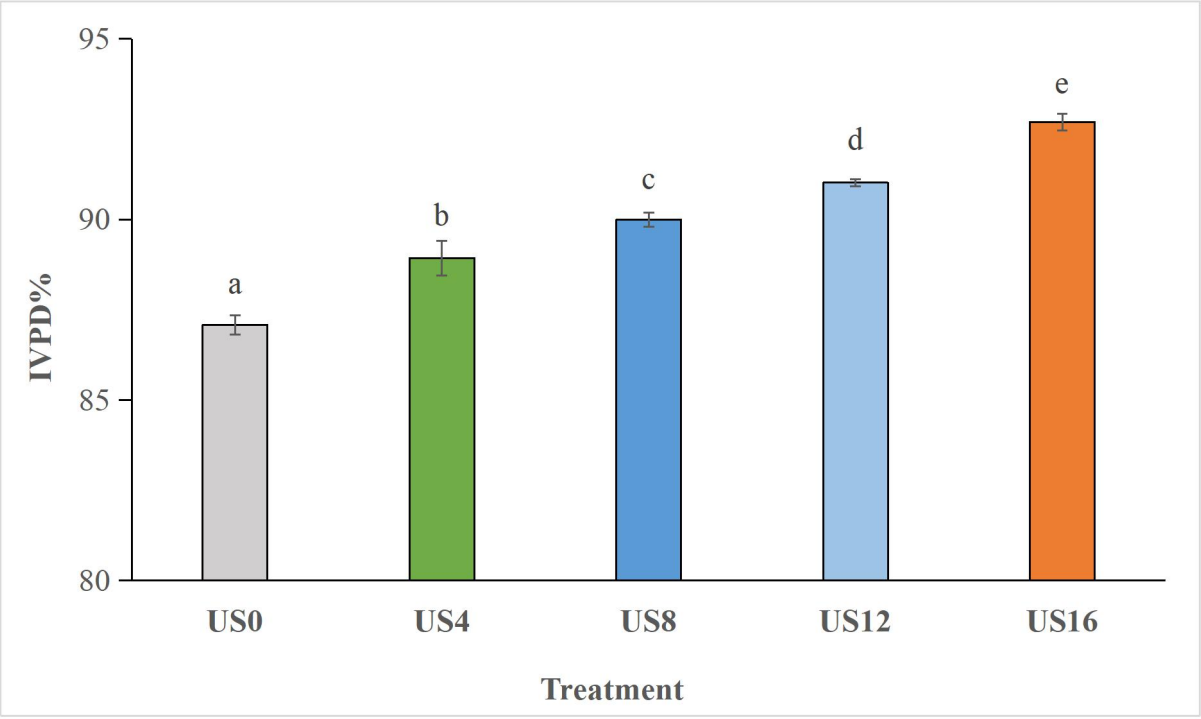

Figure 3.2. Effect of microwave combined ultrasonic processing on protein digestibility (IVPD %) of soybean protein.

3.4.5 FTIR analysis of secondary structural changes in soymilk protein

FTIR analysis is used to study the effect of ultrasonic treatment on the secondary structure of soy protein. The amide I band (1700–1600 cm-1) area was commonly used to analyze the secondary structure of the protein. It includes overlapping bands of different secondary structures, including β-sheets (1610~1640cm-1), α-helix (1650~1658cm-1), β-turns (1660~1700 cm-1), and randomly coiled (1640 ~ 1650cm-1) conformations (C. Wang et al., 2011).

The changes of secondary structures in soymilk samples after processing are shown in Table 3.2. It was noticed that β-sheets and randomly coiled turns were the major secondary structures present in soy protein, representing about 31.72% ~34.32% and 28.63% ~29.99%. A decrease in the α-helix was observed after ultrasound processing, while a rise in the β-sheets structure was present in proteins. Vanga et al. (2020a) also found that the secondary structure of soy protein after ultrasonic treatment has an increase of β-sheets conformations with a concurrent reduction in the α-helix, while Hu et al. (2013) found that when a high power ultrasound (600 W, 15-30 min) was used to process soy protein, it would lead to an increased α-helices and random coils and a loss in the β-sheets.

Besides, Jin et al. (2016) investigated the changes of corn gluten meal hydrolysates under dual-frequency ultrasonic-power pretreatment. They found a decline in the turn structure content and an increase in α-helix and β-sheets. It shows that the structural changes under ultrasonic treatment are not alike in various proteins. These results suggested that the ultrasound treatment led to a disruption of the molecule interactions and changed the protein secondary structures.

Table 3.2. Changes of secondary structure of protein under microwave combined ultrasonic-treated soymilk samples

Treatment	β-sheet % (1610-1640 cm^{-1})	Unordered % (1640-1650 cm^{-1})	α-helix % (1650-1658 cm^{-1})	β-Turns % (1660-1700 cm^{-1})
US0	31.72 ± 0.37 ^{a}	28.63 ± 0.17 ^{a}	15.77 ± 0.20 ^{d}	23.87 ± 0.15 ^{a}
US4	33.01 ± 0.59 ^{b}	29.38 ± 0.76 ^{ab}	13.73 ± 0.16 ^{c}	23.89 ± 0.59 ^{a}
US8	33.62 ± 0.58 ^{bc}	29.99 ± 1.09 ^{b}	13.13 ± 0.36 ^{b}	23.26 ± 1.31 ^{a}
US12	34.32 ± 0.59 ^{c}	28.90 ± 0.24 ^{ab}	12.45 ± 0.13 ^{a}	24.34 ± 0.70 ^{a}
US16	32.89 ± 0.39 ^{b}	29.12 ± 0.30 ^{ab}	13.50 ± 0.08 ^{bc}	24.49 ± 0.39 ^{a}

3.4.6 Allergenicity

A sandwich ELISA was used to determine Gly m 5 contents to find whether there was a reduction in the allergenicity of soymilk samples after the treatment. As shown in Figure 3.3, the content of Gly m 5 decreased gradually with the rise of processing time. Compared with US0 (99.38 μg/g), the maximum decrease of 66.33% was observed at US16 (33.46 μg/g), followed by US12 (38.11 μg/g), US8 (52.53 μg/g), US4 (61.41 μg/g). The Gly m 5 content at US16, US12, and US8 had a significant decrease ($P < 0.5$) in comparison to that in control. Whereas no significant decline was observed between US4 and US0.

As shown in SDS-PAGE (Figure 3.4), compared to the untreated sample, protein electrophoretic patterns did not have a change in molecular weight, suggesting that ultrasonic treatment did not modify the protein profiles of soymilk regardless of the sonication duration. Similarly, both Karki and Hu (2013) pointed out that sonication did not modify the soy protein profile (Karki et al., 2009). This indicates that the reduced allergenicity of Gly m 5

may be caused by the change of secondary structures of protein and destruction of its binding epitope by ultrasound treatment; further studies are needed to verify the mechanism of ultrasonication.

