EFFECTS OF ULTRASOUND AND MICROWAVE PROCESSING ON PHYSIOCHEMICAL AND ALLERGENIC PROPERTIES OF SHRIMP

BY

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ABSTRACT

Shrimp is a common species in shellfish with abundant nutrients, such as protein, amino acids, minerals, unsaturated fatty acids, vitamins, astaxanthin, and antioxidants. However, as one of "big eight" allergic foods, it can lead to a series of allergic reactions, including mild to life-threatening anaphylaxis. Tropomyosin is identified as the major allergen in shrimp, and the structures of shrimp allergens may be altered by thermal or non-thermal treatments. Compared with conventional treatments (e.g., heating, steaming), novel treatments have superior effects and maintain the original characteristics of shrimp. Novel processing methods to lower the allergenicity of shrimps commonly include microwave, ultrasound, pulsed light, cold plasma, fermentation, enzymatic hydrolysis, and combination of them.

In this study, the effects of thermal processing (Microwave treatment) and non-thermal processing (Ultrasonic processing) on the allergenicity, color attributes, antioxidant capability, microstructures, secondary structures, in vitro protein digestibility of *Litopenaeus vannamei* have been investigated. The results indicated there was a significant decrease in total soluble protein content and an increase of total antioxidant capacity compared to the untreated sample. The in vitro protein digestibility and peptide production showed enhancement with the increasing temperature and time of microwave processing or the increasing time of ultrasound processing. More and more fragments and strips were observed by SEM, and four kinds of secondary structures transfer occurred in treated samples, with the end result being an enhancement of the treatment.

Résumé

La crevette est une espèce commune dans les mollusques et crustacés avec des nutriments abondants, tels que protéines, acides aminés, minéraux, acides gras insaturés, vitamines, astaxanthine et antioxydants. Cependant, en tant que l'un des «huit grands» aliments allergiques, il peut entraîner une série de réactions allergiques, notamment une anaphylaxie légère à potentiellement fatale. La tropomyosine est identifiée comme l'allergène majeur chez la crevette et les structures des allergènes de crevette peuvent être altérées par des traitements thermiques ou non thermiques. Comparés aux traitements conventionnels (chauffage, cuisson à la vapeur, par exemple), les nouveaux traitements ont des effets supérieurs et conservent les caractéristiques originales de la crevette. De nouvelles méthodes de traitement visant à réduire le pouvoir allergène des crevettes comprennent généralement les micro-ondes, les ultrasons, la lumière pulsée, le plasma froid, la fermentation, l'hydrolyse enzymatique et leur combinaison.

Dans cette étude, les effets du traitement thermique (traitement par micro-ondes) et non thermique (traitement par ultrasons) sur l'allergénicité, les attributs de couleur, la capacité antioxydante, les microstructures, les structures secondaires, la digestibilité in vitro des protéines de Litopenaeus vannamei ont été étudiés. Les résultats ont indiqué qu'il y avait une diminution significative de la teneur totale en protéines solubles et une augmentation de la capacité antioxydante totale par rapport à l'échantillon non traité. La digestibilité des protéines in vitro et la production de peptides ont montré une amélioration avec l'augmentation de la température et du temps de traitement aux micro-ondes ou avec l'augmentation du temps de traitement par ultrasons. De plus en plus de fragments et de bandes ont été observés par SEM et quatre types de transfert de structures secondaires se sont produits dans les échantillons traités, le résultat final étant une amélioration du traitement.

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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 manuscript-based 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 as 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. Xin Dong, Jin Wang and Vijaya Raghavan. 2020. "Critical Reviews and Recent Advances of Novel Non-thermal Processing Techniques on the Modification of Food Allergens" (Online published by Critical Reviews in Food Science and Nutrition)

2. Xin Dong, Jin Wang and Vijaya Raghavan. 2020. "Effects of High-Intensive Ultrasound Processing on Shrimp Properties: Allergenicity, Secondary Structure, Antioxidant Capacity and Digestibility" (Under review)

3. Xin Dong, Jin Wang and Vijaya Raghavan. 2020. "Recent Advances of Novel Techniques on Modification of Shrimp Allergens: A review" (In Progress)

4. Xin Dong, Jin Wang and Vijaya Raghavan. 2020. "Effects of Microwave Processing on Shrimp Properties: Allergenicity, Secondary Structure, Antioxidant Capacity and Digestibility" (In Progress)

The work reported here was performed by Xin Dong and supervised by Prof. Vijaya Raghavan of the Department of Bioresource Engineering, Macdonald Campus of McGill University, and Montreal. The entire research work was carried out at the Postharvest 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 also participated in reviewing the manuscript.

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CHAPTER I

INTRODUCTION

Shrimps are popular in human diet due to high nutritive value and tasty flavor. Shrimp is identified as a rich source of proteins, with low lipids and caloric levels (Pires et al., 2018). Shrimp is also abundant in vitamin B_{12}, calcium, selenium and astaxanthin, which is often used as a natural additive due to the resistance to oxidation (Venugopal, 2008). Shrimps contain large amount polyunsaturated fatty acids, such as eicosapentaenoic (C20:5n3, EPA) and docosahexaenoic (C22:6n3, DHA) acids, which play predominant roles in human health (Bono et al., 2012). In human diets, fresh shrimps can be consumed directly or by cooking with sauce. With the beneficial properties, shrimps are popular thus existing in pastas, salads, soups, curry and stir-fried dishes (Dayal et al., 2013).

However, shrimp allergy, belonging to shellfish allergy, which is a kind of "The Big 8" food allergy (Lopata et al., 2016). It has been reported that shrimp is the most frequent offender in children and adults allergenicity and it is often associated with the anaphylaxis from mild to life-threatening (Bock et al., 2007; Sicherer et al., 2004). Over eleven kinds of allergens have been identified in shrimp, among them, tropomyosin as the main shrimp allergen is water-soluble and heat-stable (Shanti et al., 1993). According to the specific properties and structures of tropomyosin, researchers try to find the effective food processing techniques to lower the allergenicity of shrimps.

Various studies have shown that the shrimp allergenicity can be altered using different processing techniques including thermal and non-thermal methods, such as boiling, high pressure, microwave, ultrasound, pulsed light, cold plasma, fermentation, enzymatic hydrolysis. Compared with thermal techniques, non-thermal techniques have better performance in retaining the original attributes and nutritional characters. Among them, ultrasound processing gained significant attention due to its numerous advantages such as environmental-friendliness, less energy consumption, higher mass transfer rate, and original flavor maintenance of food products during processing (Wang et al., 2019). In recent years, many studies investigated the

effects of ultrasound processing on shrimp allergenicity by treating at various powers or time; and found ultrasound treatment to have great potential in lowering shrimp allergenicity . However, few studies applied microwave treatment to the modification and elimination of shrimp allergens; the main reason being shrimp tropomyosin is heat-stable and has potent ability to maintain a great allergenic potential during thermal treatment (Carnés et al., 2007; Liu et al., 2010b). A previous study found that there were no obvious effects of shrimp immunology under microwave treatment at 16-17°C for different time durations of 1, 5, 10, 20 min (Kim et al., 2006). However, no studies til now investigated the influence of microwave processing with higher applied temperature to shrimp allergens, which is imperatively needed in this field.

1.1 Hypothesis and Implications

Thermal and non-thermal food processing approaches include microwave and ultrasound treatment in the food industry. Although ultrasound has been identified as an effective way to lower shrimp allergenicity, most previous studies applied ultrasound treatments with long time durations, which led to the lower economic value and higher energy consumption. To date, few studies applied microwave treatment under high temperature to shrimps. Thus, in-depth research about microwave and ultrasound treatment in reducing or eradicating shrimp allergens is needed. In this project, the impacts of the high-intensity microwave and ultrasound processing are separately discussed on the color attributes, antioxidant capability, secondary structures, microstructures, in vitro protein digestibility, and allergenicity of shrimp samples.

1.2 Objectives

The objective of the study is to investigate and optimize the modification of shrimp allergens under microwave treatment and ultrasound treatment, and simultaneously attain the good sensory quality and physicochemical properties such as color attributes and total antioxidant capability of shrimps. Additionally, protein structures and in vitro protein digestibility of shrimp samples are to be investigated.

CHAPTER II

LITERATURE REVIEW I

Critical reviews and recent advances of novel non-thermal processing techniques on the modification of food allergens

2.1 Abstract

Nowadays, the increasing prevalence of food allergy has become a public concern related to human health worldwide. Thus, it is imperative and necessary to provide some efficient methods for the management of food allergy. Some conventional processing methods (e.g., boiling and steaming) have been applied in the reduction of food immunoreactivity, while these treatments significantly destroy nutritional components present in food sources. Several studies have shown that novel processing techniques generally have better performance in retaining original characteristics of food and improving the efficiency of eliminating allergens. This review has focused on the recent advances of novel non-thermal processing techniques including high-pressure processing, ultrasound, pulsed light, cold plasma, fermentation, pulsed electric field, enzymatic hydrolysis, and the combination processing of them. Meanwhile, general information on global food allergy prevalence, food allergy pathology and biochemical characterizations of the main allergens are also described. Hopefully, these findings regarding the modifications on the food allergens through various novel food processing techniques can provide an in-depth understanding on the mechanism of food allergy, which in turn possibly provide a strategy to adapt in the reduction of food immunoreactivity for the food industries.

Keywords: Novel non-thermal processing; modification of food allergens; fermentation; pulsed electric field

2.2 Introduction

Food allergy has been considered as a significant public health challenge occurring when certain foods are ingested (Blázquez and Berin, 2017; Polloni et al., 2017). An epidemiological survey conducted by the World Allergy Organization has reported that approximately 200 to 250 million individuals are suffering from food allergies all over the world (Pawankar et al., 2013). Global analysis have reported that there are approximately 8% of children and 2% of adults suffering from food allergy in developed countries such as United States, Canada, and Italy, and this population number is up to 6% of children and 4% of adults in developing countries such as Brazil and India (Boye, 2012; Cianferoni and Spergel, 2009; Ekezie et al., 2018; Gupta et al., 2007). Further, food allergens potentially can trigger severe allergenic reactions, even leading to death, and the prevalence is increasing at a faster rate than any other allergic disordered diseases whereas there is very limited longitudinal research conducted about this phenomenon (Sicherer and Sampson, 2010; Wu et al., 2012). It has been reported that more than 170 food types can induce an abnormal immunological response, of which specific foods (including milk, seafood, egg, peanut, soy, tree nut, and wheat) are accountable for 90% of the reported cases (Shriver and Yang, 2011). Therefore, food allergy is becoming a global challenge affecting the daily life of individuals.

To solve this global challenge related to human health, many studies have been performed to evaluate the relationship between food processing and food immunoreactivity. Most food allergens are proteins, and hence many studies have tried to modify their functional properties by changing the structural properties, which may affect the immunoreactivity of related food proteins. Thermal processing methods such as boiling and roasting are considered as the most effective method to alter food attenuation and mitigate allergens by various modifying reactions, including peptide bonds hydrolysis, denaturation, disulphide bonds restructure and interaction with other ingredients (Ekezie et al., 2018). However, the organoleptic properties and nutrient compositions of food products would be affected under thermal treatment, such as protein denaturation of eggs or milk, caramelization resulting from sugar dehydration, gas or water vapor generation by baking, enzyme inactivation due to high temperature (Meda et al.,

2017; Regier and Schubert, 2005). Moreover, the effect of thermal processing on the reduction of food immunoreactivity is difficult since specific allergens present in foods are heat-stable, such as soy, shellfish, wheat, celery, tree nuts (Besler et al., 2001; Lasekan and Nayak, 2016). In comparison with thermal processing, non-thermal processing showed numerous of advantages not only in maintaining the sensory properties (e.g., freshness, flavor, color attributes) and improving nutritional properties (phenolics and antioxidants), but also in reducing the food immunoreactivity of soy, peanuts, and shrimp (Li et al., 2011; Li et al., 2013; Meinlschmidt et al., 2016).

In this review, we have outlined several types of mechanisms causing food allergies and their related symptoms to provide some necessary information for the readers. Then, the biochemical characterizations of "big eight" food allergens are described in detail. Further, we reviewed the recent advances of novel non-thermal processing techniques on the modification of food allergens, including high-pressure processing, pulsed light, ultrasound, cold plasma, fermentation, pulsed electric field, enzymatic hydrolysis, and the combination processing of them. Hopefully, it can provide some strategies for the management of food allergy in the future.

2.3 Properties and pathology of food allergens

Most of the allergens present in foods are proteins that are from different families such as pathogenesis-related protein (PR-10), thaumatin-like proteins (TLPS), non-specific lipid transfer proteins (ns-LTPs) and profilin (Wang et al., 2017). Epitopes, the allergenic portions of proteins, are 10-70 kDa, water-soluble glycoproteins, and some of them are stable to resist denaturation during heating or acid treatment (Sicherer and Sampson, 2010). Immunoglobulin E (IgE) recognition sites, called IgE-binding epitopes in proteins, can specifically bind with related antibodies resulting in allergic reactions when individuals with allergy history are exposed to the specific allergens (Matsuo et al., 2015).

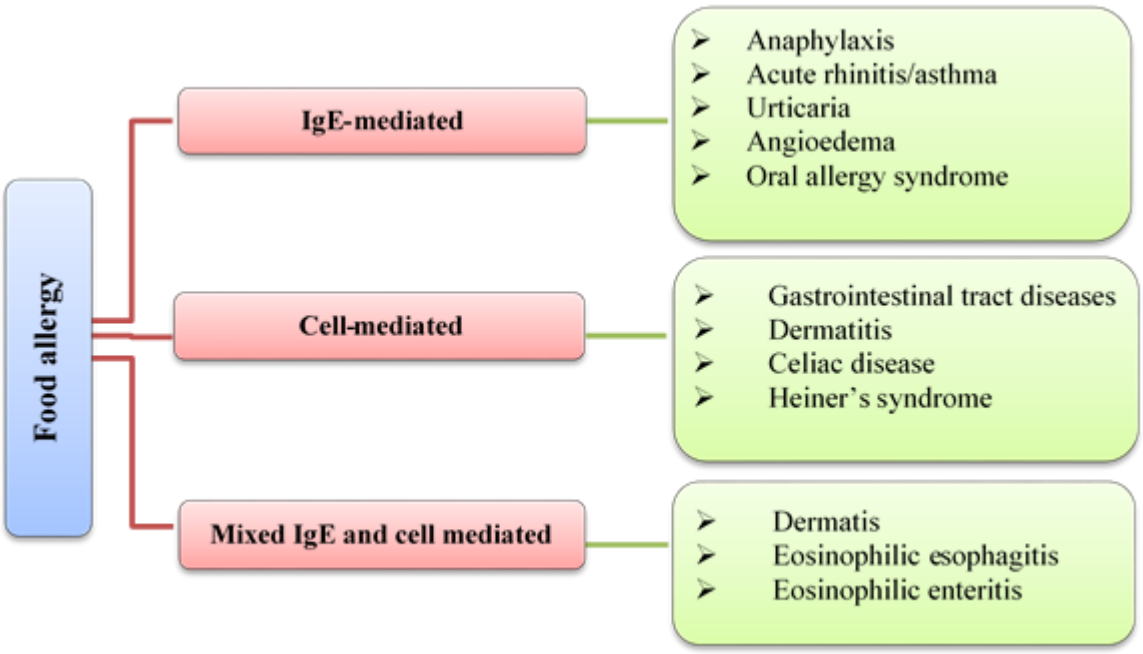

Figure 2.1. Classification of food allergy

Generally, food allergies can trigger various symptoms ranging from oral allergy syndrome (OAS) to life-threatening anaphylaxis. As shown in Figure 2.1, food allergies are classified into three categories based on their immunological mechanisms causing allergic symptoms. IgE-mediated food allergy is the first type, which is mediated by IgE antibodies causing characterized allergenic reactions (Figure 2.1). As outlined in Figure 2.2, IgE-mediated food allergies develop when an allergen penetrate the mucosa and then is captured by dendritic cells (DCs) (Li et al., 2007). This binding of allergen with DCs results in the release of specific IgE antibodies, which can specifically combine with mast cells (Wang et al., 2019). When the same allergen is exposed later, an allergic reaction occurs as pre-existing IgE binding with the target macromolecules (Faridnia and Selamat, 2011). Oral allergy syndrome (OAS) is one of the most common symptoms triggered during IgE-mediated food allergy. Generally, it may involve a sudden onset of itching, stinging pain, and vascular edema of the lips, tongue, palate, and pharynx (Price et al., 2015; Purohit-Sheth and Carr, 2005). A typical example of OAS is oral mucosal symptoms, which are caused by the intake of the fruits belonging to family Rosaceae (e.g., apple, cherry, peach) among individuals with pollen allergy (Kondo and Urisu, 2009; Sloane and Sheffer, 2001). It has been reported that oral allergy syndrome responses to a variety

of fruits, nuts, and vegetables (Table 2.1).

Table 2.1. Symptoms and related allergens causing oral allergy syndrome (OAS) and life-threatening anaphylaxis

Classification	Symptoms	Allergen sources	References
Oral allergy syndrome (OAS)	Oropharyngeal pruritis (tingling and itching of the lips); Angioedema (involving the oral mucosa, tongue, palate or throat)	Nuts (almond, hazelnut, walnut); Fruits (apple, apricot, banana, cantaloupe, cherry, kiwi, peach, pear, plum, prune, watermelon)	(Price et al., 2015) (Purohit-Sheth and Carr, 2005) (Sloane and Sheffer, 2001)
Life-threatening anaphylaxis	Lung disease (Heiner syndrome) Gastrointestinal tract diseases (Eosinophilic gastrointestinal disorders, iron deficiency anemia, celiac disease) Rhinoconjunctivitis/Asthma	Milk; Soy; Vegetable (i.e. celery); Fruits (i.e. kiwi); Cereals	(Burks et al., 2012)

The second type is cell-mediated food allergy involving the gastrointestinal tract and occurs when the cell component of the immune system is responsible for the food allergy (Figure 2.1) (Yu et al., 2016b). Clinical manifestations regarding cell-mediated food allergies include food protein-induced enterocolitis syndrome, proctocolitis syndrome, weight depreciation, celiac disease, and pulmonary hemosiderosis (Cianferoni and Spergel, 2009; Henderson et al., 2012). Further, in some clinical cases, food allergies can also be triggered by both IgE and immune cells (Figure 2.1). Mixed IgE-cells mediated food allergy generally induces the cutaneous, gastrointestinal, respiratory diseases, with symptoms such as atopic dermatitis, allergic

eosinophilic esophagitis, asthma (Sampson and Immunology, 2003). Atopic manifestations arising from IgE-independent factors mainly include delayed food-allergy-associated atopic dermatitis and eosinophilic gastrointestinal disorders (e.g., eosinophilic oesophagitis), which are often triggered by the allergens in milk (Yu et al., 2016b). Cell-mediated and mixed IgE-cell-mediated food allergy can result in predominantly abdominal symptoms (e.g., bloody stools) (Burks et al., 2012). The best strategy for the management of these food allergies is to avoid the intake of those potential allergens.