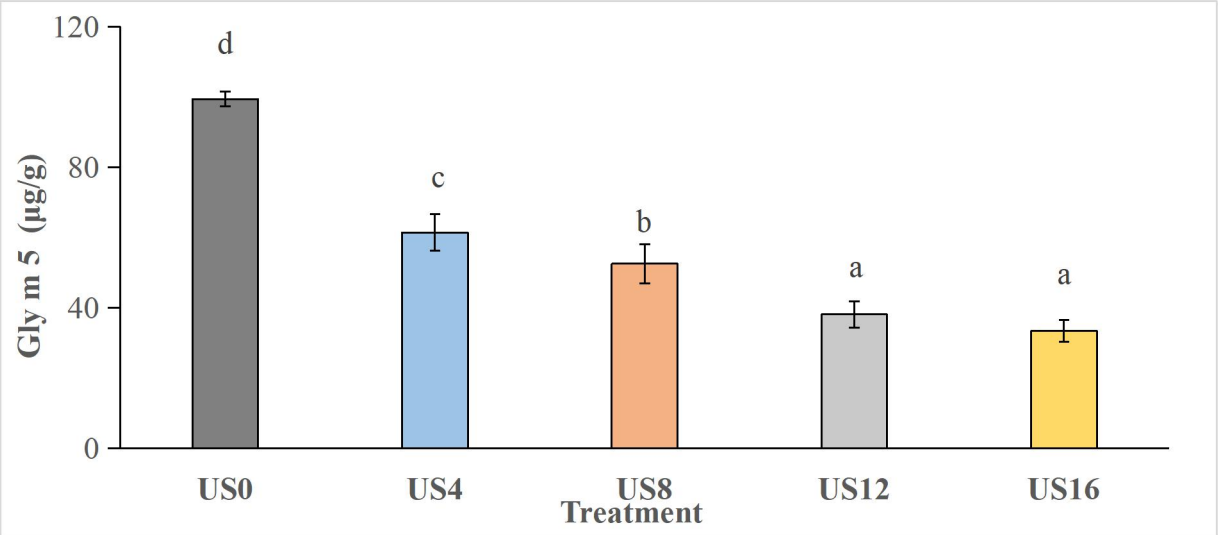

Figure 3.3. The Gly m 5 contents of soymilk samples under microwave combined ultrasonic treatment

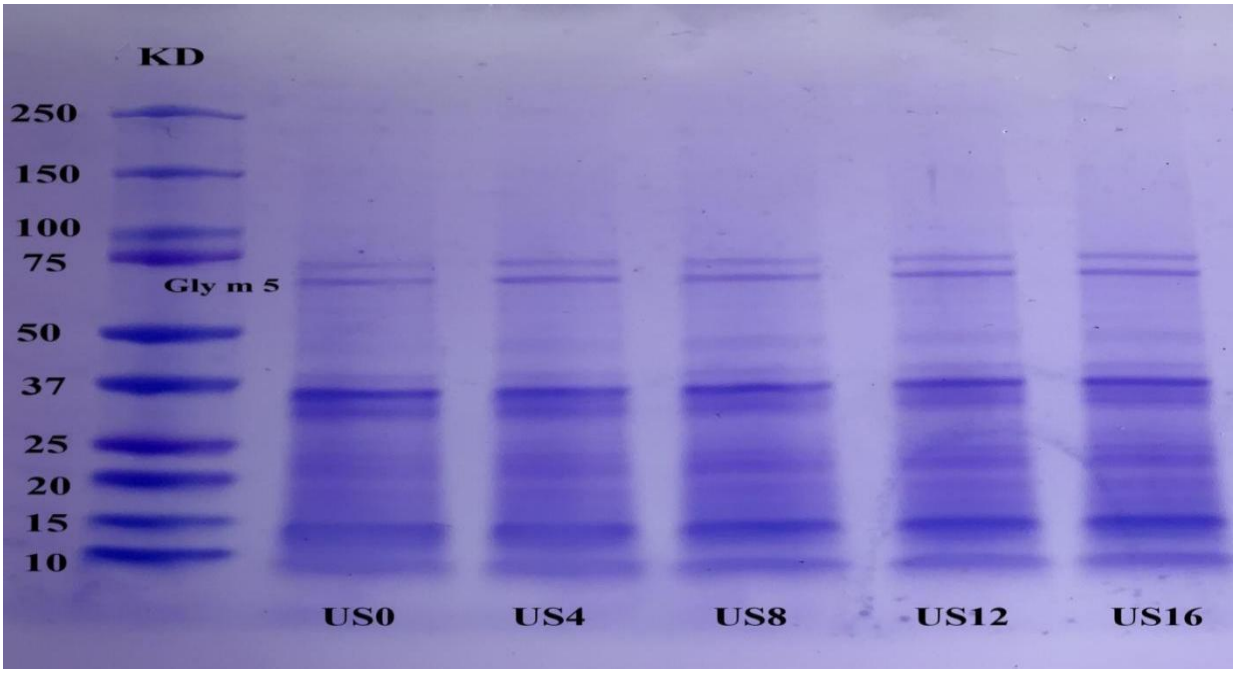

Figure 3.4. SDS-PAGE of microwave combined ultrasonic-treated and untreated proteins in soymilk sample.

3.4.7 Microstructure observation

A scanning electron microscope (SEM) was used to analyze the microstructures of ultrasound treated soymilk samples as shown in Figure 3.5.

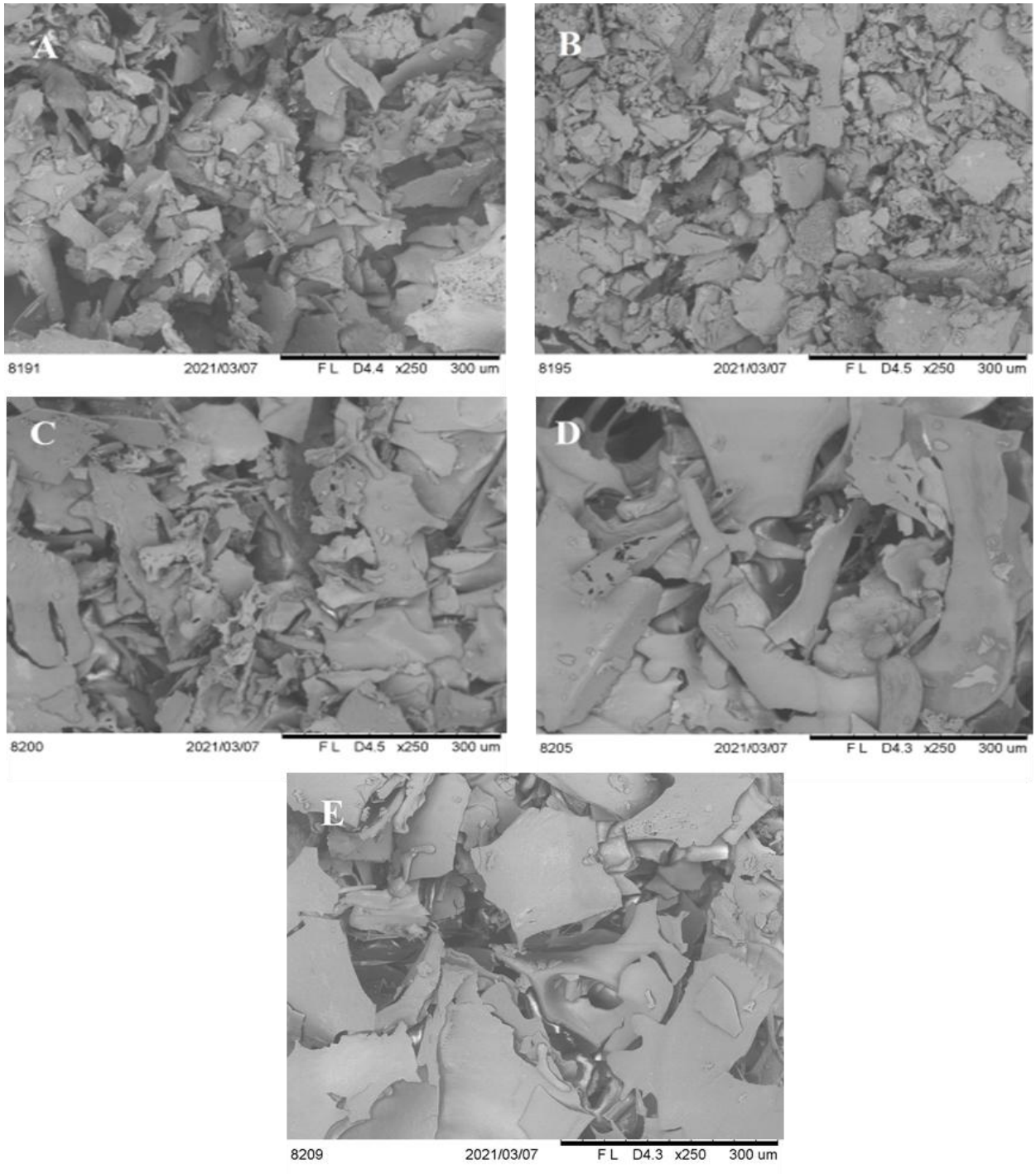

Figure 3.5. SEM photomicrographs of freeze dried soymilk (A) US0 (control sample), (B) US4, (C) US8, (D) US12, and (E) US16 (magnification at 250x)