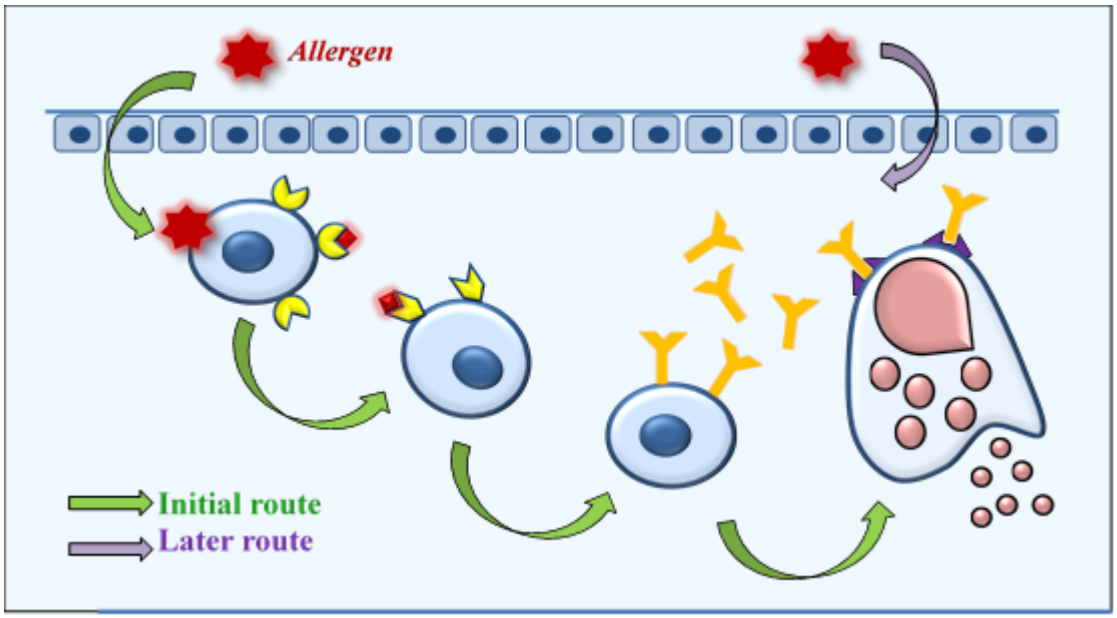

Figure 2.2. Mechanisms of IgE-mediated anaphylaxis reaction

2.4 Biochemical characterizations of major food allergens

As known above, tree nuts, peanuts, cow's milk, hen's egg, wheat, soy, fish, and crustaceans are considered as the "Big Eight" food allergens (Alves et al., 2016). In children, the most common allergens are cow's milk and hen's egg, while shellfish is the major allergen in adults (Turnbull et al., 2015). As shown in Figure 2.3, the most common allergens present in foods were outlined. Generally, these allergens are commonly water-soluble glycopeptides and have high-level resistance to heat, acid, and proteolytic enzymes (Turnbull et al., 2015).

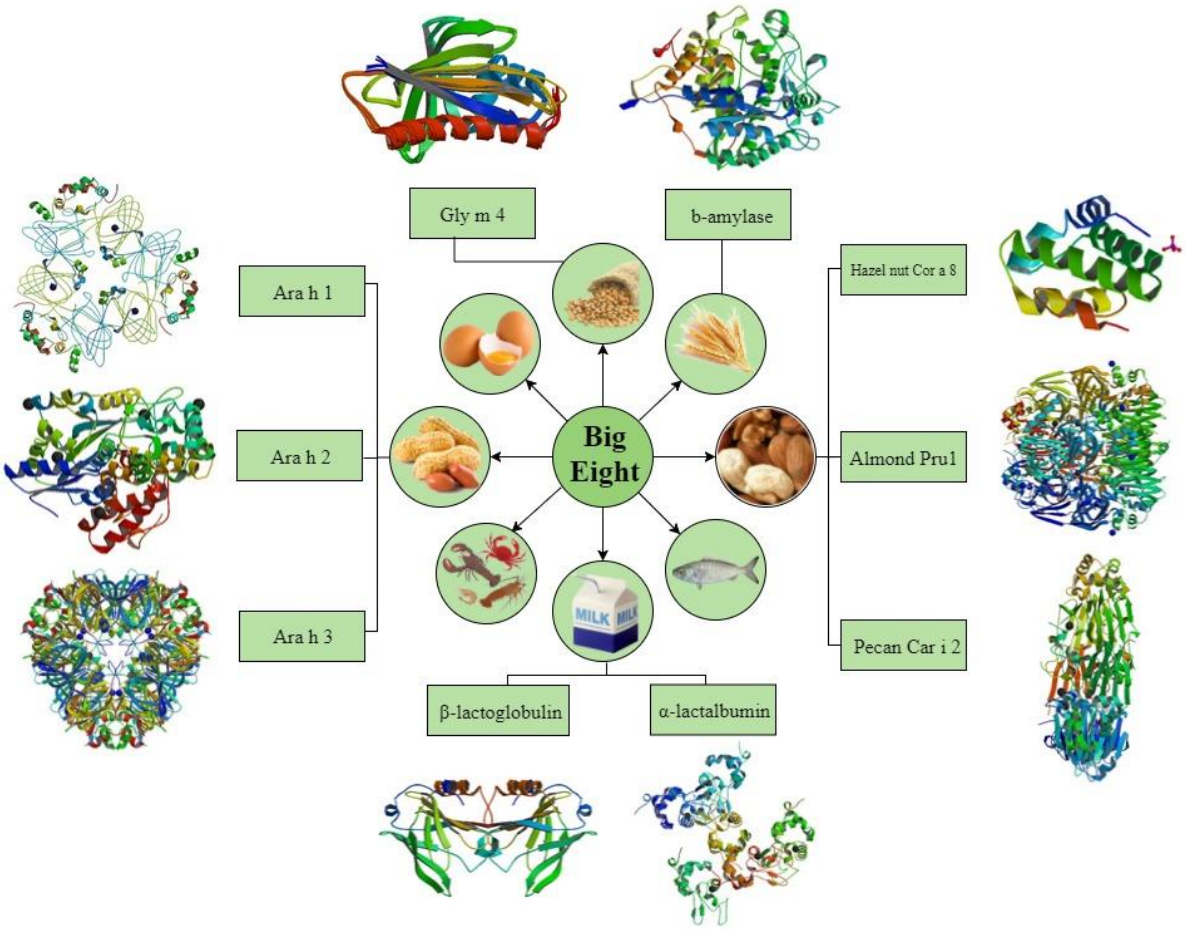

Figure 2.3. Biochemical characters of "big eight" allergens: Ara h 1, Ara h 2, Ara h 3 in peanut; β-lactoglobulin, α-lactalbumin in milk; Cor a 8 in hazelnut, Pru1 in almond, Car i 2 in pecan; Gly m 4 in soy; b-amylase in wheat (Source: protein data bank)

2.4.1 Tree nuts and peanuts

Although tree nuts and peanuts are botanically unrelated, both of them easily induce IgE-mediated allergic reactions by consuming a minimal amount (McWilliam et al., 2015). It has been reported that 20-30% of patients allergic to peanuts may suffer from one or more tree nuts allergy due to their sharing of similar protein epitopes (Maloney et al., 2008). The prevalence of tree nuts is up to 4.9% all over the world (Geiselhart et al., 2018). Tree nuts family commonly consists of the hazelnut, walnut, pecan, almond, cashew nut, Brazil nut, pistachio, and macadamia nut (Eigenmann et al., 2017). Studies have reported that 86% of the patients with hazelnut allergy are attributed to seed storage proteins, including Cor a 9 (11S globulin) and

Cor a 14 (2S albumin) (Table 2.2) (Beyer et al., 2002; Eller et al., 2016). Pathogenesis-related proteins, including Cor a 1 (PR-10), Cor a 2 (profilin), and heat-stable Cor a 8 (lipid transfer protein), also could cause immunologic reactions in some patients when consuming them (Weinberger and Sicherer, 2018).

In walnuts, eight allergens have been reported by the World Health Organization and International Union of Immunological Societies (WHO/IUIS). Jug r 1 with 15-16 kDa molecular weight was considered as the major allergen present in walnuts and five IgE epitopes identified in Jug r 1 were responsible for 75% of walnut allergy (Pedrosa et al., 2015; Sordet et al., 2009). In pecans, there were two major identified allergens, including Car i 1 (2S albumin seed storage protein) and Car i 2 (vicilin), with IgE reactivity of 79% and 24% among 25 tested patients, respectively (Geiselhart et al., 2018). Moreover, almond nuts rank the fourth source in tree nuts causing allergenic reactions, and six almond allergens were identified by the researchers (Masthoff et al., 2013; McWilliam et al., 2015). Among all the almond allergens, Amandin (360 kDa), a heat-stable protein, accounted for 65-75% of total almond proteins causing IgE-mediated reactions (Roux et al., 2001; Vanga and Raghavan, 2017).

In peanuts, two major types of allergens were identified, including albumins (water-soluble) and globulins (saline soluble) (Comstock et al., 2016; Johns and Jones, 1916). Globulins account for up to 87% of the total protein belonging to seed storage proteins (Arya et al., 2016). To date, there are 17 peanut allergens (from Ara h 1 to Ara h 17) having been reported by the WHO/IUS allergen nomenclature subcommittee. Ara h 1 (7S globulin), Ara h 2 (2S albumin), and Ara h 3 (11S globulin) were considered as the top three major peanut allergens (Jong and Zijverden, 1998). Ara h1, a 64 kDa protein, is a member of the vicilin family with unusual thermostability (Zhang et al., 2017). It has been reported that over 90% of peanut-allergic subjects have antibodies against Ara h 1 (Ramesh et al., 2016). Ara h 2 is the member of the 2S albumins belonging to the prolamin superfamily (Porterfield et al., 2009). Ara h 3 is an 11S globulin (legumins/glycinins), which is part of the cupin superfamily (Zhuang and Dreskin, 2013). Most of the tree nuts and peanuts allergens are thermal stable due to large amounts of disulfide bonds in the protein structures (Vanga and Raghavan, 2017).

Table 2.2. Summary of major allergens in "Big Eight" foods

Foods		Category	Major allergens	Biochemical name	Molecular weight	References
Tree nuts	Hazelnut	Seed storage proteins	Cor a 9	11S globulin	40 kDa	(Beyer et al., 2002; Eller et al., 2016)
		Seed storage proteins	Cor a 14	2S albumin	10 kDa	(Beyer et al., 2002; Eller et al., 2016)
		Pathogenesis-related proteins	Cor a 1	PR-10	17 kDa	(Weinberger and Sicherer, 2018)
			Cor a 2	Profilin	14 kDa	(Weinberger and Sicherer, 2018)
		Pathogenesis-related proteins	Cor a 8	lipid transfer protein	9 kDa	(Weinberger and Sicherer, 2018)
	Walnut		Jug r 1	2S albumin	15-16 kDa	(Pedrosa et al., 2015; Sordet et al., 2009)
	Pecan	Pathogenesis-related proteins	Car i 1	2S albumin	16 kDa	(Geiselhart et al., 2018)
		Seed storage protein	Car i 2	Vicilin	55 kDa	(Geiselhart et al., 2018).
	Almond	Seed storage protein	Pru du 6	Amandin, 11S globulin	360 kDa	(Roux et al., 2001; Vanga and Raghavan, 2017).
Peanut		Seed storage protein	Ara h 1	Cupin, 7S globulin	64 kDa	(Ramesh et al., 2016)
		Legumin-like storage protein	Ara h 2	Conglutin, 2S albumin	17 kDa	(Porterfield et al., 2009)
			Ara h 3	Cupin, 11S globulin	60 kDa	(Zhuang and Dreskin, 2013)
		Vicillin-type seed storage protein				
		Legumin-type				
Milk		Whey proteins	β-LG	β-lactoglobulin	36 kDa	(Hochwallner et al., 2014)
		Caseins	α - LA	α-lactoalbumin	14.4 kDa	(Hochwallner et al., 2014)
Egg		Serine protease inhibitor	Gal d 1	Ovomucoid	28 kDa	(Osborne et al., 2011; Watanabe et al., 2015)
		Storage protein	Gal d 2	Ovalbumin	45 kDa	(Osborne et al., 2011; Tong et al., 2012)
Wheat		α-amylase inhibitors	Tri a 30	Tetramericα-amylase inhibitor CM3	16 kDa	(Sandiford et al., 1995)
		Seed storage protein	Tri a 19	ω-5 gliadin	65 kDa	(Juhász et al., 2018)
Soy		Soybean hull protein	Gly m 1		7 kDa	(Gonzalez et al., 1992)
		Soybean hull protein	Gly m 2	Hydrophobic protein from soybean	8 kDa	(Gonzalez et al., 1992)
		Birch pollen–related protein	Gly m 3	Defensin	14 kDa	(Masilamani et al., 2012)
			Gly m 4	Profilin	17 kDa	(Masilamani et al., 2012)
Fish		Pathogenesis-related protein	Lep w 1	PR-10, Bet v 1 family member	12 kDa	(Ma et al., 2008; Van Do et al., 2005)
Crustacean		Muscle protein	Met e 1	β-parvalbumins	34 kDa	(Leung et al., 1996)
		Muscle protein		Tropomyosin		

2.4.2 Milk and egg

Cow's milk ranks the third most common allergic source only behind peanuts and tree nuts, responsible for 10%-19% of food anaphylactic reactions (Kattan et al., 2011). The clinical manifestations induced by cow's milk have a highly variable range from mild to life-threatening. The mild symptoms result from non-IgE mediated mechanism, which may involve skin, respiratory system, and gastrointestinal system. The severe anaphylaxis is due to the IgE-mediated mechanism, which may lead to death in some cases (Martorell-Aragonés et al., 2015; Venter et al., 2017; Villa et al., 2018). The major allergens in cow's milk are the caseins and whey proteins (α-lactoalbumin and β-lactoglobulin) (Hochwallner et al., 2014). Caseins are essentially disordered proteins with very few secondary and tertiary structures, but their functions can still perform well. Caseins are heat-resistant proteins, with a slight reduction or no effect on their immunoreactivity after thermal treatments, whereas whey proteins are heat-labile (Bhat et al., 2016; Verhoeckx et al., 2015).

To date, six egg allergens have been listed by WHO/IUIS; they are ovomucoid (Gal d 1), ovalbumin (Gal d 2), ovotransferrin (Gal d 3), and lysozyme (Gal d 4) in egg white and α-livetin (Gal d 5) and YGP42 (Gal d 6) in egg yolk. Ovomucoid (Gal d 1), the most crucial allergen, is responsible for the majority of egg allergy, although it only accounts for 11% in egg white total protein. Sharing the highest immunoreactivity is probably due to its heat-stable and low digestibility properties (Osborne et al., 2011; Watanabe et al., 2015). Ovalbumin (Gal d 2) is a storage protein which accounts for the majority of egg white protein (54%), while it is a heat-labile protein and may lose its immunoreactivity after thermal treatment such as frying and boiling (Osborne et al., 2011; Tong et al., 2012).

2.4.3 Wheat and soy

Wheat is a staple grain that contains specific proteins to trigger adverse immune responses, including both IgE and non-IgE mediated reactions. IgE mediated reactions to wheat may relate to wheat ingestion (food allergy) or wheat inhalation (respiratory allergy); non-IgE mediated food allergy reactions to wheat mainly involve chronic eosinophilic inflammation (such as

eosinophilic esophagitis or eosinophilic gastritis) (Cianferoni, 2016). There are 32 various well-classified wheat allergens, which are recognized by WHO/IUIS Allergen Nomenclature Database. The major wheat allergens include α-amylase inhibitors, acyl-coenzyme, fructose-biphosphate aldolase, wheat-flour peroxidase, prolamins, and ω-5 gliadin (Pasha et al., 2016). It has been reported that wheat α-amylase inhibitors are responsible for baker's asthma (Sandiford et al., 1995). Prolamins are described as alcohol-soluble and water-insoluble storage proteins, which could trigger dietary-allergy symptoms, including atopic dermatitis and exercise-induced anaphylaxis (WDEIA) (Shewry and Halford, 2002). ω-5 gliadin allergen in wheat also can cause similar symptoms such as heat-dependent and WDEIA (Juhász et al., 2018).

Soy allergy is rarely seen in adults, whereas usually seen in children by inducing a series of IgE-mediated allergic symptoms such as atopic dermatitis and enterocolitis (Kattan et al., 2011; Sicherer, 2011; Sicherer and Sampson, 2009). There are approximately twenty-eight potential allergens identified according to the IgE binding from soy-allergic patients (Shibasaki et al., 1980). Gly m 1, Gly m 2, Gly m 3, and Gly m 4 are considered as the major allergens in soy described by WHO/IUIS (Masilamani et al., 2012). Gly m 1 and Gly m 2 are aeroallergens present in soybean hull and may be related to allergic asthma (Gonzalez et al., 1992). Gly m 3 and Gly m 4 are birch pollen-related allergens belonging to profilin family. Other dominant allergens such as Gly m 5 (β-conglycinin) and Gly m 6 (glycinin) are storage proteins accounting for 70-80% of total seed protein (Masilamani et al., 2012).

2.4.4 Fish and crustaceans

Fish allergy is an IgE-mediated immune reaction with clinical manifestations, including acute and generalized urticaria, vomiting, nausea, diarrhea, abdominal cramps, asthma, and wheezing after the exposure to fish. In the most severe cases, life-threatening anaphylaxis can be potentially induced (Daga et al., 2018). The most common fish allergen is parvalbumin that exists in a wide range of fish species, especially β-parvalbumin (Ma et al., 2008; Van Do et al., 2005). Parvalbumin is a 12 kDa molecular-weight muscle protein, with water-soluble, heat-resistant properties. Since it is a calcium-binding protein, the IgE-binding capacity of

parvalbumin decrease with the reduction of calcium-ions (Bugajska-Schretter et al., 1998). Other important fish allergens also were found in Japanese fish, creatine kinase in long-tail tuna, triosephosphate isomerase in common sole, aldolases, and enolases in cod, albacore tuna, and salmon (Valverde-Monge et al., 2018). Aldolases and enolases are heat-labile native oligomers, and triosephosphate isomerase is essential in the glycolysis process and energy production (Kuehn et al., 2013; Pérez-Gordo et al., 2010).

Crustacean allergy is the most frequently mentioned seafood allergy leading to adverse reactions in patients (Lopata et al., 2017). The allergenic symptoms induced by crustacean are often associated with skin and respiratory systems (Warren et al., 2019). The major allergen in crustaceans, causing allergic reactions is a muscle protein tropomyosin (Leung et al., 1996). Tropomyosin is considered as a cross-reactive panallergen because it also shows clinical reactivity to molluscs and arthropods (Ayuso et al., 2002). Additional allergens identified in crustaceans commonly include arginine kinase (AK), myosin light chain (MLC), sarcoplasmic calcium-binding protein (SCP), troponin-C (TnC), and triosephosphate isomerase (TIM) (Bauermeister et al., 2011).