Results suggest that longer ultrasonic duration could induce larger structures. Ultrasonic treatment could unfold the soy protein molecules, and it may lead to an aggregation of these molecules during freeze drying. Hu found that the aggregations in dispersion could be smaller, even though the larger aggregations of soy protein samples after ultrasonication was observed in a dry state (H. Hu, Fan et al., 2013). These were consistent with the rising of total soluble protein content after ultrasonic treatment.

3.5 Conclusions

This study investigated the influence of microwave combined ultrasonic treatment on the trypsin inhibitors activity, antioxidant capability, secondary structures, microstructures, in vitro protein digestibility, and allergenicity of soymilk samples. Total soluble protein content and total antioxidant capacity increased gradually with the increase of time and reached the highest value at 16 min (US 16). Meanwhile, the decrease of trypsin inhibitor activities with the longer treatment was observed, and it is also related to higher values of in-vitro protein digestibility. A larger aggregations of soy protein were observed by SEM. Secondary structures change with the increasing time, specifically showing the upward trend of β-sheets and the downward trend of α-helix. Finally, ELISA test shows that a 16 min of ultrasound treatment significantly inhibited the IgE binding capacity of Gly m 5. These results demonstrated that microwave combined ultrasonic treatment has the potential to promote the elimination process of soybean allergenicity and it would not bring about the adverse effects of long-term hight temperature treatment of soybeans. However, further studies regarding the mechanisms of ultrasonication and microwave in causing a reduction in the allergenicity of proteins are needed.

CONNECTING TEXT

In Chapter III, we investigated the influence of microwave combined ultrasonic treatment on the trypsin inhibitor activity, antioxidant capability, secondary structures, microstructures, in vitro protein digestibility, and allergenicity of soymilk samples. In the next chapter, we will investigate the effects of microwave combined ultraviolet on similar parameters of soymilk samples.

CHAPTER IV

Effects of Microwave combined Ultraviolet Processing on the Physiochemical and Allergenic Properties of Soymilk

4.1 Abstract

This study investigates the influence of microwave combined ultraviolet treatment on the trypsin inhibitor activity, antioxidant capability, secondary structures, microstructures, in vitro protein digestibility, and allergenicity of soymilk samples. The results of microwave combined with ultraviolet processing showed it to have the capability to decrease the allergenicity of soymilk caused by one of the major allergen Gly m 5 in soybean. The percentage of reduced allergenicity was positively related to the increase of ultraviolet irradiation time; the minimum allergenicity with a 50.24% reduction was observed in ultraviolet treatment for 60 min. Meanwhile, the total soluble protein content increased by 31.07% after 60 min ultraviolet treatment, and improving the antioxidants capacity and decreasing trypsin inhibitor activity of soymilk. But in IVPD of soymilk, there was no significant change compared to the control sample. Soymilk protein denaturation and aggregation may induce the increase of β-turn and α-helix structures and the decrease of β-sheet and unordered structures in treated samples. A higher number of microstructural holes were observed in treated samples with an increase in treatment time.

Keywords: Ultraviolet, Soymilk, Allergenicity, Digestibility, Antioxidant capacity, Structure

4.2 Introduction

Soymilk, the water extract of soybean, is rich in a variety of nutrients. The biologically active factors contained in soymilk, such as isoflavones, phytosterols, etc., have a variety of health benefits, such as reducing cholesterol levels and preventing cancer (Jeng, Shih, Wu, & Sung, 2010; KWOK & Niranjan, 1995). Though its appearance is similar to milk, its physical properties and nutrients are very different. Compared to cow's milk, soymilk has low amounts of fat, carbohydrates, and calcium (Oguntunde & Akintoye, 1991; Poliseli-Scopel, Hernández-Herrero, Guamis, & Ferragut, 2012). As cow's milk allergy and lactose intolerance promoted soymilk consumption, people are increasingly inquisitive and concerned about soymilk's negative attributes. Soybean is also one of the eight major allergens. About 0.2% to 0.4% of the population would have an allergy to it and cause various allergic reactions ranging from mild symptoms to life-threatening symptoms (Authority, 2004; Savage et al., 2010). Gly m 5 is one of eight key allergenic proteins present in soybean, while ELISA is an antibody-based test that can target a single allergen and detect its content (Amponsah & Nayak, 2016). Many studies have shown that heat treatment and non-heat treatment can effectively improve the nutritional value of soymilk and reduce allergies (Vanga et al., 2020a; W. W. Yang, De Mejia, Zheng, & Lee, 2011).

Microwave has been widely used in food processing. The destruction of the cell wall is caused by microwave treatment because of the forced overheating of the remaining water molecules and continuous collisions in the matrix (Kratchanova, Pavlova, & Panchev, 2004). Meanwhile, it changes the structure of proteins, which may reduce allergenicity (Lakemont, de Jongh, Hessing, Gruppen, & Voragen, 2000).

Ultraviolet processing is commonly used for food sterilization. UV-C light penetrates the outer cell membrane of microorganisms, causing the formation of thymine dimers, which subsequently leads to the destruction of their DNA (Ruplal Choudhary & Bandla, 2012). For protein, exposure to ultraviolet light will cause free radical oxidation, which in turn induces protein cross-linking, protein fragmentation, and peroxidation of unsaturated fatty acids (Kolakowska, 2003). Changes in protein may cause allergic property changes. Due to the thermal stability of Gly m 5, microwave treatment alone may not be able to completely remove its allergies (T. Wang, Qin, Sun, & Zhao, 2014). In this study microwave treatment (85℃,10 min) combined with ultraviolet (254 nm) lighting treatment (0, 15, 30, 45, 60 min) was performed. The reduction of soymilk allergenicity was analyzed by the ELISA test. Further, physiochemical characteristics of soymilk such as trypsin inhibitor activity, secondary structure, microstructure, antioxidant capacity, in-vitro protein digestibility were also investigated.

4.3 Material and Methods

4.3.1 Sample preparation

Raw soybeans (Agrocentre Belcan, Sainte-Marie, QC, Canada) were flushed and were soaked for 24 h in water. After soaking, Soyabella (Tribest, CA, USA) was used to make the soymilk (soybean and water ratio is 1:9 (w/v)). After filtering, soymilk was stored in a fridge at 4ºC until its further use, as shown in Figure 4.1.

4.3.2 Microwave combined Ultraviolet light treatment

The Mini Wave digestion system with a frequency of 2.45 GHz (SCP Science, QC Canada) was used for microwave processing. Soymilk was heated to 85℃ for 10 min and then cooled

down to room temperature. Short Wave Ultraviolet system (Spectronics Corporation, Westbury, New York, USA) was applied using 254 nm ultraviolet lighting. Pouring 45 mL soymilk into the clean dish and moving it at 10cm away from the light source was accomplished. Then the treatment was done for 0 min (UV0), 15 min (UV15), 30 min (UV30), 45 min (UV45), and 60 min (UV60), respectively (Figure 4.1). A freeze dryer (7420020, Labconco Corporation, Kansas City, USA) was used to dry the samples, and they were stored at −20℃ until their further use. All treatments and analyses were performed in triplicates.

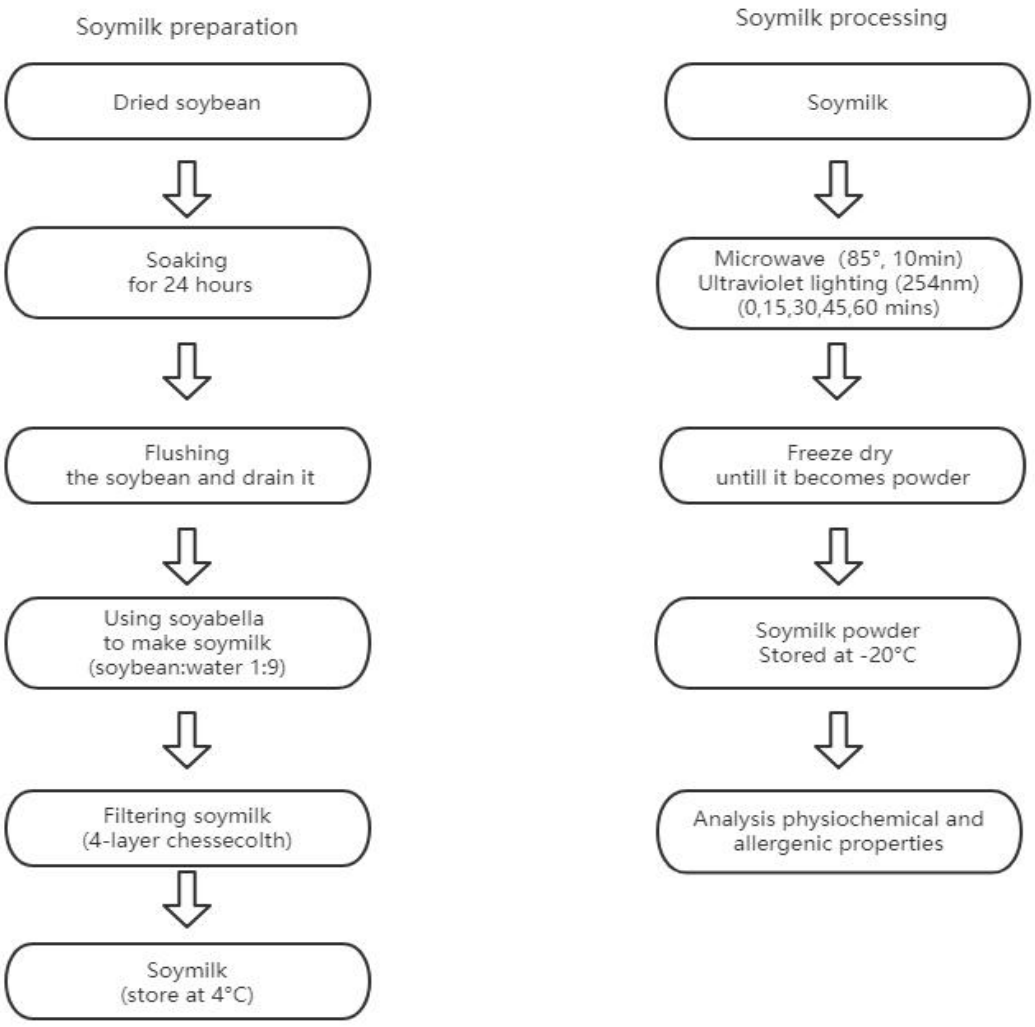

Figure 4.1 Schematic of soymilk sample preparation and processing.

4.3.3 Total soluble protein content

The total soluble protein content of soymilk samples was determined by a Pierce BCA protein assay kit (Thermo Fisher Scientific, Canada). All the steps were performed according to the protocol of the protein assay kit.

4.3.4 SDS-PAGE

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was performed according to Laemmli (Laemmli, 1970) with some modifications. After electrophoresis, gel was got Coomassie-stained for 15 min and soaked in destaining buffer (50% methanol and 10% acetic acid in water) at 45℃ until the protein band was cleared.

4.3.5 Allergenic protein content –Enzyme-linked immunosorbent assay (ELISA)

ELISA kit (Soybean Gly m 5 ELISA 2.0, Indoor Biotechnologies, USA) was used to quantify the Gly m 5 content in the soymilk samples. All the steps were performed according to the protocol of the Elisa kit. The concentration of Gly m 5 was calculated through a standard curve according to the sample absorbance at 450 nm.

4.3.6 Secondary structure

Fourier transform infrared spectroscopy (FTIR) spectrometer (Nicolet Magna 158,750 FTIR, Nicolet Instrument Corp., Madison, WI) was used to determine the secondary structures of proteins. An air-background spectrum was collected before the determination of others to avoid the air influence. The powder sample was transferred to the diamond crystal, and then there were thirty-two scan spectra at 4 cm−1 resolution was generated and recorded, and averaged in the mid-infrared region (1000–2000 cm−1). Peakfit software was used for curvefitting and analyzing the amide I band (1700–1600 cm−1).

4.3.7 Analysis of total antioxidant capacity

Using 2.5 ml methanol extraction of soymilk sample (50 mg) with a duration of 30 min was done. The mixture was then centrifuged at 4,000 x g for 10 min and the supernatant was collected for further analysis. Ferric Antioxidant Status Detection Kit (purchased from Thermo Fisher Scientific, Canada) was used for detecting the total antioxidant capacity of the extraction. By adjusting the concentration of samples and performing the steps that was provided by the kit, the sample was ready for the next assessment. Antioxidant power in the samples would be reflected by the absorbance at 560 nm.

4.3.8 Trypsin inhibitor activity

Using 25 mL of 0.01 M NaOH to mix with the soymilk powder (250 mg) at room temperature and with constant stirring for 3 h and the pH of 8.5 was reached. (Kakade, Rackis, McGhee, & Puski, 1974). Using distilled water to dilute the supernatant so that 2 mL solution could inhibit 40–60% of trypsin. Mixing of two milliliters of trypsin solution (8 mg in 200 mL of 0.001 N HCl) with 1 mL diluted supernatant in 15-mL tubes was performed. Following this step, tubes were placed into the water bath (37 ℃). 5 mL of preheated (37 ℃) benzyl-DL-arginine-para-nitroanilide solution (0.08 mg in 2 mL dimethyl sulfoxide diluted to 200 mL using pre-heated, 8.2 pH tris-buffer) was then added to the tubes and vortexed. Tris-buffer was made by Tris (hydroxymethyl) aminomethane (1.21 g) and 0.59 g of CaCL2.H20. Using 180ml of distilled water to dissolve the chemicals, and adjusting the pH to 8.2 with 1N HCI (10-15 drops); then it was increased to 200 ml with distilled water. 1 mL of 30% acetic acid was used to stop the reaction after a 10-min incubation. Centrifuging the solutions at 4000 x g for 10 min was done. Then a spectrophotometer was used to measure

the absorbance of the supernatant at 410 nm. The sample blank and trypsin standard from bovine pancreas were prepared and tested as described in Hamerstrand, Black, & Glover, 1981.

4.3.9 Microstructure

Scanning Electron Microscope (SEM) (TM3000, Hitachi High-Technologies Corporation., Tokyo, Japan) was used to observe the microstructure of each sample (Jin Wang, Wang, Ye, Vanga, & Raghavan, 2019). Stacking a few pieces of freeze-dried soymilk samples to the measuring platform. The level of magnification was set at 100 ~500x, and the images were captured with the software equipped with SEM.