2.5 Effect of novel non-thermal food processing techniques on the food immunoreactivity

As we mentioned previously, although thermal processing was considered as one of the most effective processing methods in the reduction of food immunoreactivity, the sensory (e.g., flavor and color attributes) and nutritional (e.g., ascorbic acid and antioxidants) properties were affected negatively during processing (Meda et al., 2017; Regier and Schubert, 2005). In the last decade, minimally processed products gained more attention due to health considerations. In comparison to thermal processing, non-thermal techniques are beneficial to maintain the freshness, flavor, color attributes, and nutritional properties, especially some thermal-unstable compounds such as ascorbic acid and polyphenols. Therefore, novel non-thermal processing techniques show a potential application in the reduction of food immunoreactivity. These techniques generally include high-pressure processing, pulsed light, cold plasma, ultrasound, fermentation, pulsed electric field, enzyme hydrolysis, and their combination (Rahaman et al., 2016a).

2.5.1 High-pressure processing

High-pressure processing (HPP) is also named high hydrostatic pressure processing or ultrahigh-pressure processing. HPP is a non-thermal processing technique, with the pressures generally ranging from 100 to 800 MPa. In the food industry, it is commonly used to inactivate microbes while minimizing the damage in foods, which is the main aim to improve the quality and safety of food products (Khan et al., 2018). The pressures applied to foods are uniform and simultaneous in all directions, whereas the appearance alteration of food-induced by HHP is irreversible due to the compression of air and water (Muntean et al., 2016). Under such a high-pressure condition, the structure of related proteins may be changed. Generally, conformational changes of proteins are observed when the pressure reach 200–300 MPa (Kurpiewska et al., 2019). Recently, HHP has been considered as a potential application in the reduction of food immunoreactivity due to the denaturation and aggregation of treated protein under high-pressure conditions (Gharbi and Labbafi, 2018).

Li et al. (2018) treated soy-protein solutions using HPP (200-500 MPa) for 5-20 min. The results showed that the immunoreactivity of soy protein extract significantly decreased compared to the untreated samples. The highest reduction (55.5%) was observed when the samples were treated with 300 MPa for 15 min. Such reduced immunoreactivity was probably due to the alterations of secondary and/or tertiary structures of soy allergenic proteins (Rahaman et al., 2016b). Similarly, they also treated 1% soybean β-conglycinin solutions with HPP at 200-500 MPa for 5-20 min. The results indicated that the antigenicity reduction of β-conglycinin was up to 92.7%. Related analysis proved that the alteration of the antigenicity was induced by the structural modification of β-conglycinin under HPP (Xi and He, 2018). In walnut, Yang et al. (2017b) processed the walnut samples by different pressures at 250-650 MPa for 5-25 min. The results indicated the immunoreactivity of walnut to have decreased at 550 and 650 MPa, with a maximum reduction of 46.64%. In ginkgo seed, the protein extract was treated under pressure of 100-700 MPa (Zhou et al., 2016a). Protein immunoreactivity was determined by western blotting and ELISA assay, where the results found that the immunoreactivity of ginkgo seed protein was completely inhibited when the pressure reached

to 600 and 700 MPa. This reduction or inhibition in the immunoreactivity can be explained that HPP treatment leads to the structural modification in allergenic proteins, which potentially produces the destroyed or altered IgE-binding sites. These sites have no access to be recognized by IgE antibodies or to elicit immune reactions (Shriver and Yang, 2011).

However, some studies have reported that HHP may cause an increase in the immunoreactivity of related food products. Nan et al. (2018) extracted native collagen from bullfrog skin and treated with HHP at 300-500 MPa. They found the immunoreactivity of treated collagen increased significantly ($P < 0.05$), and such increases had a positive correlation with the increasing pressures. HHP might result in more exposure to collagen antigenic determinant after treatment because of the increase of the random coil region of collagen molecules (Nan et al., 2018). In milk, Meng et al. (2017) isolated and treated bovineβ-lactoglobulin (β-Lg) with HPP at 100-500 MPa, and characterized the allergenic properties of β-Lg using ELISA test. They found the IgG-binding capacities of β-Lg significantly increased. This increase in the immunoreactivity of β-Lg might be attributed to exposure of IgG linear epitopes after HHP, which resulted in the tertiary structural changes of β-Lg (Meng et al., 2017).

In most food products, HHP showed a potential application in the reduction of food immunoreactivity. Further, studies have reported that high-pressure treatment shows a better performance in maintaining the freshness of foods with a minimum impact on the essential compounds, including vitamins, flavor compounds, and pigments (Muntean et al., 2016). Thus, HPP can be used as one of the effective treatments in improving the quality and safety in the food industry in the future.

2.5.2 Pulsed light/ Pulsed UV-light

Pulsed light (PL) is formed by a sequence of extensively short and high-energy pulses of broad-spectrum white light, which is made up of ultraviolet (UV) light (54%), visible light radiation (26%) and infrared (20%) (Shriver and Yang, 2011). The wavelengths of pulsed ultraviolet (PUV) light ranging from 200 nm to 1000 nm can lead to top power pulsed light during an extremely short time (Chung et al., 2008a). Accordingly, the intensity of PUV light is thousand-

time higher than the conventional continuous UV light (Shriver and Yang, 2011). As shown in Figure 2.4 A, the electromagnetic radiation spectrum of pulsed light is made up of three portions with various light spectrums (Forney and Moraru, 2009). In the food system, PUV

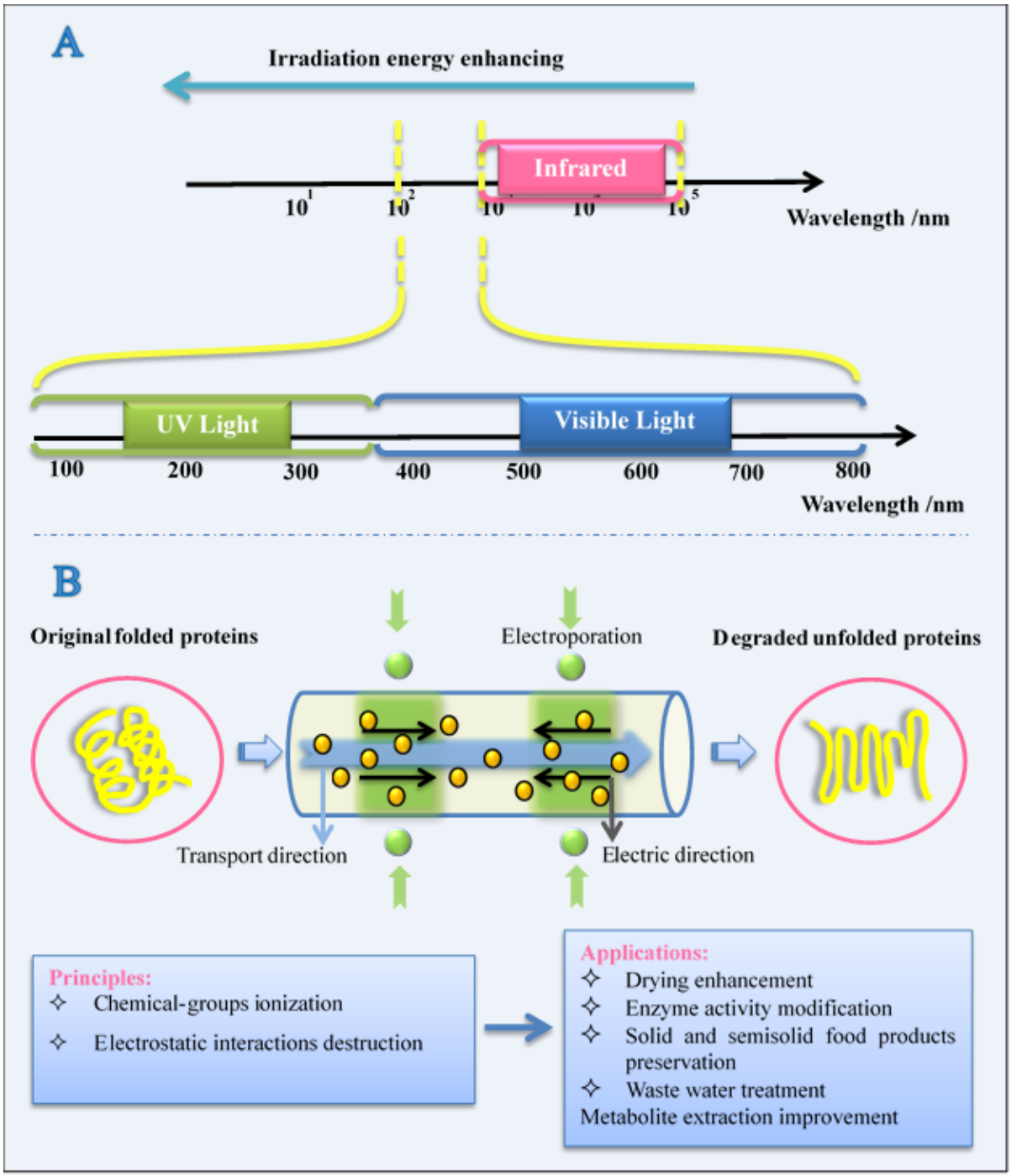

Figure 2.4. (A) Electromagnetic radiation spectrum of pulsed light; (B) The inactivation process of food allergenic proteins treated by pulsed electric field

light can be used to change the conformation of food allergens and induce protein aggregation, potentially leading to the loss of conformational epitopes (Chung et al., 2008a; Krishnamurthy et al., 2007). However, protein aggregates are also likely to be formed by the re-association of the residual peptides after treatment, which may cause more reactive conformational neo-epitopes, and it could further enhance the allergenic potential of foods (Thomas et al., 2007). The inactivation mechanism of PUV light is classified into three categories, including photochemical, photothermal, and photophysical effects, which can cause chemical conversions and protein structure alterations resulting from different heating rates and discontinuous high energy pulse disturbances (Krishnamurthy et al., 2008).

In Table 2.3, recent studies regarding the effect of UV-light on the food allergens are summarized. In soybean, Yang et al. (2010) treated soy extracts with PUV light for 2, 4, and 6 min, respectively. The results showed that PUV treatment resulted in a downward trend in the number of soy allergens (e.g., glycinin and β-conglycinin) by using SDS-PAGE and indirect ELISA analyses. IgE binding capacity of soybean allergens was decreased by 20%, 44%, and 50% when treated for 2, 4, and 6 min, respectively, compared to the untreated soy samples. This decrease may be due to the aggregation of related allergenic proteins under PUV light (Yang et al., 2010). In peanut, Yang et al. (2012a) evaluated the effect of PUV light at different distances (10.8, 14.6, and 18.2 cm) on the IgE binding of raw peanut, roasted peanut, and peanut butter extracts when treated for 1-6 min. The results found that the major peanut allergens, Ara h 1, Ara h 2, Ara h 3 (Figure 2.5 A, B, C), showed a significant reduction in the intensity of protein SDS-PAGE band with the increase of energy levels ranging from 111.6 to 223.2 J/cm^2 and processing time, while it decreased with the increase in distances away from the PUV light. The ELISA analysis reported that the IgE binding capacity of peanut extracts and peanut butter slurry decreased approximately 3 and 7 folds, respectively, compared to the untreated samples. The significant reduction in the peanut allergens is attributed probably due to the conversion of peanut protein solubility and hence the formation of precipitating compounds under PUV radiation (Yang et al., 2012a).

Table 2.3. Summary of recent studies on the effect of UV-light processing on food allergens

Food material	Target allergen	Treatment	Effect on immunoreactivity	References
Milk	α-casein, α-lactalbumin	UV-C treatment (15 min)	α-casein reduced 25%	(Tammineedi et al., 2013)
	β-lactoglobulin	UV-C treatment (15 min)	Whey fractions reduced 27.7%	(Tammineedi et al., 2013)
Shrimp	Tropomyosin	PUV sterilization (4 min)	Reduced	(Yang et al., 2012b)
Peanut	Ara h 1	PUV treatment on butter slurry (1–3 min); Raw and roasted peanuts (2–6 min)	IgE binding of peanut butter slurry reduced up to 6.7 folds; extracts reduced 12.9 folds	(Yang et al., 2012a)
	Ara h 2			(Yang et al., 2012a)
	Ara h 3			(Yang et al., 2012a)
Soy	Gly m5	PUV treatment (1–6 min)	Gly m5 reduced 100%	(Meinlschmidt et al., 2016)
	Gly m6	PUV treatment (1–6 min)	Gly m6 retained	(Meinlschmidt et al., 2016)
	Soy extracts (i.e., glycinin, β-conglycinin)	PUV treatment 2 min	Reduced by 20%	(Yang et al., 2010)
		PUV treatment 4 min	Reduced by 44%	(Yang et al., 2010)
		PUV treatment 6 min	Reduced by 50%	(Yang et al., 2010)
Egg	Ovalbumin	UV processing(10.6 kJ m^{-2})	No effect	(Manzocco et al., 2012)
	Ovomucoid	UV processing(63.7 kJ m^{-2})	No effect	(Manzocco et al., 2012)

A recent investigation demonstrated the immunoreactivity of Gly m5 and Gly m6 in soy to have reduced after treating by PUV light at distances of 8 to 10 cm to the light source for 1, 2, 4, and 6 min, respectively. The SDS-PAGE analysis showed that the gel band of Gly m5 subunits completely disappeared after 2-min PUV treatment, while the gel band of Gly m6 retained until after 6 min. The loss of bands could be attributed to protein degradation or precipitation (Meinlschmidt et al., 2016). Further, in one study, the IgE binding capacity of

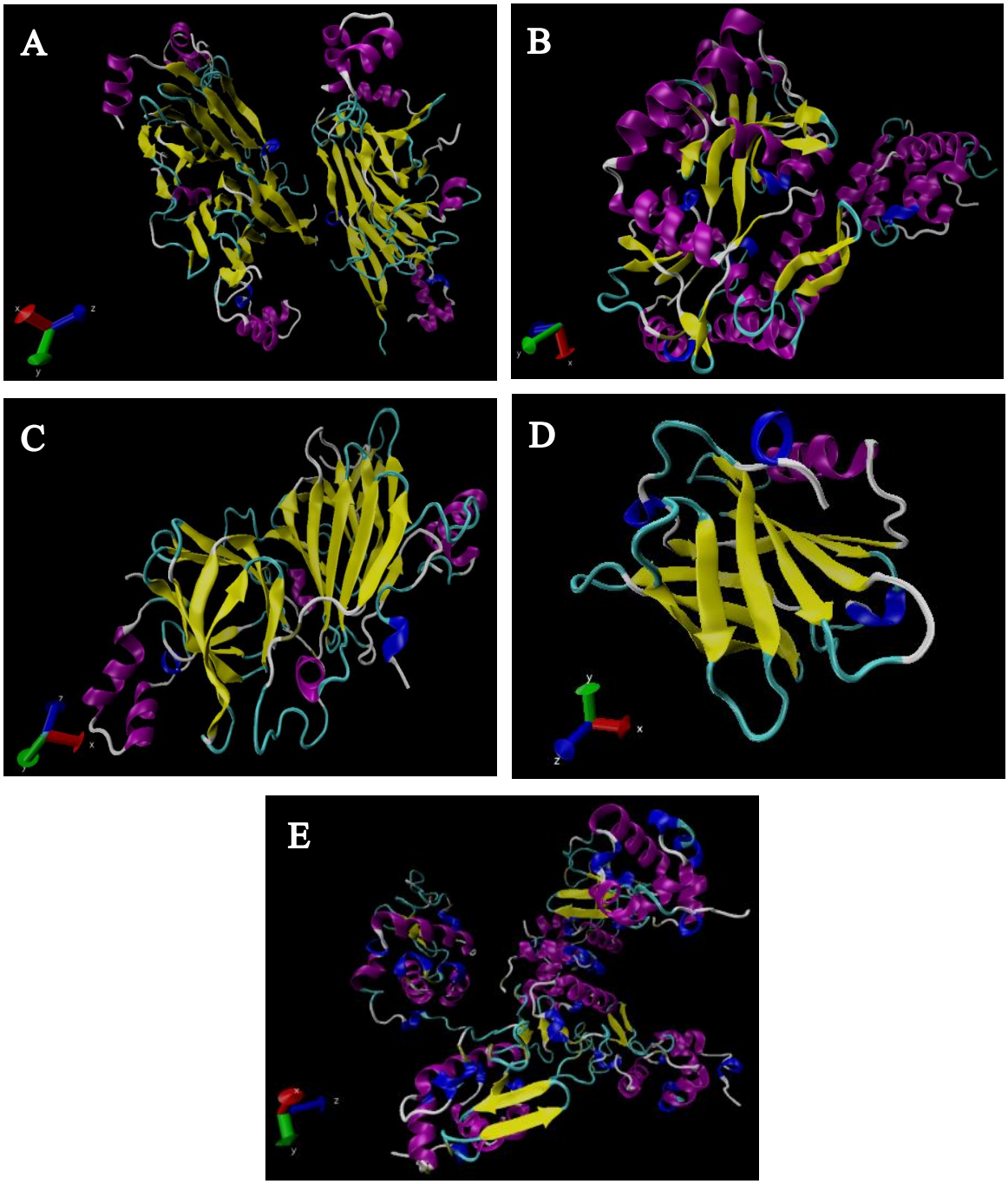

Figure 2.5. Crystal structures of peanut major allergens: Ara h 1(A), Ara h 2(B) and Ara h 3(C) and milk major allergens: β-lactoglobulin(D) and α-lactalbumin(E) (Source: protein data bank; PDB code: 3S7E, 3OB4, 3C3V, 2Q2M and 1F6S respectively) [α-helix: Purple, 3/10 helix: Blue, π-helix: Red, β-sheets: Yellow, Turns: Cyan, coils: White]

major milk allergens (α-casein, α-lactalbumin, and β-lactoglobulin) showed a significant reduction (P<0.05) after a 15-min UV-C treatment (Tammineedi et al., 2013). The

immunoreactivity reduction might have resulted from conversions in the discontinuous epitope structures of proteins, and the higher efficiency in reducing whey solution allergens by PUV light treatment may be due to the effect of higher pulses and energy (Anugu, 2009; Lee et al., 2001). In addition, Yang et al. (2012b) treated raw/ boiled shrimp extracts (5 mg/mL) by PUV light (width: 360 μs, rate: 3/s, distance from the light source: 10 cm). The results showed there was an irreversible reduction in the shrimp allergenic reactivity (Yang et al., 2012b). Thus, PUV light treatment could be considered as a potential approach to producing hypoallergenic shrimp products. The reduction of shrimp allergen was probably because some tropomyosin could be cross-linked and became some high-weight molecules under PUV light (Shriver et al., 2011).

However, Manzocco et al. (2012) reported that ultraviolet processing could cause both egg white protein aggregation and backbone cleavage, but no significant differences were found in the immunoreactivity of egg white protein compared to the untreated samples. It suggests that these modifications may not change the structure of the epitope present in egg white protein (Manzocco et al., 2012). So it can be concluded that PUV treatment could reduce allergens in soybean/soy, peanut, shrimp, milk, whereas it has no effect on allergens in eggs. Although PUV light treatment showed a potential application in the reduction of immunoreactivity of certain foods, which can be used as alternative processing technique to produce hypoallergic food products; further studies especially clinical tests and in vivo studies are in need to extend its applications in food industry in the near future (Vanga et al., 2017).

2.5.3 Pulsed electric field

Pulsed electric field (PEF) is considered as an emerging non-thermal technique in the food industry to maintain or even improve food quality. The electric field strength generally is from 0.1 to 80 kV/cm to a specific food located between or passed through two electrodes within certain nanoseconds to milliseconds (Bhat et al., 2018). The pulsed electric field is not only used to inactive enzymes and microorganisms as commonly known but also it was applied to modify food protein structure, causing the alterations of functional characteristics (Han et al., 2018). Although the exact mechanisms of PEF processing are not clear, it has been confirmed that PEF leads to the losses and

modifications of secondary and tertiary structures of proteins due to the ionization of specific chemical groups or destruction of electrostatic interactions (Wei et al., 2018; Zhao and Yang, 2008). Firstly, the specific protein molecules are polarized, which is followed by the explosion of the hydrophobic amino acids to the solvent. Eventually, unfolding proteins convert into aggregates under high-intensity electric fields (Zhao and Yang, 2009). The inactivation process of PEF applied to food allergenic proteins is shown in Figure 2.4 B (Bhat et al., 2019). PEF has attracted increasing attention and expands its applications in the food industry due to the advantages, including energy-saving mode, minimal loss of nutrients, the accessibility of liquid food systems. (Han et al., 2018).

Yang et al. (2017a) reported that high-intensity PEF treatment at 25-35 kV/cm for 60-180 μs significantly altered the conformational structures such as α-helix and immunogenic properties of ovalbumin present in egg white. Under high strength and long-time duration of PEF processing, the maximum reduction was observed after treatment at 35 kV/cm for 180 μs (Yang et al., 2017a). Therefore, PEF processing has the potential to decrease the immunoreactivity of ovalbumin. Vanga et al. (2015) evaluated the effect of the oscillating and static electric field (2450 MHz, 0.05 V/nm) at different temperatures (300, 380, 425 K) on the structure of Ara h 6 (a major peanut allergen) using the simulation of molecular dynamics. The result showed that the electric fields induced the alteration of protein structure at these three given temperature conditions, which may further impact its functional properties (Vanga et al., 2015). Sequentially, Vanga et al. (2016) applied an electric field with the intensity of 10, 15, and 20 kV for different time durations of 60, 120, and 180 min to raw peanut flour samples. They found the α-helix secondary conformational changes to have increased with time of increasing treatment, which is due to more formation of new random coils and aggregates during processing (Vanga et al., 2016). However, Johnson et al. (2010) investigated the effect of PEF (electric field strengths: 0-35 kV/cm, frequency: 2 Hz, energy input: 0-130 kJ/kg) on purified allergens including Ara h 2,6 (peanut 2S albumins) (1 mg/mL) in native peanuts, Mal d 3 (0.5 mg/mL) and Mal d 1 (0.5 mg/mL) in apples. The results demonstrated no significant changes in the secondary structures of these allergens after pulsed electric field treatment, which currently could not be accurately explained due to insufficient research analysis (Johnson et al., 2010). In celery, no obvious changes were found in the reduction of allergens after pulsed electric field

processing at 10 kV with a frequency of 50 Hz (Paschke, 2009). In comparison to thermal processing and other non-thermal treatments, the effect of PEF on the modification of related food allergens is very limited. Therefore, the optimization of processing conditions regarding PEF processing needs further studies.

2.5.4 Cold plasma

Cold plasma is considered as a novel, non-thermal technology which has been mainly used in microbial inactivation and food decontamination due to the less energy consumption and lower temperature requirements compared to the conventional processing methods (Ekezie et al., 2018). The plasma processing system is mainly made up of a high-frequency plasma generator and ceramic electrode (Figure 2.6 A) (Chizoba Ekezie et al., 2018). As the fourth state of matter, plasma is a partially ionized gas consisting of reactive species, such as ions, UV photons, electrons, free radicals, molecules, and excited atoms. These reactive species can interact with proteins and alter their conformations (Ekezie et al., 2018; Inagaki, 2014; Tolouie et al., 2017). Many studies have reported that plasma treatment showed a potential application in the reduction of food immunoreactivity.

Recently, Venkataratnam et al. (2019) treated dry, defatted peanut flour and whole peanut under cold atmospheric plasma with a voltage of 80 kV for various treatment times (0, 15, 30, 45, 60 min). The results revealed the antigenicity reduction of defatted peanut flour and a whole peanut up to 43% and 9.3% by competitive ELISA analysis; the secondary structure modifications were also observed by circular dichroism (Venkataratnam et al., 2019). In one study, tropomyosin, a significant shrimp allergen, was treated with direct dielectric discharge cold plasma for 5 min with a voltage and frequency of 30 kV and 60 Hz, respectively. The results showed that the immunoreactivity in shrimp to have reduced 76% after cold plasma treatment (Shriver, 2011). Similarly, Nooji (2011) utilized the same plasma to wheat for 5 min and then

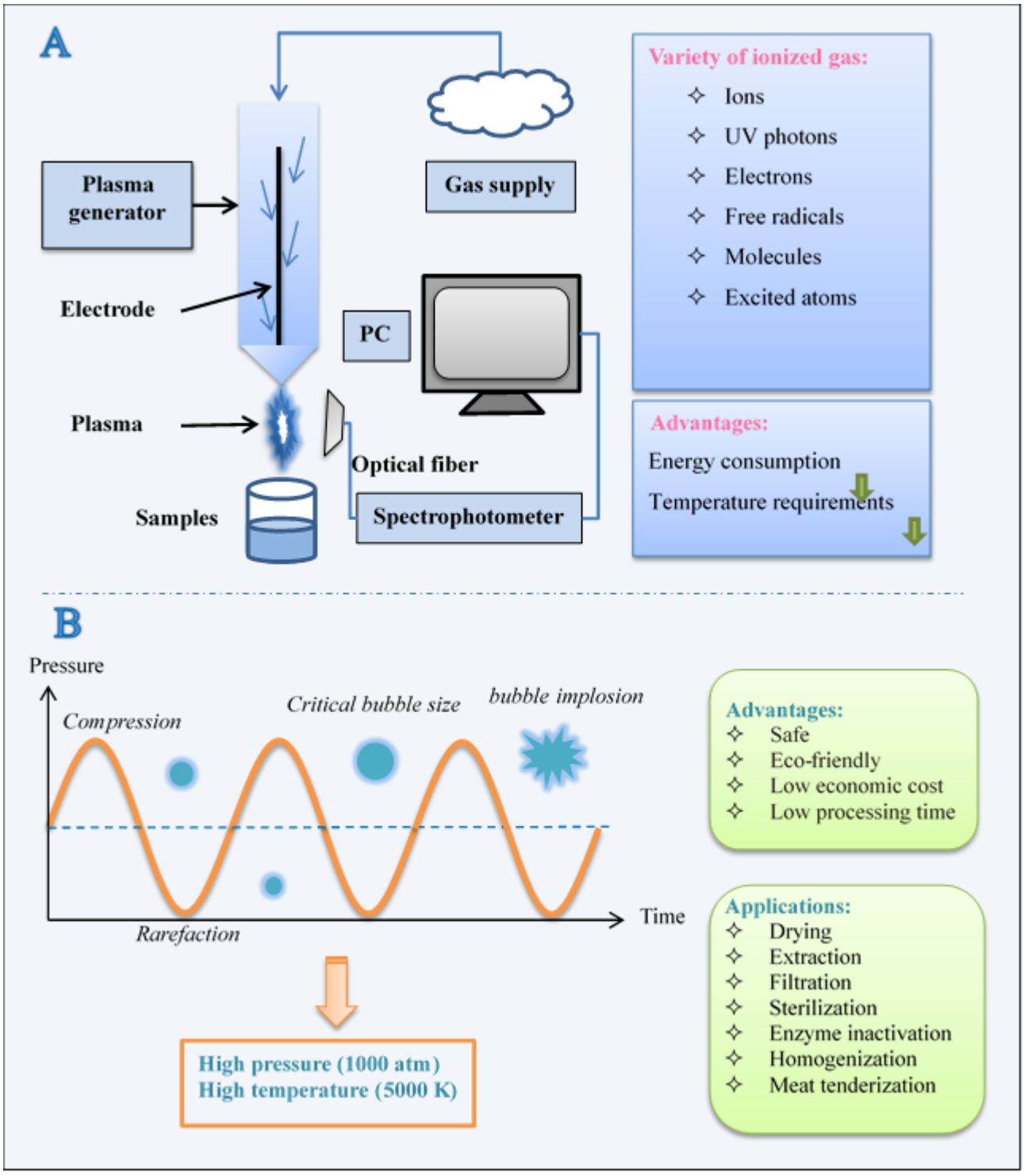

Figure 2.6. (A) Mechanisms and advantages of cold plasma processing system; (B) Mechanisms and applications of ultrasound processing system

found the reduction of immunoreactivity up to 37%. Because of the conformational binding epitope, it masked or disrupted the free radicals generated under plasma exposure, leading to

the removal of IgE-mediated reaction, further inducing the abnormal immunological response (Nooji, 2011). In another study, protein extracts from untoasted soybeans were treated by direct cold atmospheric pressure plasma (CAPP) for 0-10 min at a peak-to-peak (pp) voltage of 9, 10, and 11 kVpp and a frequency of 3.0 kHz. The result showed that the reduction of Gly m5 (the significant soy allergens) immunoreactivity under CAPP was almost completed as determined by sandwich ELISA utilizing specific mouse monoclonal anti-Gly m5 antibodies, which might be due to the conformational and linear epitope modification or antibody binding site destruction (Meinlschmidt et al., 2016).

Further, Tammineedi et al. (2013) treated α-casein of milk with cold atmospheric plasma at 13.56 MHz radiofrequency and at a rate of 30.7 L/min of argon gas for 5, 10, and 15 min, respectively. The results demonstrated that no significant changes in food immunoreactivity were observed after cold atmospheric plasma treatments, which might attribute to the low plasma power or indirect exposure of the sample to the cold atmospheric plasma system (Tammineedi et al., 2013). Therefore, the effects of cold plasma on the immunoreactivity of foods are different in various food sources, and the mechanism is not clear, which implied that more related studies are needed. Until now, it is known that cold plasma could decrease allergens in soybean, peanut, shrimp, wheat, but there was no difference in allergen amounts in treated milk found. Moreover, many studies have shown cold plasma processing to decrease the quality of food due to lipid oxidation acceleration, reduction in vitamins, and sensory attributes loss during processing (Dasan and Boyaci, 2018; Misra et al., 2011; Pankaj et al., 2018). Thus, cold plasma treatment may not be an ideal food processing method, and more novel processing techniques are to be explored.

2.5.5 Ultrasound processing

Ultrasound is specified as sound waves bearing a frequency of up to 20 kHz (Awad et al., 2012). It is considered as one of the emerging and efficient food processing technologies widely used for different applications, including homogenization, cutting, extraction, inactivation of microorganisms or enzymes, drying enhancement, surface cleaning, depolymerization, and so on (Feng et al., 2011). As shown in Figure 2.6 B, the ultrasound mechanism and cavitation

phenomenon are outlined. The sonication bubbles are formed from mechanical waves compressing, refracting intermittently, and collapsing at critical bubble sizes under the application of high energy. The implosion of bubbles results in localized high pressures up to 1000 atm and high temperatures up to 5000 K. These extreme parameters can induce the alteration of allergens' structure (Soria and Villamiel, 2010).

Table 2.4. Summary of recent studies on the effect of ultrasound processing on food allergens

Food	Target allergen	Treatment	Immunoreactivity	References
Soy	Proteins	37 kHz, 10 min	Reduced 24%	(Choudhary et al., 2013)
Milk	α-casein	500 W and 20 kHz (10–30 min)	No effect	(Tammineedi et al., 2013)
	β-lactoglobulin	500 W and 20 kHz (10–30 min)	No effect	(Choudhary et al., 2013)
	α-lactalbumin	500 W and 20 kHz (10–30 min)	No effect	(Stanic-Vucinic et al., 2012)
Peanut	Ara h1,	50 Hz for 5 h	Reduced 84.8%	(Li et al., 2013)
	Ara h2	50 Hz for 5 h	Reduced 4.88%	(Li et al., 2013)
Shrimp	Boiled shrimps proteins	30 kHz, 800 W (0–50°C, 0–30 min)	Reduced 40%-50%	(Li et al., 2011)
	Raw shrimps Proteins	30 kHz, 800 W (0–50°C, 0–30 min)	Reduced up to 8%	(Li et al., 2011)
	Tropomyosin	100–800 W, 15 min	Reduced	(Zhang et al., 2018)
	Shrimp and allergens	30 Hz, 800 W (30–180 min)	Reduced up to 75 %	(Li et al., 2006)
Crayfish	Arginine kinase	200 W, 30°C (10–180 min)	No effect	(Chen et al., 2013)

Table 2.4 summarizes the recent studies on the effect of ultrasound processing on the food allergens. In soy, Choudhary et al. (2013) treated soy proteins with high-intensity ultrasound at

37 kHz for 10 min. The results showed the immunoreactivity of soy proteins decreased by 24% compared to the untreated samples, which may be attributed to the change of secondary and tertiary conformation in soy proteins (Choudhary et al., 2013). In roasted peanut, studies have reported that the major allergen Ara h 1 and Ara h 2 content reduced by 84.8% and 4.88%, respectively, when treated with ultrasound at 50 Hz for 2 h (Li et al., 2013). The reduction in peanut allergens is due to the structural changes of IgE binding epitopes located on the α-helical regions of Ara h 1 and Ara h 2 under the high-intensity ultrasound treatment for a long time duration (Barre et al., 2007; Wu et al., 2001).

Li et al. (2006) treated shrimp and purified shrimp allergen with high-intensity ultrasound at 800 W, 30 Hz for 30-180 min. The results showed that the immunoreactivity of shrimp allergen decreased by up to 75% determined by ELISA with polyclonal antibodies. Moreover, it was observed that there was a linear relationship between protein immunoreactivity and treated time (Li et al., 2006). Similarly, Zhenxing et al. (2006) sealed peeled shrimp (*Penaeus vannamei*) muscle in a plastic bag and then treated it by ultrasound (30 Hz, 800 W) with temperatures of 0 and 50°C for 1.5 h. The results showed that shrimp immunoreactivity decreased under the treatment of high-intensity ultrasound at 50°C (Zhenxing et al., 2006). Furthermore, Li et al. (2011) treated boiled and raw shrimps using ultrasound under 30 kHz, 800 W with the temperatures of 0°C and 50°C for 0, 2, 8, 10, and 30 min, respectively. The results found that the immunoreactivity of the boiled shrimps decreased by approximately 50% and 40%, respectively, after ultrasound treatment at 0°C and 50°C for 10 min. As for the raw shrimps, the immunoreactivity of samples increased within the first 10 min treatment at 0°C and then decreased, while the immunoreactivity of samples treated at 50°C reduced slightly (8%). Thus, the ultrasound showed a noticeable effect on reducing the immunoreactivity of boiled shrimp samples compared with the raw ones (Li et al., 2011). In one study, the major shrimp allergen, tropomyosin (TM), was treated with a high-intensity ultrasound at 100-800 W for 15 min. The results based on immunoblotting and ELISA tests showed that ultrasound significantly reduced the immunoreactivity of shrimps due to the degradation of TM molecules under high-intensity treatment (Zhang et al., 2018b). However, Chen et al. (2013) treated arginine kinase (AK), a

major shellfish allergen with ultrasound (200 W) at 30°C for 10-180 min. The results found that ultrasound treatment rarely induce the degradation of crayfish AK and reduction of IgE-binding activity (Chen et al., 2013). Besides, Tammineedi et al. (2013) treated milk proteins using ultrasound treatments at 500 W power and 20 kHz frequency for 10, 20, and 30 min, respectively. No significant differences were observed in the intensity of the SDS-PAGE gel band of α-casein, β-lactoglobulin, and α-lactalbumin (Figure 2.5 D, E), which might attribute to the low intensity and short ultrasound processing time. Similar results targeting the immunoreactivity of cow's milk under ultrasound treatments were also observed in other studies (Choudhary et al., 2013; Stanic-Vucinic et al., 2012; Tammineedi et al., 2013).

Ultrasound processing gained significant attention due to its numerous advantages such as environmental-friendliness, less energy consumption, higher mass transfer rate, and original flavor maintenance of food products during processing (Wang et al., 2019). Moreover, ultrasound processing shows a potential application in the reduction of immunoreactivity in soy, peanut, shellfish, which can be used as alternative non-thermal processing in the future. It is also noticed that ultrasound treatments seem to be more efficient when combined with heat, instead of being used solely. Thus, ultrasound can be considered as an alternative non-thermal processing in the reduction of food immunoreactivity, whereas the optimization of processing conditions is needed (e.g., time duration, frequency, and temperature).

2.5.6 Fermentation treatment

Fermentation, as one of the oldest techniques in the food industry, is still very popular today because the amount of daily consumed foods are fermented. The main examples are certain protein-abundant foods, such as dairy foods (yogurts, cheeses), alcoholic drinks (wine, beer), fermented vegetables (sauerkraut and pickles), and fermented meats (sausages and salami) (Soccol et al., 2013). Fermentation processing is induced by specific microorganisms acting on food substrates. One of the most common examples is that sugars could be converted to alcohol, carbon dioxide, and organic acids under anaerobic conditions. In this process, microorganism strains and related conditions (e.g., temperature, pH, and substrate concentration) significantly affect the specific products liberated (Pérez Díaz et al., 2017). Other than the applications of fermented food

products, recent studies have found that the linear and conformational epitopes of allergenic proteins can be modified and destroyed by hydrolysis during fermentation.

Yang et al. (2018) investigated the impacts of solid-state fermentation by mixing yeast, *Lactobacillus casei* and *Bacillus subtilis* on the immunoreactivity of soybean meal (SBM). The results found that the IgE binding capacity of the SBM was significantly reduced after fermentation. This reduction might be due to the degradation of protein into peptides with low molecular weights, which results in the damage of some allergenic sequences of β-conglycinin and glycinin present in soybeans. Similar results have been observed by Chung et al. (2008b), the lower immunoreactivity of soybeans was found in fermented soybeans by *Bacillus subtilis* KFCC 11293 and *Lactococcus lactis subspcould* compared to the untreated samples (Chung et al., 2008b). Hence, fermentation is effective in the reduction of soybean allergens (Yang et al., 2018). Moreover, mixed cultures may have a more significant effect on such processing. In a study, lower immunoreactivity was observed when wheat flour was fermented by cultured-yeast mixtures, compared with by the homo-hetero fermentative lactic acid bacteria. The results might be due to the performance of mixed cultures with strong proteolysis capabilities to induce their broad specificity (De Angelis et al., 2006; Leszczyńska et al., 2009). In milk, Yao et al. (2014) studied the IgE-binding capacity changes of four proteins including α-lactalbumin (LA), β-lactoglobulin (LG), α-caseins (CN), and β-C fermented by *Lactobacillus rhamnosus* GG. The results indicated the IgE-binding capacity of four proteins in fermented milk to have been significantly reduced by up to 70% compared to the untreated milk, which might be due to the alteration of allergens or the cleaving of allergenic protein epitopes (Yao et al., 2014). In addition, Kim et al. (2008) performed a study using a competitive indirect enzyme-linked immunosorbent assay (Ci-ELISA) to explore the immunoreactivity changes in saeujeot (a variety of shrimp salted and fermented). The saeujeots were fermented with different salt concentrations (25, 15, and 10%) at various temperatures (25, 15, and 5 °C). They found that the IgE-binding capacity of shrimps was significantly decreased when fermented with a low salt concentration at high temperatures. These significant reductions in the immunoreactivity of fermented foods may be due to the hydrolysis and degradation by certain microbes, which in turn can lead to modification or destruction of sequential and conformational epitopes of food proteins

and cause the further reduction of food immunoreactivity (Zhou et al., 2016b). In conclusion, the significant reduction can be observed in allergens from soybean, wheat, shrimp, and even milk by fermentation treatment. Therefore, fermentation, especially long-time fermentation, shows a potential application in reducing food immunoreactivity.