4.3.10 Simulated vitro gastrointestinal digestibility

An IVPD was performed using pepsin and pancreatin as described in previous studies (Hejazi, Orsat, Azadi, & Kubow, 2016; Vilela, Lands, Chan, Azadi, & Kubow, 2006) with some modifications. In the extracting stage, mixing 0.25 g of soymilk powder with 10 mL of phosphate buffer (0.01 M, pH 7.0) was done. In the second-stage digestion, pH of extraction was adjusted to 1.5 using 1 M HCl. Then 0.1 mL of pepsin solution was added (10 mg pepsin/mL in 0.01 M HCl) and incubated for 30 min in the water bath (37 ℃). Adding 100 µL, 1.0 M NaOH solution to the sample stopped the digestion. The pH of the sample was adjusted to 7.8 with 1.0 M NaOH solution, and 300 µL of pancreatin solution (10 mg/mL in sodium phosphate buffer, pH 7.0) was added to start the third-stage digestion, then incubation in the water bath (40 ℃) for 1 h was done. Finally, the overall digestion stages were stopped by adding 100 µL of Na2CO3 solution (150 mM). The Pierce BCA Protein Assay kit was used to measure the protein content of soymilk samples before and after the digestion.

4.3.11 Statistical analysis.

Using the analysis of variance (ANOVA) package of the SPSS software (SPSS Inc., Ver.18, Chicago, IL, USA) analysis of the experimental data was done. Duncan, multiple range test to separate the means and to establish significance ($p \leq 0.05$) was done and recorded.

4.4 Results and Discussion.

4.4.1 Protein determination and Total antioxidant capacity (TAC)

As shown in Table 4.1, the results of total soluble protein and total antioxidant capacity (TAC) in soymilk samples are summarized. It is to be noted that the total soluble protein content of microwave combined ultraviolet samples increased up to 31.1% compared to the control. Highest soluble protein content was observed in the UV60 sample (208.23 mg/g), while UV45 showed 205.45 mg/g and UV30 showed 201.16 mg/g. Statistically. they had no significant difference compared to UV60. Compared to the control sample, UV15 showed 184.34 mg/g, which was a statistically significant increase. In reviewing the literature, we did not find any data on UV treatment relating to the increase of soluble protein content. In BCA determination, the reaction was noted to be strongly influenced by four amino acid residues (cysteine, cystine, tyrosine, and tryptophan) in the amino acid sequence of the protein (Smith et al., 1985). The increase of protein content may be due to UV destructing the disulphide bonds (S-S), inducing structural change in the protein that exposed more amino acid residues, which might have influenced the results (Pattison, Rahmanto, & Davies, 2012). Kristo et al. (2012) found that the UV treatment could increase the concentration of total and accessible thiol groups in 1% whey protein isolate solutions, and Semagoto et al. (2014) showed the milk protein concentrate powder exposure to UV, resulting in more protein carbonyls. These

shows that UV does affect the structure of proteins, and further discussion is presented in Section 4.4.4.

The comparison of the total antioxidant capacity of treated and untreated samples is shown in Table 4.1. There was no significant change in FRAP values. It remained almost constant in the irradiated and control samples. Similar results were reported by Alothman et al. (2009), who found that the antioxidant capacity of pineapple did not change after UV-C treatment. UV-C treatment has been shown to cause a significant increase in the antioxidant capacity of blueberries and a decrease in the antioxidant capacity of apple juice (Perkins-Veazie, Collins, & Howard, 2008; Q. Xiang et al., 2020). It seems that different commodities exhibit diversified changes in the antioxidant capacity when exposed to UV radiation; in the case of soymilk, UV-C light does not induce changes in antioxidant capacity.

Table 4.1. Changes of bioactive compounds in microwave combined ultraviolet-treated soymilk samples

Treatment	Soluble protein (mg/g)	TAC (μMol/g)	Gly m 5 (μg/g)	TIA %
UV0	158.87 ± 6.71 ^{c}	22.08 ± 0.49 ^{a}	141.52 ± 6.63 ^{a}	55.61 ± 0.56 ^{a}
UV15	184.34 ± 3.33 ^{b}	21.71 ± 0.28 ^{a}	130.51 ± 2.66 ^{b}	51.42 ± 0.60 ^{b}
UV30	201.17 ± 7.33 ^{a}	21.66 ± 0.52 ^{a}	107.42 ± 7.38 ^{c}	48.86 ± 0.19 ^{c}
UV45	205.45 ± 3.93 ^{a}	22.06 ± 0.15 ^{a}	99.47 ± 3.99 ^{c}	45.92 ± 0.47 ^{d}
UV60	208.23 ± 0.72 ^{a}	21.50 ± 0.02 ^{a}	70.42 ± 6.48 ^{d}	42.44 ± 0.66 ^{e}

Note: different letters in the same column are significantly different ($p<0.05$) from each other.

4.4.2 Trypsin inhibitor activity

The changes of trypsin inhibitor activity in the Ultraviolet teated soymilk samples have been outlined in Table 4.1. The TIA of soymilk reduced to 51.4% after 15 min ultraviolet processing compared to the control sample (55.6%). As the processing time was further increased to 30, 45 min, the activity of TI present in soymilk was reduced to 48.9% and 45.9%, respectively. The lowest TIA of soymilk samples was found when the processing time were increased to 60 min (42.4%). Katchman et al. (1948) has previously evaluated the effect of ultraviolet processing on soybean trypsin inhibitor; he found soybean trypsin inhibitor to have been inactivated by ultraviolet light. Kim et al. (1985) found that Kunitz Soybean trypsin inhibitor consists of 3 very close isoforms, all of which contain 181 amino acid residues and 2 intramolecular disulfide bonds. Tetenbaum et al. (2001) suggested that changes in disulfide bonds play a crucial role in inactivating trypsin inhibitors. Therefore, the ultraviolet treatment for the soymilk sample would inactivate the trypsin inhibitor by destroying the disulfide bonds in it.

4.4.3 In-Vitro Protein Digestibility

Ultraviolet treatment seems to have no significant effect on soymilk proteins digestibility. The In-Vitro Protein Digestibility value in the range of 84.62% ~84.67% is shown in Figure 4.2. UV treatment inactivated TI, and it also led to aggregation of soy protein. SDS-PAGE results is shown in Figure 4.3. It is noticeable where aggregation on the top of the lane and other bands occurs. Pattison suggests that the locations of both inter-and intramolecular S-S bonds are vital for the level of aggregation of high and low molecular weight proteins (Pattison et al., 2012). Similarly, Gennadios et al. (1998) treated soy protein film, which also

found soy protein aggregation. And Kristo et al. (2012) also used Size-exclusion chromatography to determine whey proteins aggregation and generation of oxidation products induced by UV-C. Besides, Chen et al. (2013) also mention that oxidation might deteriorate the digestive proteolysis susceptibility of soy protein. Commonly, decreasing TIA would lead to a higher digestibility of soy protein, but the aggregation and oxidation of protein induced by UV treatment may affect the protein digestibility. For more clarity, further studies are needed.