2.5.7 Enzymatic hydrolysis

Enzymatic hydrolysis treatment has obtained significant attention due to its efficient, selective, reproducible, and environmental-friendly characteristics, and is regarded as a promising technique to produce hypoallergenic foods (Zhang et al., 2018a). In the food processing industry, there are more than 55 enzyme varieties applied during the processing of related products (van Oort, 2009). Many studies have reported that enzymatic hydrolysis treatment shows a potential reduction in the immunoreactivity of the proteins or their extracts by changing the structure of allergic proteins. The mechanism of this technique is to modify or destroy the linear epitopes of allergens by proteolytic or cellular enzymatic hydrolysis. During processing, the structures of the proteins are broken down into peptides and/or amino acids (Ekezie et al., 2018; Kasera et al., 2015).

Enzymatic hydrolysis processing technique has been used to produce easy-digested food for infants for over 60 years (Knights, 1985). For instance, proteases are often applied to produce hydrolyzed formula milk by hydrolyzing proteins in milk, which is beneficial to improve the digestibility of milk powder (Fleischer et al., 2016). Further, many studies have found that enzyme treatment can reduce the allergic risk of food. Yu et al. (2011) reported that enzymatic treatment could significantly reduce (up to 100%) the amount of two major allergens (Ara h 1 and Ara h 2) in roasted peanut kernels. Specifically, the roasted peanut kernels were treated with a-chymotrypsin and trypsin for 1-3 h; the results showed that Ara h 1 and Ara h 2 were hardly determined by ELISA test (Yu et al., 2011). Similarly, Yu et al. (2013) treated peanut butter samples with trypsin, alpha-chymotrypsin, or the combination of these two enzymes at 37°C for 3 h or at room temperature for 24 h. The results demonstrated that the immunoreactivity of Ara h 1 and Ara h 2 reduced by 100% under the combination of alpha-chymotrypsin and trypsin (1: 1) at 0.04% of enzyme-to-peanut butter ratio. At the same total

enzyme concentration, the efficiency of Ara h 1 and Ara h 2 under the combination of two enzymes was decreasing, and it was higher than trypsin treatment individually, followed by alpha-chymotrypsin treatment individually (Yu et al., 2013). Beta-lactoglobulin (β-Lg) is a principal component in milk. Duan et al. (2014) divided 24 BALB/c mice into three groups and injected them subcutaneously with native bovine β-Lg and its hydrolysates on days 0, 7, and 14, respectively. The results suggested that the spleen lymphocyte proliferation level of the sensitized mice induced by β-Lg hydrolysates was lower than that of the intact one, which implied enzymatic hydrolysis treatment could reduce the immunoreactivity of β-Lg. The reason for this outcome might be the three-dimensional structure of β-Lg to have been destroyed by the hydrolysis processing, accompanying specific epitopes removed subsequently (Duan et al., 2014). Likewise, Kulis et al. (2012) performed an in vivo test in mice, and the allergenic activity of cashew proteins decreased by pepsin digestion (Kulis et al., 2012). In wheat flour, studies found that the immunoreactivity of gliadin was eliminated when treated with sequential *alcalase-papain* hydrolysis at 53°C, pH of 8.0 for 62 min (Li et al. (2016). Further, Kasera et al. (2015) investigated that protein extracts from legumes-kidney beans, peanut, and black gram were hydrolyzed by *flavourzyme* and *alcalase*. The results showed that the immunoreactivity of hydrolysates to have been significantly reduced compared to the untreated one (Kasera et al., 2015).

However, enzymatic hydrolysis treatments have conflicting results when evaluating their effects on the immunoreactivity of plant foods, since sometimes it contributes to increasing its immunoreactivity. In lentil protein extracts, studies found that the allergic proteins residual still can be detected using IgE immunoblotting and ELISA test when hydrolyzed with *flavourzyme* and *alcalase* (Cabanillas et al., 2010). Palosuo et al. (2003) applied pepsin and trypsin to wheat and found the IgE-binding ability of the ω-5 gliadin (a major allergen in wheat) was stable after digestion. It was probably because hydrolyzed peptides are cross-linked by tissue transglutaminase (tTG) to large allergen complexes, which may lead to sensitized individuals suffering in anaphylactic reactions (Palosuo et al., 2003). Kiyota et al. (2017) observed the reactivity of tomato allergen profilin (Sola I 1) after papain treatment by using competitive

indirect ELISA; the results showed that Sola I 1 reactivity was retained after treatment (Kiyota et al., 2017). Panda et al. (2015) applied four enzymes (alcalase, trypsin, chymotrypsin, bromelain, or papain) separately to soybean protein isolates by IgE immunoblot evaluation using the sera from eight soybean-allergic patients. The results indicated the enzymatic hydrolysis process had no effect on the immunoreactivity reduction, and chymotrypsin or bromelain have the potential to enhance soybean immunoreactivity (Panda et al., 2015). Thus, enzymatic hydrolysis treatment can effectively lower the allergens in food products, including beans, wheat, cashew, especially peanut, which could be mitigated completely. However, enzyme treatment has high requirements for the specificity of different proteins, so there are limitations for enzyme application in the reduction of food immunoreactivity.

2.5.8 Combination of processing techniques

As discussed above, the application of a single processing technique may not show a significant effect on the reduction in the immunoreactivity of certain foods. The combinations between various processing technologies provide a new strategy for reducing the immunoreactivity of food (Ekezie et al., 2018). Studies reported that no significant differences in peanut structure were observed after high-pressure treatment at room temperature or even at 80°C (Van der Plancken et al., 2012). However, the combination of high pressure and enzymatic treatment could easily lose the protein structure of peanut allergens, and such conformational changes of peanut allergens may further induce their property modifications (Dong et al., 2011). Chung et al. (2013) found the combination of high pressure (500 Mpa) and polyphenol oxidase significantly to have significantly reduced the content of major peanut allergens and the IgE binding capacity by two times compared to either polyphenol oxidase or high-pressure treatment alone. The reduction of allergenic capacity of peanut might be because high pressure can enhance the susceptibility of proteins/allergens in enzyme hydrolysis (Chung et al., 2013). In soybean, a similar study found that high pressure associated with enzymatic hydrolysis treatment (flavourzyme) could efficiently reduce the immunoreactivity of soy proteins. The hydrolysis processing was accelerated by the combination with high pressure, which in turn attributed to modifying the epitopes of allergens (Meinlschmidt et al., 2017).

The combination of high pressure and the high temperature was also applied to reduce the allergenic potential of food products. In milk, Kleber et al. (2007) found the antigenicity of β-lg to have been shown a downward trend under combination treatment of high pressure (200-600 Mpa) and high temperature (60-68°C), while no significant differences were observed under high pressure or thermal treatment (Kleber et al., 2007). In whey protein, the combination of microwave irradiation (MWI) and chymotrypsin hydrolysis improved the enzymatic hydrolysis of whey protein and reduced the residual antigenicity of the hydrolysates, which might be due to the conformation rearrangements of proteins induced by MWI treatment (Izquierdo et al., 2008; Porcelli et al., 1997).

Additionally, studies have reported that ultrasound combined with enzymatic hydrolysis or germination treatment showed a potential application in the reduction of food immunoreactivity. In peanut, Li et al. (2013) treated roasted peanut with ultrasound (0-2.0 h) and enzyme treatment (1-9 h with α-chymotrypsin or trypsin). The results found that ultrasound-enzymatic treatment not only significantly raised the amount of soluble peanut protein and lowered IgE binding of the peanut extracts but also eliminated two main allergens (Ara h 1, Ara h 2) in peanuts. This reduction in peanut immunoreactivity may be due to ultrasound-enzyme treatment to have cleaved the roasted peanut protein sequences at some epitopes. Similarly, Yang et al. (2015) treated soybean seeds with ultrasound treatment at 0-300 W, and then germinate them for 5 days. The results showed IgE-binding capacity of soybean protein under ultrasound-germination treatment significantly decreased by 51.39% compared to the untreated samples, which might be due to the disruption or elimination of epitopes in proteins during the ultrasound and germination treatment. Likewise, Yu et al. (2016a) germinated peanut kernel for three days and then treated them with high-intensity ultrasound at a frequency of 100 kHz. The results showed that allergenic proteins were degraded completely (Yu et al., 2016a).

It can be seen that the immunoreactivity of milk is more difficult to be reduced than that of other food allergens like soybean, peanut, and wheat. The combined treatment of high pressure and high temperature may not perform as effectively as the combination of high pressure and enzymatic hydrolysis. Besides, both enzymes combined ultrasound and enzyme combined high

pressure show a potential effect in reducing food immunoreactivity. Therefore, the combination treatment generally shows a better performance in reducing food immunoreactivity compared to the single processing technique.

2.6 Conclusion and future trends

Food allergy as a global health issue is expected to be addressed by using some innovative non-thermal processing techniques, such as high-pressure processing, pulsed light, cold plasma, ultrasound, fermentation, pulsed electric field, enzymatic hydrolysis treatment, and combination of them. As discussed above, these novel processing techniques showed potential applications in the reduction of food allergens to produce hypoallergenic foods for individuals living with food allergies. As discussed above, ultrasound processing could be considered as alternative non-thermal processing in the reduction of food immunoreactivity due to its lower energy consumption, higher nutritional compounds maintenance compared to other treatments. Indeed, multiple food allergens normally can be reduced by altering sequential or conformational epitopes and even eliminating them at certain optimal conditions. Thus, in order to improve the efficiency in the reduction of food immunoreactivity, the optimization of processing conditions (e.g., duration, energy, frequency, and temperature) in ultrasound treatment is needed. Further, the immune responses of individuals with food allergy history to these processed or modified foods are unknown, and further clinical studies are still in need. To date, the best therapeutic strategy suggested by the doctors is to avoid exposure to food allergens or related food sources.

CONNECTING TEXT

In the review reported in Chapter II, we have seen the recent advances of novel non-thermal processing techniques including high-pressure processing, ultrasound, pulsed light, cold plasma, fermentation, pulsed electric field, enzymatic hydrolysis, and combination processing. Further, general information on global food allergy prevalence, food allergy pathology, and biochemical characterizations of the main allergens was also described. In the next chapter, we will focus on the epidemiology worldwide and the main allergenic properties of shrimp allergy and its evaluation when subjected to several novel processing methods to lower the allergenicity of shrimps.

CHAPTER III

LITERATURE REVIEW II

Recent Advances of Novel Techniques on Modification of Shrimp Allergens: A review

3.1 Abstract

Shrimp is a common species of shellfish with abundant nutrients, such as protein, amino acids, minerals, unsaturated fatty acids, vitamins, astaxanthin, and antioxidants. However, as one of "big eight" allergic foods, it can lead to a series of allergic reactions, including mild to life-threatening anaphylaxis. Tropomyosin is identified as the major allergen in shrimp, and the structures of shrimp allergens may be altered by thermal or non-thermal treatments. Compared with conventional treatments (e.g. heating, steaming), novel treatments have superior effects and maintain the original characteristics of shrimp. This review discusses the epidemiology worldwide and main allergenic properties of shrimp allergy and evaluates several novel processing methods to lower the allergenicity of shrimps, including microwave, ultrasound, pulsed light, cold plasma, fermentation, enzymatic hydrolysis, and their combination.

Keywords: Novel processing techniques; shrimp allergy; thermal processing; non-thermal processing

3.2 Introduction

Seafood products play a significant role in the food industry and used for increasing human consumption worldwide (Arruda and immunology, 2013). Shellfish constitutes a diverse group of species subdivided into more than 50,000 crustacean species and 100,000 mollusk species (Pedrosa et al., 2015). Shrimp is the most valuable species in crustaceans belonging to the order *Decapoda* and suborder *Dendrobranchiata* (Martin and Davis, 2001). Whiteleg shrimp (*Litopenaeus vannamei*) is the most widely cultured shrimp species all over the world, and the consumption are over 900,000 tons annually (Liu et al., 2010b).

Shrimps are popular in the human diet due to its high nutritive value and tasty flavor. Shrimp body (% in wet weight) is made up of moisture (75.66%–77.01%), crude protein (18.89%–20.06%), crude lipids (0.74%–0.84%), and ash (1.58%–1.67%). Shrimp is identified as a rich source of proteins, with low lipids and caloric levels (Pires et al., 2018). Shrimp is also abundant in vitamin B12, calcium, selenium, and astaxanthin, which is often used as a natural additive due to the resistance to oxidation (Venugopal, 2008). Shrimps contain a large amount of polyunsaturated fatty acids, such as eicosapentaenoic (C20:5n3, EPA) and docosahexaenoic (C22:6n3, DHA) acids, which play predominant roles in human health (Bono et al., 2012). In human diets, fresh shrimps can be consumed directly or by cooking with the sauce. With the beneficial properties, shrimps are popular in pasta, salads, soups, curry and stir-fried dishes (Dayal et al., 2013).

However, the increasing consumer demand for shrimps results in more reporting of shrimp hypersensitivity reactions, such as certain immediate symptoms involving the skin, gastrointestinal, respiratory, and cardiovascular systems (Jeebhay et al., 2001; Lopata and Potter, 2000; Samson et al., 2004). As one of the most frequent causes of food allergy worldwide, shrimp allergy is and belongs to the shellfish allergy, a kind of "The Big 8" food allergy, which is one of the leading allergenic food in adults whereas often does not outgrow during childhood (Lopata et al., 2016). Tropomyosin is a muscle protein and acts as the main shrimp allergen. A study revealed that children with shrimp allergy have higher specific IgE antibody levels, show more intense binding to shrimp peptides, and a greater epitope diversity

than in adults, suggesting that sensitization to shrimp might decrease by age (Ayuso et al., 2010). Liu et al. (2010a) also found that the estimated prevalence of clinical shrimp allergy varied by age but was 1% overall (Liu et al., 2010a). Moreover, it is a prevalent, long-lasting disorder usually persisting throughout life, since few options are available for treatment, avoidance is the only therapy recommended (Ayuso et al., 2008). In this review, we outline the global prevalence of shrimp allergy, characteristics of the shrimp allergens, and the novel processing methods to modify their functional structures, which may be helpful to related studies and food industry.

3.3 Global shrimp consumption and prevalence of shrimp allergy

Shrimp farming has expanded significantly since the 1970s all over the world. Global production by shrimp farming increased from not more than 9000 metric tons in 1970 to about 1 million metric tons in 2000, which is equivalent to almost one-third of total world shrimp supply (Biao et al., 2007). Asia, accounting for around 80% of world shrimp culture production, plays a leading role in shrimp farming (Fuchs et al., 1999). The main regions of increasing shrimp production by aquaculture are in particular some areas of China, Ecuador, Mozambique, Southeast Asia (especially, in countries such as Thailand), in addition to catches in different areas around the world, mainly Food and Agriculture Organization of the United Nations (FAO) classified Areas which include Southern-Eastern Pacific and Western Central Pacific (Ortea et al., 2012). Accordingly, shrimp consumption has increased in many countries over the past decades. In 2009, Americans' consumption of fish and shellfish reached an average of 15.8 pounds per capita, with shrimp consumption being the top choice at 4.1 pounds (Woo et al., 2011). As shrimp has become a more and more accessible food, the number of reports of allergic reactions in both consumers and seafood processors has increased (Lopata et al., 2010b). One of the most widely consumed shrimp species is *Solenocera melantho* (red shrimp), and only one study shows the relevance of *S. melantho* tropomyosin as an vital allergen (Gámez et al., 2011).

However, shrimp is the most frequent offender in children and adults allergenicity, and it is often associated with anaphylaxis from mild to life-threatening (Bock et al., 2007; Sicherer et

al., 2004). It has been reported that the common symptoms triggered by shrimp allergy generally include urticaria, angioedema, gastrointestinal, pulmonary, systemic anaphylaxis, generalized pruritus, and other symptoms (Daul et al., 1987). In North America, a Canadian survey targeted at 10,596 households in 2008 and 2009, which estimated the prevalence of perceived shellfish allergy at 1.60% and the prevalence of probable allergy was 1.42%, whereas the data of confirmed allergy prevalence was much lower than the actual situation due to the discontinuous tests and the difficulty in acquiring individual's data (Ben-Shoshan et al., 2010). In particular, the prevalence of shellfish allergy in the US is approximately 1.3% in which shrimp was most commonly reported, with the lower rate of anaphylaxis to shrimp in children than in adults (Chokshi et al., 2015). Sixty percent of shrimp allergies are found in adults (Food Allergy Research and Education), of which 2% of the United States population is affected.

In European countries, Castillo et al. (1996) studied 142 food-sensitized patients from Gran Cranaria, Spain. Of these individuals, 120 reported clinical symptoms following ingestion of one or more foods; and shrimp was the most common allergenic food with 48 patients suffering. In Norway, an investigation of 217 adults who thought they had food allergy intolerance had breathlessness as a symptom reported by 7.3% of them suffered from shrimp and crab allergy (Woods et al., 2001). In Germany, it was found that 76 of 89 patients (85.4%) allergic to Atlantic shrimp, which played an increasing role in leading to allergic asthma (Crespo et al., 1995).

In Asia, a questionnaire survey conducted towards 546 children aged 3–7 years living in Chiang Mai, Thailand, with 452 questionnaires (82.8%) returned demonstrated shrimp was the most common cause of current food allergy (33.3%) (Lao‐araya and Trakultivakorn, 2012). Similarly in Malaysia, a study involving 148 adults with symptoms of nasal congestion and rhinorrhea and 113 adult Malaysian control subjects without rhinitis symptoms also found the most commonly implicated foods were shrimp (48%) when the participants were exposed to 11 common foods in Malaysian diet (Gendeh et al., 2000). In Japan, a study examined 2053 Japanese university healthy students and found the shrimp allergy proportion to be 9.4%, which is only lower than the egg allergy with the highest proportion of 19.4% (Yoneyama and Ono,

2002). In China, 763 patients with bronchial asthma in Guangzhou were examined with skin test and found 70.3% of patients among them suffered by shrimp allergy (Lai et al., 1985). In Taiwan, a survey of 1,070 cases of bronchial asthma, age from 3 to 70 years participants, was done. The results showed there were 34.9% of patients allergic to shrimp; the age between 11-20 years old showed the highest incidence rate of shrimp allergy (Lai et al., 1985).

As mentioned above, shrimp allergy has become a global issue related to human health. It is urgent to provide some novel efficient approaches for the management of shrimp allergy. Until now, the processing techniques based on thermal or non-thermal treatments showed a potential strategy in reducing shrimp allergens by converting their original protein structures. In this review, we describe the allergen varieties, properties, sources, and further include the recent advances of novel processing techniques on the modification of shrimp allergens, including thermal processing (microwave treatment), non-thermal processing (e.g., pulsed light, ultrasound, cold plasma, and fermentation), chemical processing (enzymatic hydrolysis treatment), and the combination processing of them. Hopefully, it can clarify the mechanisms of these processing methods and provide strategies to reduce shrimp allergens.