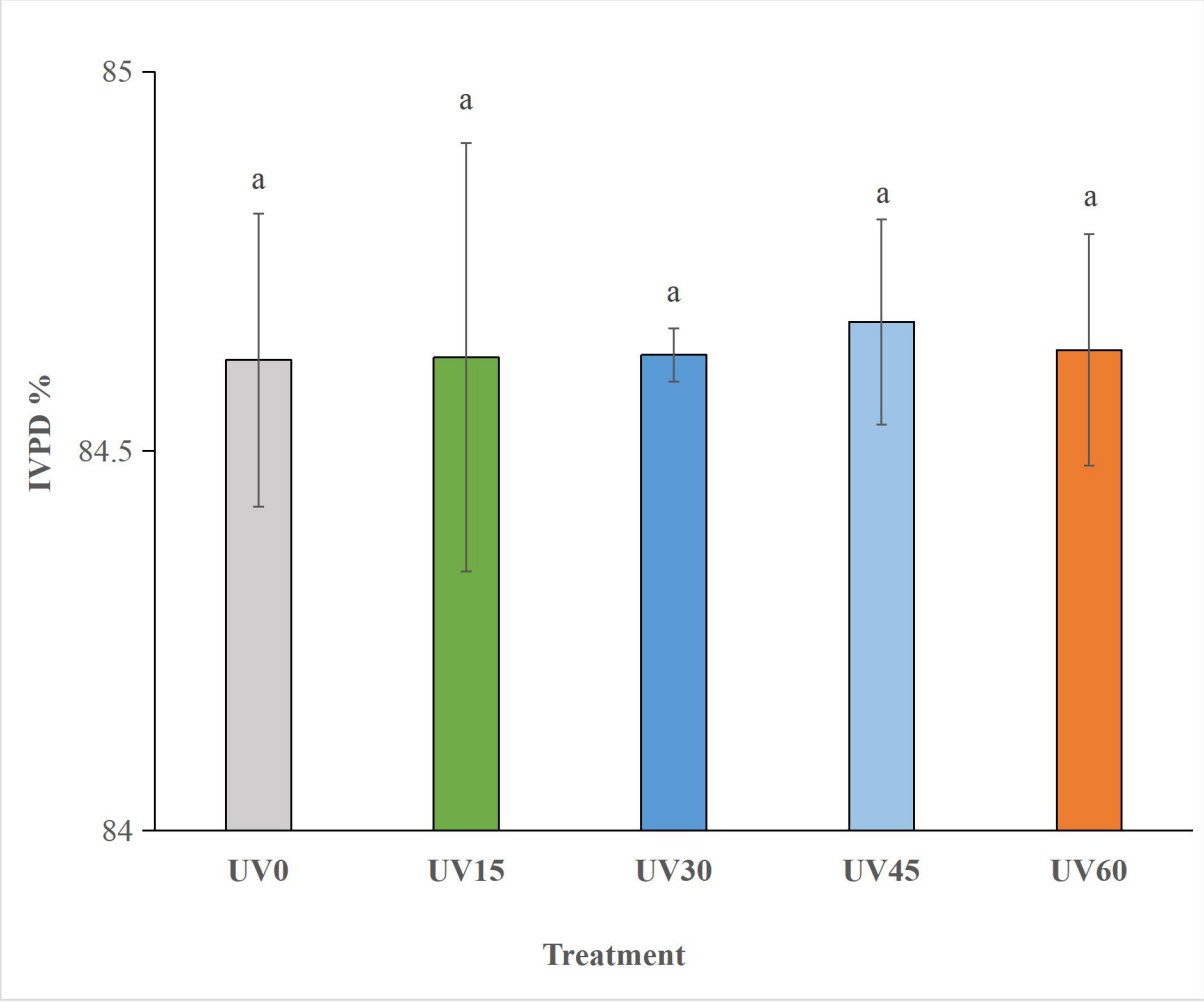

Figure 4.2. Effect of microwave combined ultraviolet processing on protein digestibility (IVPD %) of soybean protein

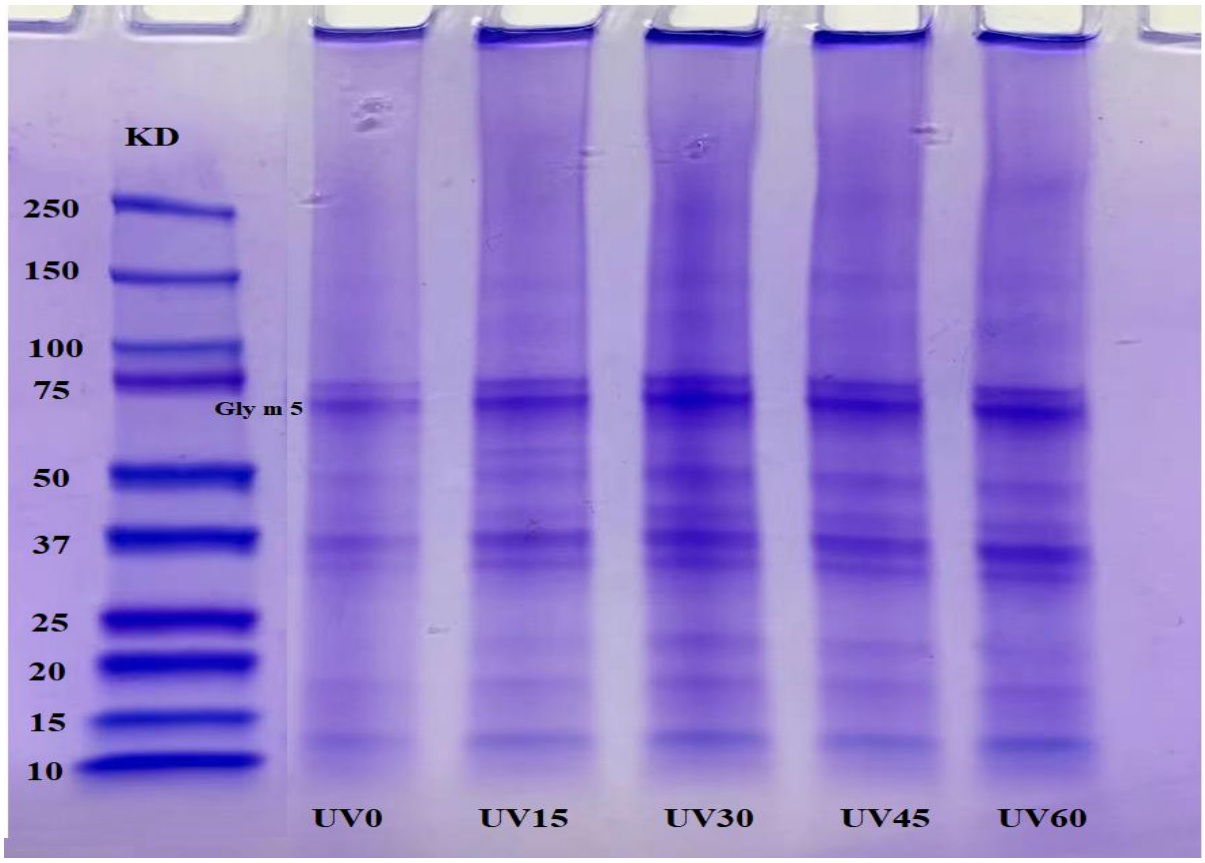

Figure 4.3. SDS-PAGE of microwave combined ultraviolet-treated and untreated proteins in soymilk sample

4.4.4 FTIR analysis of secondary structural changes in soymilk protein

FTIR spectroscopic analysis was used to test the effects of microwaves on the secondary structure of soy proteins. Table 4.2 shows the protein structures of soymilk samples subjected to ultraviolet treatments. It was noticed that β-sheet representing the most significant proportion (30.22% ~32.31%), and a remarkable decrease of β-sheet proportion compared to the control. The second dominant structure in soy protein is random coils, which Shows a trend of rising first and then falling. The highest value (29.4%) was found in UV15 treatment, then it dropped to a minimum value (28.5%) after 60 min ultraviolet treatment. Turns account for 24.06 ~25.51% of protein structures after treatment, which all increased compared with the percentage of the control (24.5%). The least structure proportion was α-helix in samples,

with the least value of 14.6% at UV0 and the highest value of 15.8% at UV60. Wu et al. (2017) reported that UV treatment change the secondary structure of β-lactoglobulin. In their studies, β-lactoglobulin solution exposure to ultraviolet light at 15 min and 30 min by UV equipment at 254 nm with a radiation dose of 11.8 Wm^{-2} were done. The results revealed that there is a decrease in the α-helix and random coil contents, while an increase in the β-sheet and β-turn contents, compared with the control. While Hu et al. (2016) using UV treatment (254 nm, 5 min and 15 min, 11.8 Wm^{-2}) to α-Casein solutions, they found the UV-C treatments to decrease the contents of β-sheet structures and increase the content of α-helix and β-turn structures compared with the control. This indicated that the protein secondary structure would be changed due to the UV treatment.