3.4 Properties of main allergens

The common shrimp species include North Sea shrimp (*Crangon crangon*), Brown shrimp (*Penaeus aztecus*), Greasy back shrimp (*Metapenaeus ensis*), Sakura shrimp (*Sergia lucens*), Kuruma shrimp (*Marsupenaeus japonica*), Juvenile white shrimp (*Litopenaeus vannamei*), Pacific white shrimp (*Litopenaeus vannamei*), Indian prawn shrimp (*Fenneropenaeus indicus*), Speckled shrimp (*Metapenaeus monoceros*), Tiger shrimp (*Penaeus monodon*), etc. (Ayisi et al., 2017; Faisal et al., 2019).

Shrimp species have been the most studied in terms of allergen characterization, particularly the black tiger shrimp (*Penaeus monodon*) and the white leg pacific shrimp (*Litopenaeus vannamei*) which are respectively the most and second-most widely cultured shrimp species in the world (Guo et al., 2019; Landsman et al., 2019). Tropomyosin (TM) as the main shrimp allergen was the first allergen identified in shellfish based on molecular characterization and is

associated with actin filament of muscle cells and in some non-contractile cells (Wai et al., 2014). TM was first described as a crustacean allergen in shrimp in 1981 (Hoffman et al., 1981). In 1993, Shanti et al. (1993) noted the presence of TM as a soluble allergen in the heat-stable fraction that dominated the allergenicity of the extract (Shanti et al., 1993). TM is a major shrimp allergen and accounts for 6% of total shrimp proteins (Ahmed et al., 2018). Tropomyosin has specific allergenic regions called epitopes that are bound by Immunoglobulin E (IgE) antibodies, which result in allergenic reactions (Silke, 2017). The highly conserved amino acid sequence is present in tropomyosin in invertebrate organisms, and up to 8 IgE-binding regions in shrimps are present in muscle and non-muscle cells. There is a slightly acidic isoelectric point in tropomyosin, which has minor glycan modifications and is water-soluble (Lopata et al., 2010a). TM has a coiled-coiled secondary structure, with the isoelectric point and molecular weight (MW) of 4.5 and 36 kDa, respectively (Haddad et al., 1979). It consists of 286 amino acids and is abundant in glutamic acid (Glu), leucine (Leu), alanine (Ala), lysine (Lys), and arginine (Arg) residues. The most plentiful amino acids present in TM epitopes are glutamic acid (Glu), tyrosine (Tyr), arginine (Arg), phenylalanine (Phe), and serine (Ser) (Leung et al., 2014). The IgE-binding peptide sites varied in length from 8 to 15 amino acids long, depending on the region and the subject studied (Lehrer et al., 2003).

In continuation, other allergens have been characterized subsequently, such as arginine kinase (AK), myosin light chain (MLC), and a calcium-binding sarcoplasmic protein (SCP) (Pedrosa et al., 2015), and hemocyanin (Ayuso et al., 2011; Giuffrida et al., 2014; Piboonpocanun et al., 2011), troponin C (Ayuso et al., 2011; Bauermeister et al., 2011), triosephosphate isomerase (Bauermeister et al., 2011), and certain novel potential allergens identified including fatty-acid-binding protein (FABP) (Ayuso et al., 2011), alpha-actinin, beta-actin, and ubiquitin (Gámez et al., 2014). It has been identified that there are eleven main allergens in shrimps, and their molecular weights are shown in Table 3.1.

To date, the muscle protein tropomyosin (Lit v 1) was the only major cross-reactive allergen identified in different shrimp species (Daul et al., 1994). Arginine kinase (Pen m 2, Lit v 2), a minor shrimp allergen, has been described in Pacific white shrimp and Black tiger prawn,

respectively (García-Orozco et al., 2007; Yu et al., 2003). It was identified that MLC is a new

Table 3.1. Main allergen molecular weights in shrimp

Allergen	Molecular weight	References
Tropomyosin (TM)	36 kDa	(Wai et al., 2014)
arginine kinase (AK)	40-kDa	(García-Orozco et al., 2007)
myosin light chain (MLC)	20 kDa	(Ayuso et al., 2008)
calcium-binding sarcoplasmic protein (SCP)	20 kDa	(Shiomi et al., 2008)
hemocyanin	75 kDa	(Piboonpocanun et al., 2011)
troponin C	16.8 kDa	(Bauermeister et al., 2011)
triosephosphate isomerase	27 kDa	(Bauermeister et al., 2011)
fatty-acid-binding protein (FABP)	43 kDa	(Gámez et al., 2014)
alpha-actinin	94–99 kDa	(Gámez et al., 2014)
beta-actin	46 kDa	(Gámez et al., 2014)
Ubiquitin	6-7 kDa	(Gámez et al., 2014)

major shrimp allergen, Lit v 3 (Ayuso et al., 2008). Further, Shiomi et al. (2008) observed a 20 kDa allergen by purifying black tiger shrimp and identified it as a sarcoplasmic calcium-binding protein (SCP), Lit v 4.0101, based on the determined amino acid sequences of 2 enzymatic fragments (Shiomi et al., 2008). It was reported that AK and the SCP are minor allergens sensitizing only 10%-15% of Italian shrimp-allergic patients, but they are clinically relevant (Giuffrida et al., 2014). Moreover, the new allergen hemocyanin with high molecular weight has been identified in shrimp-allergic patients. It was shown that hemocyanin is a

clinically relevant shrimp allergen (associated with systemic reactions to shrimp with anaphylaxis) and possibly cross-reacting to house dust mites (Asero et al., 2012; Giuffrida et al., 2014). The shrimp varieties have different main allergens, as shown in Figure 3.1, which is the summary of allergic proteins characterized in various shrimp species (Daul et al., 1994; Leung et al., 1994; Shanti et al., 1993; Woo et al., 2011).

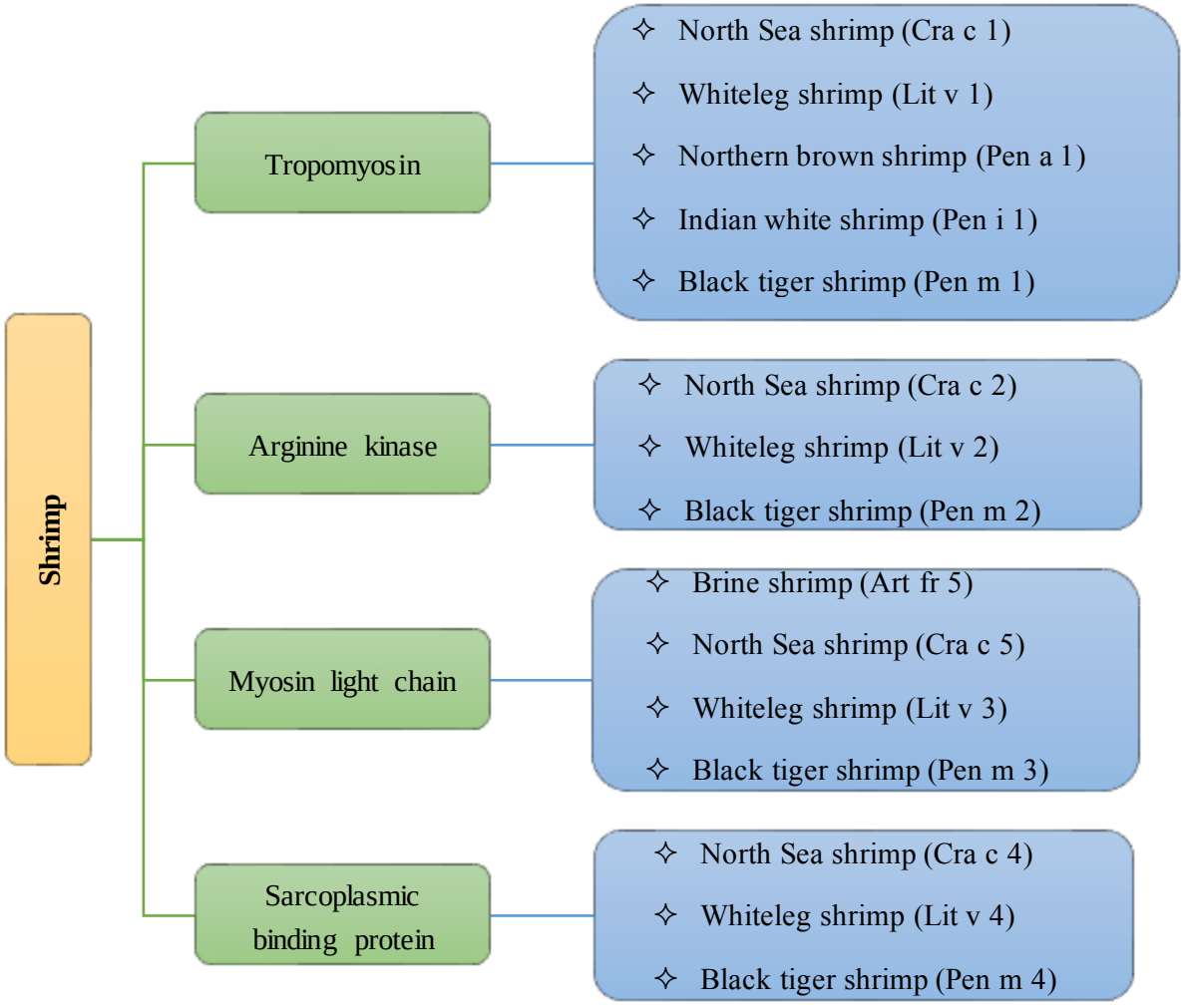

Figure 3.1. Allergenic proteins characterized in various shrimp species

3.5 Effect of novel techniques on shrimp allergenicity

3.5.1 Thermal processing

Thermal processing is commonly seen in the food processing industry, such as cooking, boiling, baking, roasting, frying, grilling, pasteurization, and sterilization. Regarding shrimp allergens,

specific thermal techniques have been applied for attenuation or modification of them by denaturation and interaction reactions (Ekezie et al., 2018b). Allergenic proteins can be modified by interactions with food ingredients under thermal conditions during the Maillard reaction (Renzone et al., 2015). For example, it has been found that Maillard modification can enhance the IgE-binding capacity of tropomyosin in allergenic shellfish (Nakamura et al., 2005). Several studies showed some food allergens as sensitive to heat, which helps in the alteration of their allergenicity. For example, a major peanut allergen, Ara h 1 showed a significant decrease after thermal treatment compared to Ara h 2 peanut allergen, which is attributable to a higher degree of denaturation and/or aggregation of Ara h 1 during high-temperature processing (Montserrat et al., 2015). A similar result was also observed in walnuts when treated with heating; the allergenicity of walnuts significantly decreased using ELISA and in vivo test, which could be resulted from the loss or the denaturation of protein under high temperatures (Vencia et al., 2018). Whereas, the major shrimp allergen tropomyosin is stable towards heat, and it can maintain their original properties even at 100 °C for 20 min due to their stable and integral α-helical coiled-coil dimeric conformation (Kamath et al., 2013). Although these traditional thermal processing techniques showed a potential application in reducing the allergenicity of some food sources; however, to date, few studies further demonstrated the degradation of tropomyosin under certain thermal processing conditions. The limitation of thermal methods also involves organoleptic properties and nutrient compositions of food material, which are easily affected under such high temperatures with a longer residue time (Maeno et al., 2013; Ozawa et al., 2011). Therefore, novel thermal processing techniques are to be explored.

3.5.1.1 Microwave treatment

Microwaves can produce electromagnetic waves bearing frequency in the range of within 300 MHz-300 GHz (Chandrasekaran et al., 2013). Studies have reported that the most common microwave frequencies used in food applications are 2450 ± 50 and 915 ± 25 MHz (Coronel et al., 2008). Microwave heating has been widely applied in the food processing industry, including drying, pasteurization, sterilization, thawing, and baking of food materials (Gupta

and Leong, 2008). As shown in Figure 3.2, dipole rotation and ionic polarization are two important factors involved during microwave heating. For the principle of dipole rotation, the presence of moisture leads to dielectric heating because of the dipolar nature of water. The permanently polarized dipolar molecules try to align themselves to the orientation of the electric field appropriately with an oscillating electric field incident on the water molecules. Under the influence of the high-frequency electric field, the internal friction produced from the interactions between the fast-rotating polar molecules and another molecule in food, which occurs at a million times per second, resulting in the heating of the food material eventually. Since the ions crash with each other in such high-frequency electric field, their kinetic energy is transferred to thermal energy (Doona, 2010; Oliveira and Franca, 2002; Tewari and Juneja, 2008).

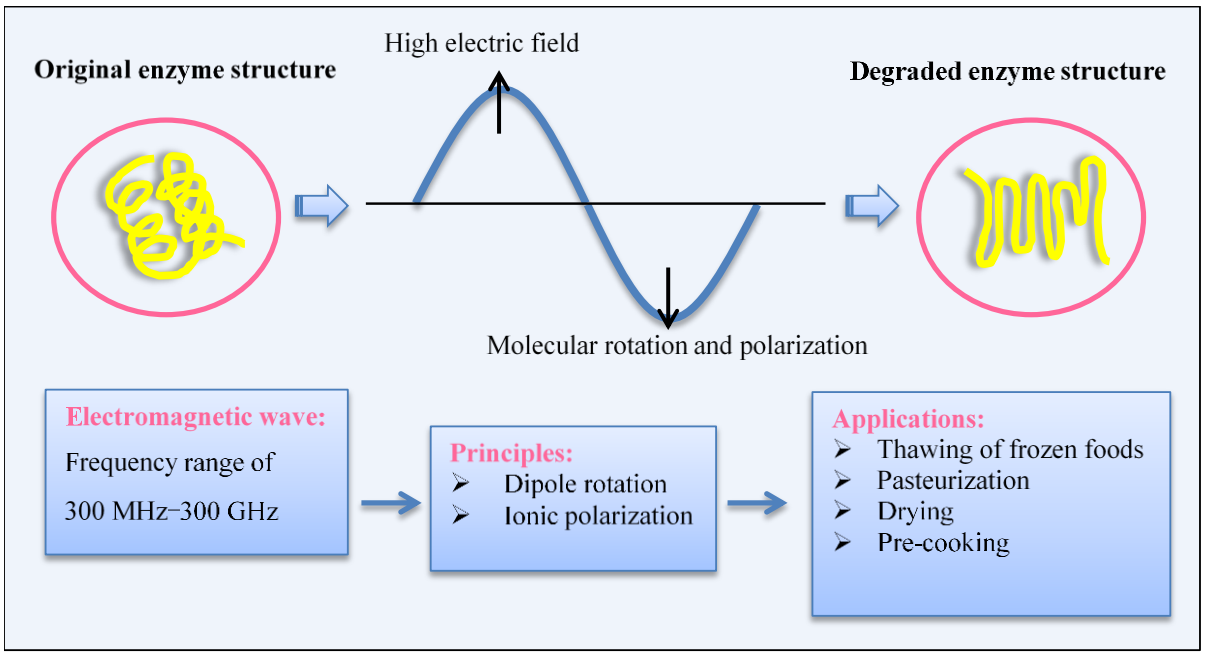

Figure 3.2. Molecular rotation mechanism and applications of microwave heating process

Microwaves have the potential to alter the native structure of proteins, and therefore, might potentially affect the ability of certain proteins to be recognized by IgE of sensitized subjects (Jiménez-Saiz et al., 2015). In comparison to the conventional thermal techniques, microwave heating shows a high heating rate, more uniform heating effect, less energy consumption, easy to operate, and environment-friendly (Salazar-González et al., 2012). Also, microwave heating

has a less negative influence on the flavor and nutritional compounds of food sources during processing (Vadivambal and Jayas, 2010). However, the chemical components including textural and organoleptic properties of food products would be changed under microwave heating, such as protein denaturation of eggs or milk, caramelization resulting from sugar dehydration, gas or water vapor generation by baking, enzyme inactivation due to high temperature (Meda et al., 2017; Regier and Schubert, 2005).

Kim et al. (2006) treated shrimp with 2450 MHz microwave at 16-17 ℃ for 1, 5, 10, 20 min; the results showed the shrimp allergenicity determined by competitive indirect ELISA changed little (Kim et al., 2006). Few studies applied microwave treatment to the modification and elimination of shrimp allergens; the main reason is shrimp tropomyosin is heat-stable and has a potent ability to maintain a great allergenic potential during thermal treatment (Carnés et al., 2007; Liu et al., 2010b). Therefore，novel non-thermal processing techniques are in need.

3.5.2 Non-thermal processing

Non-thermal processing, a technique without heating involved, is investigated frequently in recent years. Compared with thermal processing, non-thermal techniques have better performance in retaining the original attributes and nutritional characters. Thereby, novel non-thermal approaches have attracted researchers and were applied to look through the effects on shrimp allergenicity, mainly for shrimp tropomyosin, which is a heat-stable allergic protein. The modification by non-thermal procedures such as pulsed light, ultrasound, cold plasma, fermentation, and enzymatic hydrolysis treatment needs to be evaluated.

3.5.2.1 Ultrasound processing

Ultrasound is a kind of sound wave with a frequency of approximately 20 kHz (Awad et al., 2012). It is considered as one of the emerging efficient food processing technologies widely used for different applications, including homogenization, cutting, extraction, inactivation of microorganisms or enzymes, drying enhancement, surface cleaning, depolymerization (Feng et al., 2011). In Figure 3.3, the mechanism of ultrasound-assisted extraction and cavitation phenomenon are depicted. The sonication bubbles are formed from mechanical waves

compressing, refracting intermittently, and collapsing at critical bubble sizes under the high energy applied. The implosion of bubbles results in localized high pressure up to 1000 atm and high temperature up to 5000 ºK. These extreme parameters can induce the alteration of allergen structures (Soria and Villamiel, 2010).

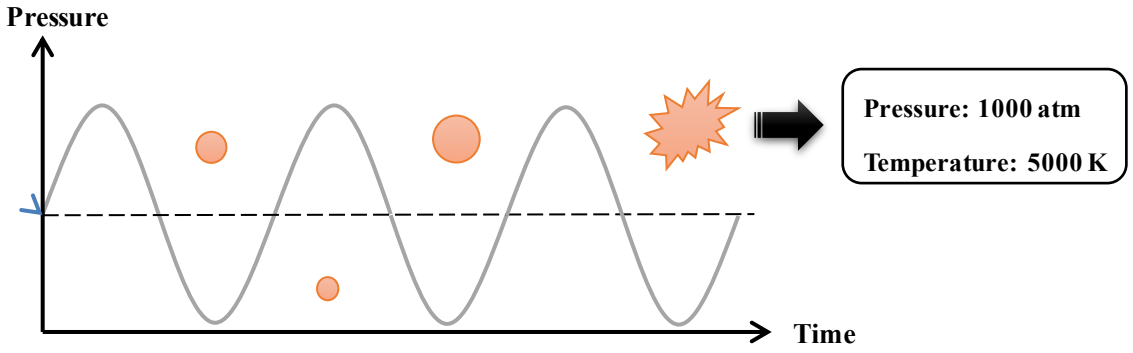

Figure 3.3. Mechanisms of ultrasound assisted extraction principle and cavitation phenomenon

Li et al. (2006) applied ultrasound treatment (30 Hz, 800 W) for 30-180 min to shrimp and purified shrimp allergen, respectively. The results demonstrated the allergenicity of shrimp allergen declined by a maximum of 75% analyzed by ELISA with polyclonal antibodies, and the allergenicity of shrimp samples has a linear relationship with treatment time (Li et al., 2006). Zhenxing et al. (2006) also employed high-intensity ultrasound (30 Hz, 800 W) to shrimp (*Penaeus vannamei*) muscle for 180 min with temperatures at 0 and 50°C; and found there was a reduction of shrimp allergenicity when samples were treated with 50°C heating (Zhenxing et al., 2006). Similarly, the same ultrasound treatments (30 kHz, 800 W) with 0°C and 50°C were treated to boiled and raw shrimps for 0, 2, 8, 10, and 30 minutes, respectively (Li et al., 2011). For boiled shrimps, the allergenicity decreased by 50% and 40% after ultrasound treatment at 0°C and 50°C for 10 min; for raw shrimps, the allergenicity at 0°C increased during the first 10 min and followed by a reduction, while the allergenicity at 50°C showed a bit decrease (8%). Therefore, allergenicity reduction by ultrasound treatment to boiled shrimp samples was more effective than the raw samples (Li et al., 2011). Besides, Zhang et al. (2018b) treated tropomyosin by a 100-800 W ultrasound for 15 min; and found ultrasound could significantly decrease shrimp allergenicity determined by immunoblotting and ELISA where TM molecules

were degraded under high-intensity ultrasound (Zhang et al., 2018b).

It is obvious that ultrasound processing is a potent approach for shrimp allergen mitigation with numerous advantages, such as environmental- friendliness, lowering heating and concentration gradients, simplified procedure with high fields, extracting selectively, etc. However, most of the studies mentioned above have a long treating time, which is not effective and economical (Chemat and Khan, 2011; Yang et al., 2015). Thus, further studies are needed to provide better strategies.

3.5.2.2 Pulsed light/ pulsed UV light

Pulsed light (PL) whose wavelengths is from 200 nm to 1000 nm is formed by short and intensive pulses of white light with broad-spectrum, and is made up of 54% ultraviolet (UV) light, 20% infrared and 26% visible light radiation (Shriver and Yang, 2011). Such efficient pulsed UV light has over a thousand-time intensity than the ordinary UV light (Chung et al., 2008; Shriver and Yang, 2011). The conformation of shrimp allergens can be altered under PUV light due to the loss of conformational epitopes (Chung et al., 2008). The photothermal effect, photochemical effect, and photophysical effect are three kinds of inactivation reactions by PUV light, which lead to the change of allergen structure, heat transfer rates (Krishnamurthy et al., 2008).

Yang et al. (2012) applied PUV light (a rate of 3 pulses/s, a width of 360 μs, a distance of 10 cm from the light source) to 10 mL of raw or boiled shrimp extract (5 mg/mL). The results demonstrated that the allergen reactivity of shrimp extracts could pronouncedly decrease without returning to their original reactive state, thereby it can be regarded as a potential approach for hypoallergenic shrimp production (Yang et al., 2012). Such results were probably caused by tropomyosin treated by PUV light cross-linked with some heat-sensitive proteins (Shriver et al., 2011). Using the same parameters, Shriver et al. (2011) treated Atlantic white shrimp (*Litopenaeus setiferus*) extract with PUV for 4 min; changes in the tropomyosin levels were determined by SDS-PAGE and IgE binding of the treated extract which was analyzed via immunoblot and ELISA. The results showed that PUV treatment to have decreased the levels

of tropomyosin and IgE binding and reduced the reactivity of tropomyosin (Shriver et al., 2011). Although studies up to now have reported PUV light treatment to have demonstrated the potential capability to reduce shrimp allergenicity, more studies especially clinical tests and in vivo studies have to be conducted to verify the effect of PUV light in mitigation of shrimp allergic proteins (Vanga et al., 2017).

3.5.2.3 Cold plasma

Cold plasma is an electrically energized matter in a gaseous state generated at 30–60 °C under atmospheric or vacuum, and is widely used to heat-labile food production due to the low temperature and energy level (Fernández and Thompson, 2012; Phan et al., 2017; Thirumdas et al., 2015). In recent years, it is gradually as a novel technique to microbial inactivation and food decontamination, whose mechanism is to interact with food proteins and further cause the conformation changes (Ekezie et al., 2018a; Tolouie et al., 2017).

Cold plasma processing can modify original characters of foods to desired ones without changing food texture and nutritional properties, whereas few studies have applied cold plasma treatment to reduce shrimp allergens, which may be probably due to its expensive cost (Misra and Jo, 2017). A study treated tropomyosin with direct dielectric discharge cold plasma for 5 min at a voltage of 30 kV and a frequency of 60 Hz. The results showed that a 76% reduction in the allergenicity of shrimp was observed after cold plasma treatment, and the IgE binding to tropomyosin and shrimp extract were also reduced under cold plasma treatment (Shriver, 2011). It is effective for the reduction on shrimp allergenicity, but literature focused on cold plasma impact to shrimp allergens is scanty, which implied that more studies concentrated on cold plasma to shrimp allergens are needed.

3.5.2.4 Fermentation

Fermentation plays an essential role in the food quality improvement and microbiological stability in food industry (Faridnia and Selamat, 2011). It takes place when microorganisms act on food substrates, one of the most common examples is that sugars could be converted to alcohol,

carbon dioxide, and organic acids without oxygen. Such a process is mainly affected by kinds of microorganisms and surroundings, such as temperature, pH, substrate concentration. It was reported that fermentation not only was widely applied to the food industry, but also could be used to modify the structure of allergenic proteins.

Kim, et al. (2008) performed a study to observe the changes in allergenicity of saeujeot (salted and fermented shrimp) using a competitive indirect enzyme-linked immunosorbent assay (Ci-ELISA). 10%, 15%, 25% of salt concentrations with temperatures at 5℃, 15℃, 25℃ was applied to shrimp samples. The results showed that a low salt concentration with high temperatures could significantly decrease the IgE-binding capacity of shrimp proteins due to the hydrolysis and degradation of proteins and further leading to the reduction of food allergenicity (Zhou et al., 2016). Therefore, fermentation with a high temperature might have a positive effect on reducing shrimp allergenicity, whereas more studies in this field should be conducted.

3.5.3 Chemical treatment

3.5.3.1 Enzymatic hydrolysis

Enzymatic hydrolysis is a promising technique to alleviate shrimp allergenicity by altering the structure of allergic proteins. Its mechanism is that enzymes extremely hydrolyze the linear epitopes of shrimp allergens into peptides and/or amino acids under certain optimal conditions (Ekezie et al., 2018a; Kasera et al., 2015). In recent years, such a technique is preferred in lowering shrimp allergenicity because of its effective, selective, energy-saving characteristics (Zhang et al., 2018a).

In a study, shrimp tropomyosin was digested with pepsin (30μg/ml) in an acid environment (pH=2) for 30, 60, and 120 min at 37°C, and the IgE binding was evaluated by ELISA and Dot-blot assay. The indirect ELISA and Dot-blot results indicated that TM decreased 83.7% in the human IgE binding under pepsin hydrolysis, which implied the sensitivity to have significantly reduced by pepsin digestion (Mejrhit et al., 2017). Liu et al. (2011) investigated the effect of enzymatic digestion (pepsin, trypsin, and chymotrypsin) on shrimp TM for 4 h (Liu et al., 2011).

The results showed the allergic protein was partially digested, and its IgE-binding ability was decreased shown by using ELISA analysis (Liu et al., 2011). WU and HU (2010) eliminated the allergens in shrimp (*Litopenaeus vannamei*) by separately hydrolyzing with trypsin and papain. The results demonstrated the IgE- binding capability to have reduced by indirect ELISA determination (WU and HU, 2010).

The degradation of allergic protein takes place through enzymatic hydrolysis, which further results in the reduction of shrimp allergy risk. The studies until now have not concluded the optimal conditions of enzyme treatment; thus the research in this area is needed.

3.6 Conclusion and future trend

Until now, many studies focus on the processing impact on the reduction of shellfish allergens, whereas a few studies concentrate on shrimp allergens. Shrimp allergy has an increasing prevalence worldwide and possibly leads to mild to severe anaphylaxis. The major allergic protein tropomyosin is heat-stable. Due to the limitations (i.e., not effective to heat-stable allergens, reduction of nutrients, changes of original sensitive properties) of thermal techniques, more studies investigated the modification of secondary and tertiary protein structures by non-thermal techniques, but the efficiency of these processing methods need to be improved and well-studied. Among them, ultrasound processing has been discussed more and probably demonstrated the most efficient effect in reducing allergenicity. Nonetheless, in-depth research in novel processing methods for reducing or eradicating shrimp allergens is still needed, which perhaps could provide strategies for better management and development of shrimp hypoallergenic products and positive health for the population.

CONNECTING TEXT

In Chapter III, we have seen that the epidemiology worldwide and the main allergen properties of shrimp allergy; also several novel processing methods to lower the allergenicity of shrimps, including microwave, ultrasound, pulsed light, cold plasma, fermentation, enzymatic hydrolysis, and their combination are reviewed. In the next part of the thesis, an experimental study on the effect of ultrasonic processing (0, 5, 10, 15, 20 min) on the color attributes, antioxidant capability, secondary structures, microstructures, in vitro protein digestibility, and allergenicity of shrimp samples is investigated.

CHAPTER IV

Effects of High-intensity Ultrasound Processing on the Physiochemical and Allergenic Properties of Shrimp

4.1 Abstract

Shrimp is one of the common seafood with high nutritional value and delicious flavor. However, many individuals are allergic to shrimps, and no therapies for shrimp-allergic patients have been provided utill now. This study aims to evaluate the impacts of the high-intensity ultrasound processing (0, 5, 10, 15, 20 min) on the color attributes, antioxidant capability, secondary structures, microstructures, *in-vitro* protein digestibility, and allergenicity of shrimp samples. The results indicated that there was a positive correlation between the decrease of allergenicity and the increase of the ultrasound processing time, and the best hypoallergenic effect showed at 20 min with 76% of tropomyosin reduction. At 20 min, the total soluble protein content decreased by 28.26%, while peptide content and total antioxidant capability increased by 0.81% and 71.29%, respectively. Ultrasound treatment also strengthened the *in-vitro* digestibility of shrimp proteins, although the secondary structure and microstructure were altered as per FTIR analysis and microscopy. β-sheets and α-helices were found to increase with time processing, accompanied by the decrease of turns and unordered conformations. More fragments, strips, and holes were observed after 20-min ultrasound processing, which indicated the physical essence of ultrasound treatment in improving the extraction of bioactive molecules. The findings in this study provide a better understanding of ultrasound application in both lowering allergenicity and retaining the nutritional characteristics of shrimps.

Keywords: Ultrasound treatment, ELISA, Shrimp, Digestibility, Microstructure

4.2 Introduction

Shrimp, belonging to crustacean species in seafood, is an excellent source for human health. Shrimps are rich in proteins (up to 20%), well-balanced amino acids, large amount of minerals (e.g., calcium and selenium) and highly unsaturated fatty acids (HUFA) such as eicosapentaenoic (20:5n3, EPA) and docosahexaenoic (22:6n3, DHA) acids, (Akonor, Ofori, Dziedzoave, & Kortei, 2016; Sriket, Benjakul, Visessanguan, & Kijroongrojana, 2007). Shrimp is also abundant in antioxidants, including vitamin B12, phosphorus, astaxanthin, and a fat-soluble carotenoid (Venugopal, 2008). These bioactive compounds can boost the immune system and reduce the risk of cardiovascular disease, stroke, and diabetes (Smith & Guentzel, 2010).

However, shrimp belonging to the shellfish family, which is one of the "big eight" allergenic foods, has become a major public health concern leading to a series of allergic reactions from the mild symptoms to life-threatening anaphylaxis (Hoffmann-Sommergruber, 2000; Silke, 2017). Patients suffering in shrimp allergy are cross-reactive with other seafood such as crabs and clams. An investigation were conducted among 33 individuals with shrimp allergy history and found the main symptoms included urticarial/angioedema (28%), gastrointestinal (13%), pulmonary (9%), systemic anaphylaxis (7%), generalized pruritus (2%), and other symptoms (2%) (Daul, Morgan, Waring, McCants, Hughes, & Lehrer, 1987). Shrimp is the most frequent offenders in children and adults (Sicherer, Muñoz-Furlong, & Sampson, 2004). In North America, the prevalence of shellfish allergy is approximately 1.3%, in which shrimp is the most commonly reported allergen (Chokshi, Maskatia, Miller, Guffey, Minard, & Davis, 2015). In Europe, a study reported shrimp is the most common allergenic food with 48 patients suffering from 142 food-sensitized patients surveyed in Spain (Castillo, Delgado, Quiralte, Blanco, & Carrillo, 1996). In Asia, a questionnaire survey also reported that shrimp is the most common food causing allergic reactions (33.3%) in children aged 3-7 years in Thailand (Lao‐araya & Trakultivakorn, 2012). Thus, shrimp allergy has become a challenge causing health-related issues all over the world.

The major shrimp allergen is tropomyosin (TM), which was first described as a crustacean

allergen in 1981 (Ahmed, et al., 2018; Hoffman, Day, & Miller, 1981). TM is a heat-stable protein that can maintain its activity even after being boiled in water (Besler, Steinhart, & Paschke, 2001). It is estimated that at least 80% of individuals with shrimp allergy history showed allergic reactions to this allergen producing shrimp-specific IgE antibody. TM has also been described as an essential allergen in other crustaceans such as lobsters, crabs, and mollusks, as well as other arthropods (house dust mites and cockroaches) (R Ayuso, Lehrer, & Reese, 2002). This cross-reactivity may be explained by the highly conserved amino acid sequences (Rosalia Ayuso, Reese, Leong-Kee, Plante, & Lehrer, 2002).

Novel techniques have been applied to the modification of shrimp allergens by denaturation and interaction reactions (Ekezie, Cheng, & Sun, 2018; Rahaman, Vasiljevic, & Ramchandran, 2016). To reduce the content of shrimp allergen, many methods have been used such as ultrasound processing (X. Li, Li, Lin, & Samee, 2011; Zhang, Zhang, Chen, & Zhou, 2018; Zhenxing, Caolimin, & Jamil, 2006), thermal treatment (Wal, 2003), pulsed UV light (S. Shriver, Yang, Chung, & Percival, 2011), gamma irradiation (Zhenxing, Hong, Limin, & Jamil, 2007), cold plasma (S. K. Shriver, 2011), fermentation (Kim et al., 2008), etc. Among them, ultrasound processing showed the most efficient impact in reducing the allergenicity of shrimp. Li et al., (2006) treated shrimp with ultrasound treatment (30 Hz, 800 W) for 30-180 min. The results demonstrated the allergenicity of shrimp allergen showed a decreasing trend with the rise of ultrasound processing duration. The maximum reduction was up to 75% analyzed by ELISA test (Li et al., 2006). In another study, Zhang et al. (2018) treated tropomyosin with 100-800 W ultrasound for 15 min and found the allergenicity of tropomyosin was significantly decreased using immunoblotting and ELISA test, which was associated with the degradation of TM under a high-intensity ultrasound (Zhang et al., 2018).

However, most ultrasound treatments were conducted under a long-time duration accompanying high energy consumption. The objective of this study is to evaluate the effect of high-intensity ultrasound processing (0, 5, 10, 15, and 20 min at 400 W) on the allergenicity, total soluble protein content, secondary structure, and microstructure of shrimp. The total antioxidant capacity, color, and in-vitro protein digestibility of shrimp samples are also

measured. The experimental results may further provide strategies for better management and development of shrimp hypoallergenic and positive health attributes to the population.

4.3 Materials and Methods

4.3.1 Sample preparation

Frozen shrimps (Litopenaeus vannamei, whiteleg) purchased from a local supermarket (Liantai Chinese Market, Montreal, Canada) were stored at -20ºC until their further analysis. The average length and weight of shrimps were around 8.79 ± 0.42 cm and 32.54 ± 0.85 g, respectively. As shown in Figure 4.1, the shrimps were thawed, beheaded, and deshelled. The experimental samples were prepared by mincing the edible portion with double distilled water by a ratio of 1:3 (per gram/per milliliter) using a juicer.

4.3.2 Ultrasound treatment

Ultrasound processing was conducted using a Branson Sonifier 450 (Branson Ultrasonic Corp., Danbury, CT, USA) with a probe at a 20-kHz frequency, 400 W. The sonication probe was immersed into the liquid samples maintained in a glass jar. As shown in Table 4.1, 200 mL of samples were treated by ultrasonic processer with the duty cycle at 50% for 0 min (US0), 5 min (US5), 10 min (US10), 15 min (US15), and 20 min (US20), respectively. After the treatments, some samples were stored at 4°C for color and microstructure analysis. The leftover processed samples were dried by a freeze dryer (7420020, Labconco Corporation, Kansas City, USA) for 48 h and then were stored at −20°C until further utilities (Figure 1). All treatments and analyses were performed in triplicates.

4.3.3 Total soluble protein content

The shrimp sample (0.5 g) was mixed with PBS buffer (0.1 M, pH=7.0). After incubation at room temperature for 30 min, the mixture was centrifuged at 4,000 x g for 10 min. The supernatant was collected for further measurement. In the present study, a Pierce BCA protein assay kit (purchased from Thermo Fisher Scientific, Canada) was used to determine the total soluble protein of shrimp samples according to the protocol (Jin Wang, Vanga, & Raghavan,

2019a).