Table 4.2. Changes of secondary stricture of microwave combined ultraviolet-treated soymilk samples

Treatment	β-sheet % (1610-1640 cm^{-1})	Unordered % (1640-1650 cm^{-1})	α-helix % (1650-1658 cm^{-1})	β-Turns % (1660-1700 cm^{-1})
UV0	32.31 ± 0.27 ^{e}	28.65 ± 0.09 ^{a}	14.55 ± 0.07 ^{a}	24.49 ± 0.24 ^{a}
UV15	31.94 ± 0.12 ^{d}	29.39 ± 0.17 ^{c}	14.61 ± 0.18 ^{a}	24.06 ± 0.25 ^{a}
UV30	31.16 ± 0.11 ^{c}	28.99 ± 0.12 ^{b}	14.90 ± 0.09 ^{b}	24.95 ± 0.21 ^{b}
UV45	30.76 ± 0.10 ^{b}	28.61 ± 0.15 ^{a}	15.54 ± 0.21 ^{c}	25.08± 0.29 ^{cb}
UV60	30.22 ± 0.11 ^{a}	28.50 ± 0.13 ^{a}	15.78 ± 0.10 ^{c}	25.51 ± 0.25 ^{c}

Note: values with different letters in the same column are significantly different ($p < 0.05$) from each other.

4.4.5 Allergenicity

The tendency of allergenicity was implied by the content of soybean major allergen Gly m 5 detected through the use of sandwich enzyme-linked immunosorbent assay (ELISA). As shown in Figure 4.4, microwave combined ultraviolet treated Gly m 5 exhibited a gradually weak immune response trend with the increasing time, with the Gly m 5 content decreasing from 141.52 μg/g in control to 70.42 μg/g at UV60. The Gly m 5 contents in UV45 (99.47 μg/g) and UV30 (107.24 μg/g) had no significant difference. While the value of US15 compared to the control samples, had a significant decrease at 130.51 μg/g. The decrease of Gly m 5 might be due to the ultraviolet rays destroying disulphide bonds (S-S) (Pattison et al., 2012), causing the change of conformational or linear epitopes of Gly m 5 molecules.

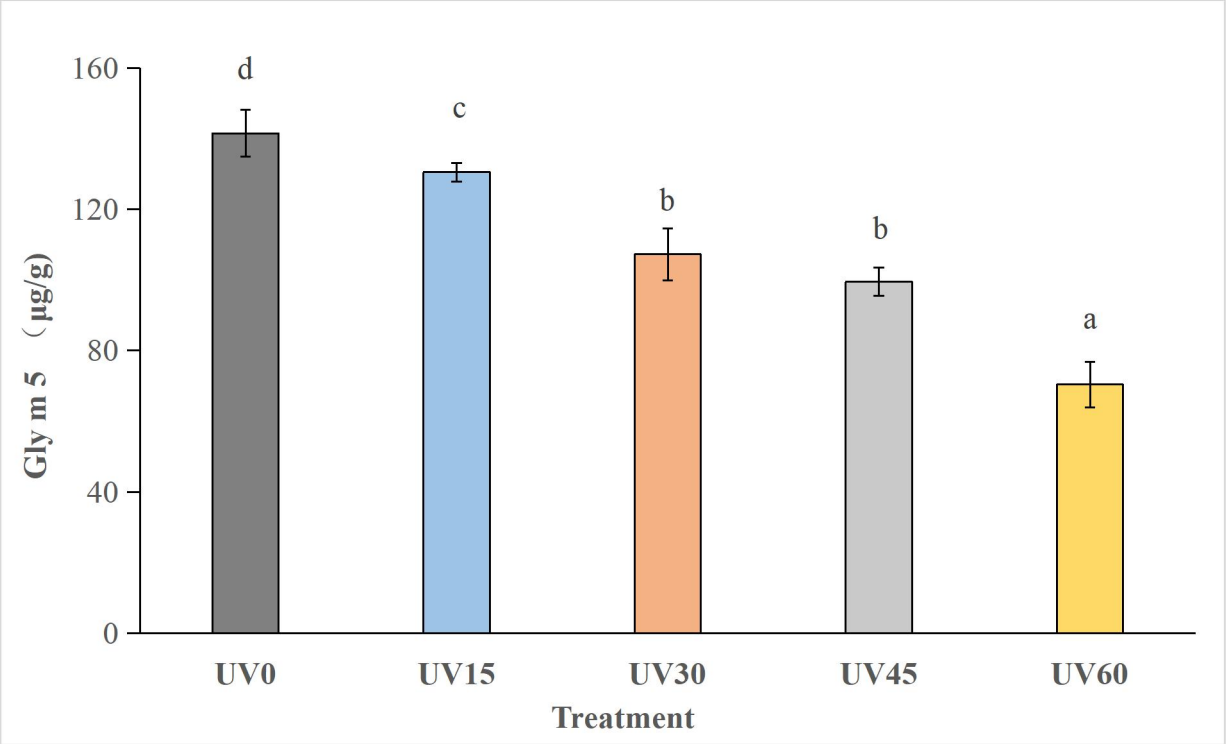

Figure 4.4. The Gly m 5 contents of soymilk samples under microwave combined ultraviolet treatment,

Ultraviolet has the potential to alter the structure of proteins and, therefore might potentially have an impact on the allergenicity of specific proteins. Hu et al. (2016) described the allergenicity of α-casein in milk tested by ELISA markedly reduced after UV irradiation at 254 nm, 15 min treatment compared to the control samples. Similar results reported by Tammineedi et al. (2013) observed the effects on whey proteins by UV-C treatments (254 nm, 15 min), and the allergenicity of whey protein was reduced by 27.7%. However, Manzocco et al. (2012) found no differences in immunoreactivity between egg whites exposed to UV light (254 nm, 5 ~30 min) and the untreated ones. Based on the values of the ELISA test, it was suggested that UV treatment might be helpful to eliminate the allergenicity of Gly m 5 in soymilk, despite different effects observed in other different foods. But, further studies about the mechanism of allergenicity reduction of Gly m 5 through by UV are needed.

4.4.6 Microstructure observation

Figure 4.5 shows the SEM micrographs of microwave combined UV-treated soymilk sample. The sample (A to E) differed in their microstructure. With the increasing time of UV treatment, more numerous small pinholes were shown in soymilk samples. A similar result was reported by Fathi et al. (2018). They found sesame protein isolate film treated by UV-C to have more holes, and cracks in the SEM image as shown. Also, Díaz et al. (2016) found a similar microstructure in the UV-treated whey protein concentrate film. These observations could be correlated with the degree of protein denaturation of soymilk due to UV irradiation.

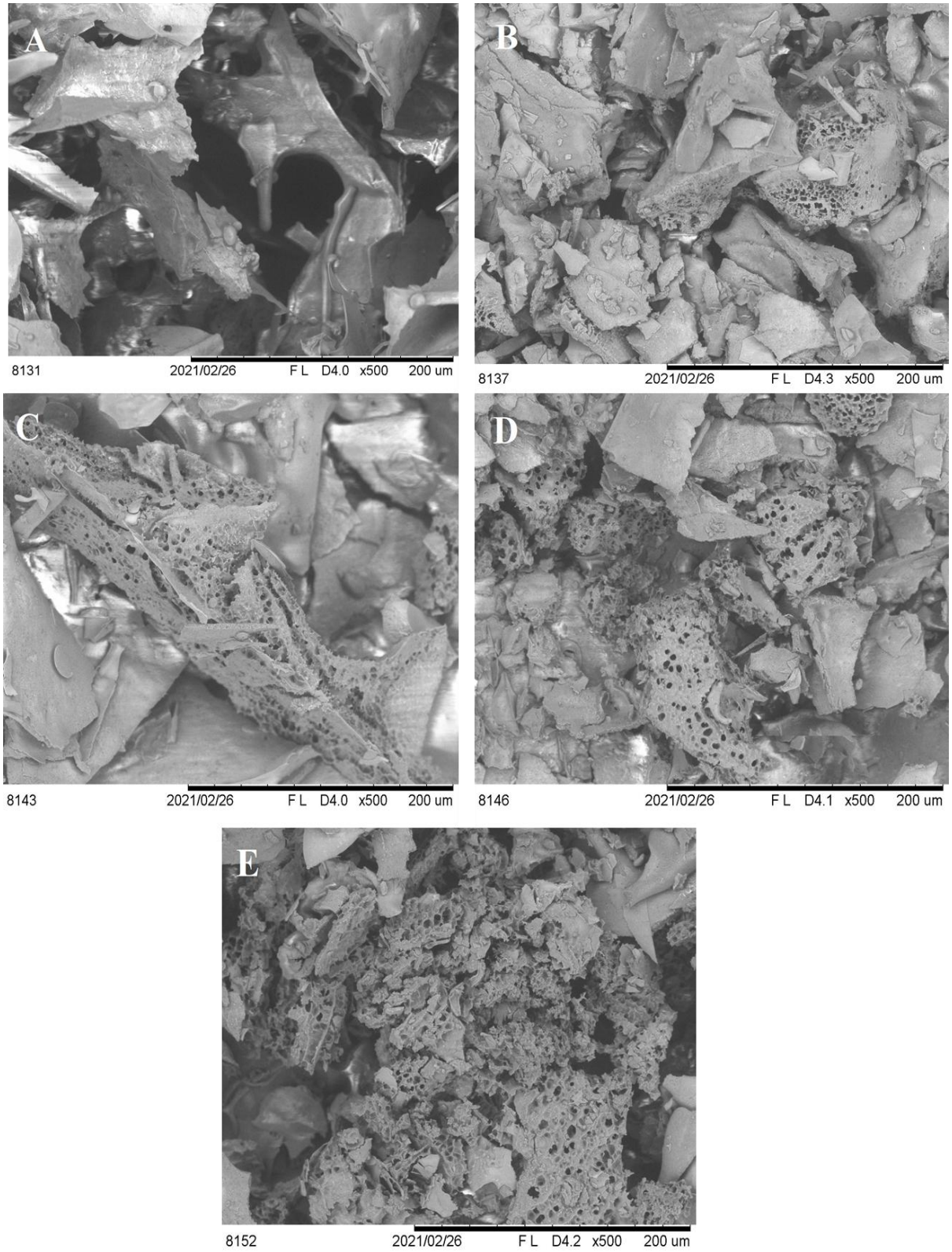

Figure 4.5. SEM photomicrographs of freeze dried soymilk (A) UV0 (control sample), (B) UV15, (C) UV30, (D) UV45, and (E) UV60 (magnification at 500x)

4.5 Conclusions

In the present study, we found microwave combined ultraviolet processing to have the capability to decrease the allergenicity of soymilk caused by one of the major allergen Gly m 5, which would do great help to the population with soybean sensitivity. The percentage of reduced allergenicity was positively related to the increase of ultraviolet irradiation time; the minimum allergenicity with a 50.24% reduction was observed at UV60. Meanwhile, the total soluble protein content increased by 31.1% after 60min ultraviolet treatment, and improving the antioxidants capacity and decreasing trypsin inhibitor activity of soymilk. But for IVPD of soymilk, there was no significant change compared to the control sample. Soymilk protein denaturation and aggregation may induce the increase of β-turn and α-helix structures and the decrease of β-sheet and unordered structures in treated samples. Numerous microstructural holes were observed in treated samples with increasing treated time. Research should be conducted on microwave combined with ultraviolet methods for different temperatures, irradiation doses, and time to understand the denaturation process of soymilk to allergen proteins better.

CHAPTER V

SUMMARY AND CONCLUSIONS

Soybean is one of the eight major allergens, which could cause some cross-reactivity, with other related allergens, such as birch pollen, leading to an increasing trend worldwide. Soybean can cause a series of allergic symptoms, and it even could lead to death. With the increasing demand for soybean products, the problem of allergenicity of soybean protein needs to be solved urgently. In Chapter II, we reported major soybean allergens in soybean such as Gly m Bd 28K, P34 (Gly m Bd 30K), and β-conglycinin α -subunit (Gly m Bd 60K). In the food industry, some physical or chemical treatments are often used to reduce the allergenicity of soybean by changing the protein structure or IgE binding site of allergens. However, different processing methods have shown varying effects on the modification of proteins and their functional characteristics.

In our first set of research objectives, we investigated the influence of microwave combined with ultrasonic treatment (MW, 85℃ 10 min, US 0-16 min) on the trypsin inhibitors content, antioxidant capability, secondary structures, microstructures, in vitro protein digestibility, and allergenicity of soymilk samples. Total soluble protein content and total antioxidant capacity increased gradually with the increase of time and reached the highest value at 16 min of ultrasonication. Meanwhile, the decrease of trypsin inhibitor activities with the longer treatment was observed, and it also led to higher values of in-vitro protein digestibility. Larger aggregations of soy protein were observed by SEM. Secondary structures change with the increasing time, specifically showing the upward trend of β-sheets and the downward trend of α-helix. Finally, ELISA test results have shown that a 16 min of ultrasonic treatment

significantly inhibited the IgE binding capacity of Gly m 5. These results demonstrated that microwave combined ultrasonic treatment has the potential to promote the elimination process of soybean allergenicity and it would not bring about the adverse effects of long-term hight temperature treatment of soybeans. However, further studies regarding the mechanisms of ultrasonication and microwave in causing a reduction in the allergenicity and other properties of proteins are needed, like the determination of sulfhydryl, disulfide bond and peptide content of soybean protein and structural responses of soybean allergen to ultrasonic treatment based on molecular dynamics simulations.

In our second set of research objectives, similar parameters of soymilk samples were tested and analyzed after microwave combined ultraviolet treatment (MW, 85℃ 10 min, UV, 0-60 min). It was found that microwave combined with ultraviolet processing to have the capability to decrease the allergenicity of soymilk caused by one of the major allergen Gly m 5, which would do great help to the population with the soybean-sensitive condition. The percentage of reduced allergenicity was positively related to the increase of ultraviolet irradiation time; the minimum allergenicity with a 50.24% reduction was observed in UV60. Meanwhile, the total soluble protein content increased by 31.1% after 60 min ultraviolet treatment, and improved the antioxidant capacity and decreased trypsin inhibitor activity of soymilk. But for the IVPD parameter of soymilk, no significant change compared to the control sample was noted. Soymilk protein denaturation and aggregation may induce the increase of β-turn and α-helix structures and the decrease of β-sheet and unordered structures in treated samples. Higher numbers of microstructural holes were observed in treated samples with increasing treated time. Research should be further conducted on microwave combined

with ultraviolet methods for different temperatures, irradiation doses and time to better understand the denaturation process of soymilk to allergen proteins.

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