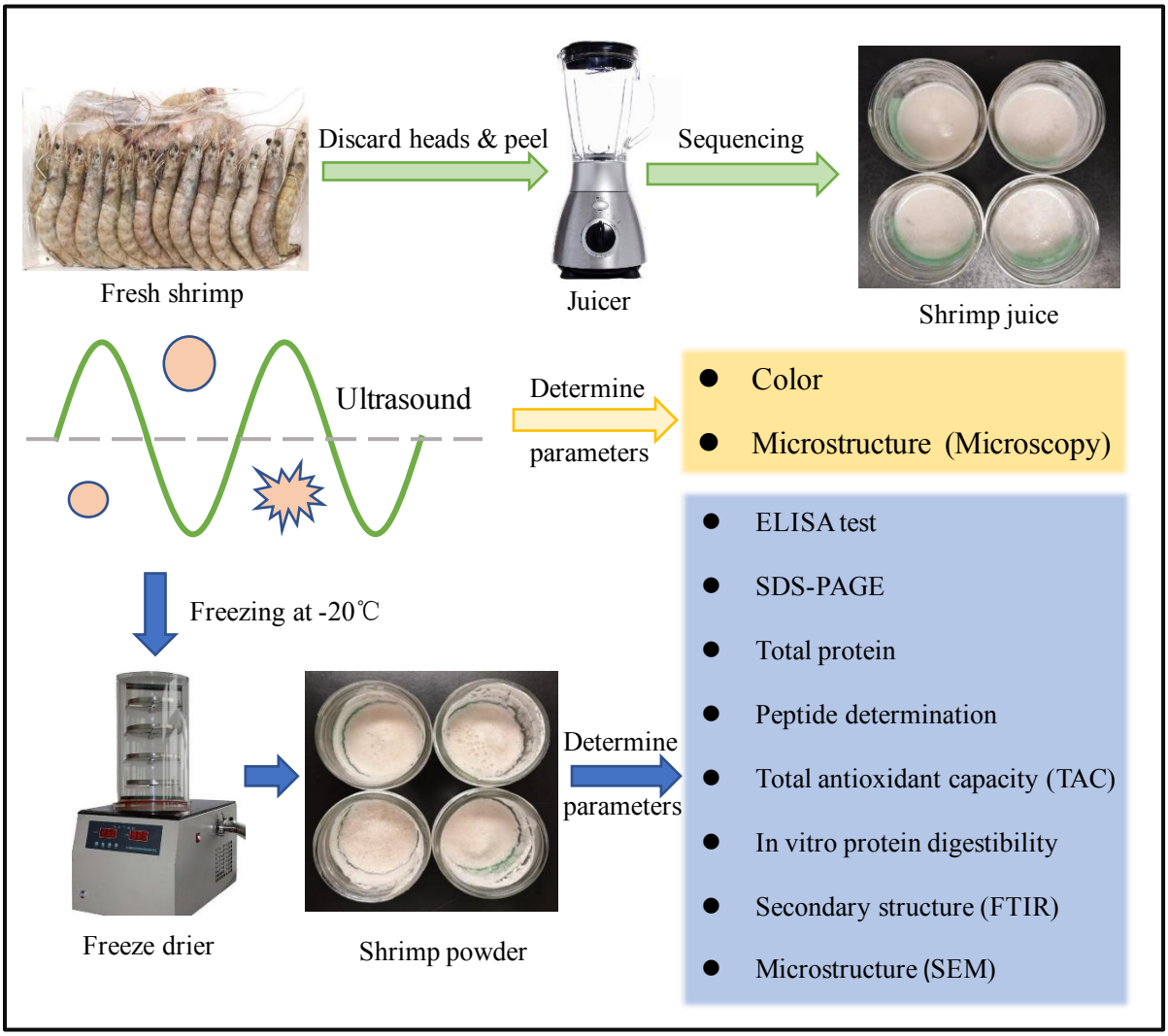

Figure 4.1. The overview experiment scheme including (A) Frozen shrimps, (B) Fresh shrimp juice, (C) Freeze dried shrimp samples

4.3.4 SDS-PAGE measurment

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method described by Zhang et al. (2018) with some modifications. The extracted protein solution was mixed with sample buffer (10 µL β-mercaptoethanol, 90 µL 4x Laemmli sample buffer) at the ratio of 1:1 (v/v). After heating at 95°C for 5 min in a water bath, the mixture was cooled down at room temperature. Then, ten µL of denatured protein samples were loaded in each lane of the gel. Molecular weight marker (10-250 kDa) (Bio-Rad,

Philadelphia, PA, USA) were loaded for five μL in the gel. Electrophoresis was performed in a vertical unit (Mini-PROTEAN® Tetra System, BIO-RAD, Philadelphia, PA, USA) at 80 V. After electrophoresis, protein gel was stained with 0.1% Coomassie Brilliant Blue R-250 (Sigma-Aldrich Chemical, St. Louis, MO, USA) for 10 min, then destained with a solution containing 90 mL of 50% methanol and 10 mL of acetic acid. The related figure was obtained using a digital camera.

Table 4.1 Ultrasound treatment index applied to shrimp samples

Treatment	Amount/ mL	Time duration/ min	Duty cycle /%	Frequency and power
US0	50	0	50%	20 kHz, 400w
US5	50	5	50%	20 kHz, 400w
US10	50	10	50%	20 kHz, 400w
US15	50	15	50%	20 kHz, 400w
US20	50	20	50%	20kHz, 400w

4.3.5 Sandwich ELISA

Sandwich ELISA (Shrimp Tropomyosin ELISA 2.0, Indoor Biotechnologies, USA) was applied to determine the tropomyosin derived antigenicity as described by Faisal, Buckow, Vasiljevic, and Donkor (2019) with some modifications. The ELISA kit contains a microtiter plate coated with the anti-tropomyosin monoclonal antibody, and reagents including tropomyosin allergen standard (500 ng/mL), rabbit anti shrimp tropomyosin antiserum, peroxidase-conjugated goat anti-rabbit lgG, wash buffer (10x), assay buffer (10x), TMB developing substrate, and stop solution. All the steps were followed as the protocol provided. The absorbance of tropomyosin in each sample was recorded at 450 nm. The concentration of tropomyosin was calculated according to the standard curve obtained by the standard.

4.3.6 Secondary structure

Fourier transform infrared spectroscopy (FTIR) was used to study the changes in secondary

structures present in shrimp proteins. The powder sample (0.1 g) was transferred to the diamond crystal at room temperature. The data was analyzed by Windows-based OMNIC software (Version 8, Thermo Nicolet Co., Madison, WI) connected to the FTIR spectrometer (Nicolet Magna 158,750 FTIR, Nicolet Instrument Corp., Madison, WI). Thirty-two scan spectra at a spectral resolution of 4 cm^{-1} will be recorded and averaged in the mid-infrared region (4000–500 cm^{-1}). To avoid the influence of air, a background spectrum without the sample was collected before each determination.

A curve-fitting procedure was applied using OriginPro (Version 9, Origin Lab Corporation, Northampton, MA, USA) to quantify the conformational changes of shrimp proteins in the amide I band. The sum of squares of the differences was minimized between experimental spectra and the computed spectra developed by the summation of the component curves. The component bands were determined using the second derivative spectrum generated by the software, and the results were given in terms of the percentage of secondary structures at corresponding wavelengths.

4.3.7 Analysis of total antioxidant capacity

As described by Wang et al. (2019), a ferric-reducing/antioxidant power (FRAP) assay was performed to test the total antioxidant activity of shrimp samples. Briefly, the FRAP working solution was prepared by mixing ferric chloride solution (20 mM), acetic acid buffer (300 mM), and 2,4,6-tripyridyl-S-triazine (TPTZ) (10 mM) in the ratio of 1:1:10 (v/v/v), respectively, at room temperature. The extraction of antioxidants from shrimp samples was done by mixing samples with methanol. FRAP solution (200 mL) and the extract (67 µL) were added to the microwells of plates, respectively. After a 5-min incubation with slight shaking at 37 °C in the dark, the color change was measured at a wavelength of 593 nm by a spectrophotometer. Standard solutions were prepared from a 1000 µM ferrous sulphate solution to make a standard curve, and the antioxidant activity of shrimp samples was presented as µmol Fe (II)/100 mg of samples.

4.3.8 Color measurement

A colorimeter (CR-300 Chroma, Minolta, Japan) with 2° N skylight as the light source was utilized to determine the color attributes of shrimp samples. The calibration procedure was conducted by placing the hand shank on a specific white tile (Y = 93.35; x = 0.3152; y = 0.3212) until the value on-screen was the same as that on the tile. Three parameters would be recorded: L* (light /dark), a* (red/green) and b*(yellow/blue) in terms of the Commission Internationale de l'Eclairage (CIE) Lab color parameters, whose range were from 0 (black) to 100 (white), − 60 (green) to + 60 (red), and − 60 (blue) to + 60 (yellow), respectively (Pathare, Opara, & Al-Said, 2013; Xiao, Gao, Lin, & Yang, 2010). The total color difference (TCD), hue angle (h), chroma (C), and color index (CI) were evaluated according to the equations below (1)-(4) (Ordóñez-Santos, Martínez-Girón, & Arias-Jaramillo, 2017):

$$TCD = \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2} \quad (1)$$

$$h = \tan^{-1}\frac{b^*}{a^*} \quad (2)$$

$$C = \sqrt{a^{*2} + b^{*2}} \quad (3)$$

$$CI = \frac{180 - h}{L^* - C} \quad (4)$$

Where a_0^*, b_0^*, and L_0^* represent the color parameters of the sample without ultrasound treatment and were immediately measured after homogenization; a*, b*, and L* represent the samples treated by ultrasound treatment and were determined promptly after every step. Each sample was measured eight times. The mean of these values ensured repeatability.

4.3.9 Microstructure

According to the method described by Statakos et al. (2016), 20 µL of diluted shrimp samples were transferred to the glass slide and then were stained using 0.1% of toluidine blue (obtained from Sigma-Aldrich (Quebec, Canada)) solution for 2 min (Stratakos et al., 2016). The mixture was observed under an optical microscope equipped with a digital camera (Leica DM500, Leica Microsystems Inc., Canada). The images were captured using imaging software (Leica

LAS EZ, Leica Microsystems Inc., Canada) at a 10× objective.

A Scanning Electron Microscope (SEM) (TM3000, Hitachi High-Technologies Corporation., Tokyo, Japan) was applied to observe the microstructural alteration of each sample (Jin Wang et al., 2019a). The freeze-dried shrimp samples were transferred to the measuring platform. The level of magnification was set at × 500, and the related figures were captured using the software equipped with SEM.

4.3.10 *In-vitro* protein digestibility

The *in-vitro* protein digestibility of shrimp proteins was conducted following a two-stage procedure suggested by Hejazi and Orsat (2016) and Vilela, Lands, Chan, Azadi, and Kubow (2006) with slight modifications. In the first stage of the digestion, shrimp powder was dissolved in the double distilled water to a final protein concentration of 3 mg/mL, and the pH of the sample solution was adjusted to 1.5 using 0.1 N HCl. The flasks were put in a water bath at 37°C, and digestion was started by adding freshly prepared pepsin solution (5 mg pepsin/mL in 0.01 M HCl) to reach an enzyme to substrate ratio of 1: 100. After 30 min, the digestion was stopped by adding 1.0 M NaOH solution moderately. The pH of the solution was adjusted to 7.8, using 1.0 M NaOH to start the second stage of the digestion. Digestion was started by adding prepared pancreatin stock solution (5 mg/mL in sodium phosphate buffer, pH 7.0) to reach an enzyme to substrate ratio of 1: 30 and incubated at 40ºC. A 150 mM Na_2CO_3 solution was used to stop the reaction after 60 min of digestion. The total protein contents of shrimp samples after the first-stage and second-stage digestion were determined using the BCA method (Beazley, 2017). The *in-vitro* protein digestibility was calculated using the following equation:

$$\text{IVPD}\% = \frac{\text{initial protein} - \text{final undigested protein}}{\text{initial protein}} \times 100 \qquad (5)$$

4.3.11 Determination of peptide content

The peptide content was determined using the OPA reagent (Church, Porter, Catignani, & Swaisgood, 1985). The OPA reagent was prepared by 25 mL of 100 mM sodium tetraborate in

water, 2.5 mL of 20% (wt/wt) sodium dodecyl sulfate, 40 mg of OPA dissolved in 1 mL of methanol, 100 uL of β-mercaptoethanol and ddH_2O to a final volume of 50 mL. The digested samples at different stages (50 μL) were incubated with 950 μL OPA reagent solution for 2 min at room temperature. The absorbances were recorded at 340 nm using a plate reader (Thermo Fisher Scientific, Canada). A standard curve was constructed using leucine-glycine as a standard peptide.

4.3.12 Statistical analysis

The experimental data were analyzed using the analysis of variance (ANOVA) of the SPSS software (SPSS Inc., Ver. 18, Chicago, IL, USA). The Duncan, multiple-range test, was applied to separate the means and to establish significance that was accepted at $p \leq 0.05$.

4.4 Result and Discussion

4.4.1 Color evaluation

Color, as one of the appearance parameters, has a direct effect on the sensory quality and market value of shrimps. The lovely color of shrimps after appropriate processing treatments can visually attract customers and stimulate consumption. As shown in Table 4.2, the color parameters (L*, a*, b*, TCD, C, h, and CI) of shrimp samples with and without US treatment are summarized. The lightness (L*) of all 5 samples showed a downward trend, suggesting the severer browning oxidation reactions of samples happened as time went on. Such results are similar to the study of kiwifruit treated by ultrasound (Jun Wang, Xiao, Ye, Wang, & Raghavan, 2019). Besides, there was a significant decrease in the red/green value (a*) of US5, US10, US15, and US20 treated samples when compared with the control ($P < 0.05$). The tendency of decrease might be due to an increase in the number of carotenoid degradation products, as observed by Martínez-Alvarez, López-Caballero, Montero, and del Carmen Gómez-Guillén (2020). In contrast, the blue-yellow value (b*) in treated samples increased slightly except for US15, showing a decline compared to the control. The changes might be due to globin denaturation and/or heme displacement or release, as stated by Kaur, Rao, and Nema (2016).

The total color difference (TCD) revealed the differences in the visual color between each sample. Shrimp samples after ultrasound treatments showed a significant upward trend with the increasing process time, accompanying with the significant differences in all treatments ($P < 0.05$). This result is the same as that in kiwifruit treated by ultrasound J. Wang, Wang, Ye, Vanga, and Raghavan (2019). For C value, all ultrasound-treated samples decreased, and a significant difference in US15 was observed ($P < 0.05$); this result was also similar as the results reported by L. E. Ordóñez-Santos, J. Martínez-Girón, and M. E. Arias-Jaramillo (2017) in gooseberry juice. Similarly, for h value, there was a decrease in the samples treated for 10-20 min. A similar result has been reported in ultrasound-treated grape juice (Lieu & Le, 2010) and kiwi juice (Wang, et al., 2019). The reason of hue values reduction was reported by Ordóñez-Santos, J. Martínez-Girón, and Arias-Jaramillo (2017), which may be due to the formation of hydroxyl groups after ultrasound treatment.

As shown in Table 4.2, CI value in all treated samples increased gradually with the rise of processing time. Compared with the control, CI value in treated samples showed a significant difference ($P < 0.05$) other than US5. It indicates that ultrasound treatment could improve the color attributes of shrimp samples through reduction of hue value, increasing CI value, and C value.

Table 4.2 The color parameters L*, a*, b*, TCD, C, h, and CI of shrimp samples under US treatment

Treatment	L	a	b	C	h	CI	TCD
US0	29.17±0.46 ^{a}	0.4±0.21 ^{a}	1.54±0.19 ^{ab}	1.6±0.21 ^{a}	1.32±0.12 ^{a}	6.48±0.12 ^{d}	-
US5	28.52±0.92 ^{a}	0.09±0.07 ^{b}	1.59±0.11 ^{a}	1.59±0.11 ^{a}	1.51±0.04 ^{a}	6.64±0.25 ^{d}	1.23±0.72 ^{d}
US10	26.45±0.85 ^{a}	0.02±0.22 ^{b}	1.58±0.22 ^{a}	1.59±0.23 ^{a}	-0.41±1.52 ^{b}	7.27±0.29 ^{c}	2.76±1.15 ^{c}
US15	23.73±0.7 ^{a}	0.04±0.16 ^{b}	1.37±0.16 ^{b}	1.38±0.16 ^{b}	0.36±1.52 ^{ab}	8.05±0.28 ^{b}	5.47±0.37 ^{b}
US20	20.18±0.57 ^{a}	-0.02±0.12 ^{b}	1.57±0.16 ^{a}	1.57±0.15 ^{a}	0.01±1.62 ^{b}	9.68±0.32 ^{a}	9±0.54 ^{a}

Note: Different letters in the same column reveal significant differences ($P < 0.05$).

4.4.2 Total antioxidant capacity (TAC)

As shown in Table 4.3, the influences of ultrasound treatment on the antioxidant capacity of shrimp samples are outlined. The total antioxidant capacity of shrimp samples significantly increased with the rise of processing duration from 5 min to 20 min. The highest total antioxidant capacity of shrimp was observed in US20 treated samples (50.53 μmol/100 mL), followed by US15 (46.28 μmol/100 mL) and US10 (42.70 μmol/100 mL) whereas no significant differences in the total antioxidant capacity were observed between US5 (35.03 μmol/100 mL) and US0 (29.50 μmol/100 mL).

These results indicate that ultrasound processing with a longer time can contribute to the increase of total antioxidant capacity. Similarly, in red raspberry puree, the total antioxidant capacity increased by 17.3% when a 20-kHz ultrasound was applied for 10 min (Golmohamadi, Möller, Powers, & Nindo, 2013). Moreover, the enhancement of total antioxidant capacity was also reported in carrot-grape juice treated by ultrasonication (20 kHz, 525 W) for 2-6 min (Nadeem, Ubaid, Qureshi, Munir, & Mehmood, 2018). These increases mainly could be resulted from the increase of antioxidants (e.g., phenolics, ascorbic acids) after ultrasound processing (Tang, Li, Zhang, Chen, Liu, & Tsao, 2015). Moreover, inactivation reactions of some oxidation related enzymes resulted from the sheer force, such as polyphenol oxidases, occurred during the processing period, which would also result in an increase of total antioxidant capacity in food products (Cheng, Soh, Liew, & Teh, 2007).

4.4.3 Total soluble protein determination

The total soluble protein content of shrimp samples was significantly affected by ultrasound processing (Table 4.3). The results showed that the total protein content of shrimp samples significantly decreased after treatment ($P < 0.05$). Specifically, the total protein content at US20 treated samples was decreased by 28.26% compared to the untreated sample, followed by US15 (26.97%), US10 (21.52%), and US5 (10.58%).

Similarly, the total protein of kiwifruit significantly decreased ($P < 0.05$) after the same ultrasound treatment for 4-16 min (Jin Wang, Vanga, & Raghavan, 2019b). Kang et al. (2016) also found a decrease in beef proteins by 20-kHz ultrasound treatment for 30-120 min. These decreases might be associated with the breakdown of hydrogen bonds and peptide chains represented in the proteins under ultrasound treatment, which in turn could alter the protein structure (e.g., secondary or tertiary structure) (de São José et al., 2014). Thus, ultrasound treatment has a potential application to modify the structure of the protein or rupture their peptide chains, which may further affect the allergenicity of related allergens.

Table 4.3 Changes of bioactive compounds in ultrasonic-treated shrimp samples

Treatment	Total protein (mg/g)	TAC (μmol/100 mg)	Tropomyosin (ng/mg)
US0	255.16±8.68 ^{a}	29.50±1.61 ^{b}	1.50±0.13 ^{a}
US5	228.17±1.49 ^{ab}	35.03±2.86 ^{b}	1.24±0.14 ^{ab}
US10	200.25±11.43 ^{bc}	42.70±8.41 ^{ab}	0.88±0.11 ^{b}
US15	186.34±33.06 ^{c}	46.28±21.25 ^{ab}	0.38±0.087 ^{c}
US20	183.04±5.67 ^{c}	50.53±3.71 ^{a}	0.36±0.065 ^{c}

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

4.4.4. *In-vitro* Protein Digestibility and peptide determination

As shown in Figure 4.2, the *in-vitro* digestibility of shrimp proteins increased with the rise of processing time. During the first 15 min ultrasound processing, a slight increase whereas no significant difference was observed in the *in-vitro* digestibility of shrimp proteins (79.61%) when compared with the initial level (76.42%). Whereas the *in-vitro* digestibility of shrimp proteins significantly increased to 83.95% after 20-min ultrasound processing.

During the determination of *in-vitro* protein digestibility, two-stage digestion, including pepsin digestion and pancreatin digestion, was conducted in the present study. Pepsin tended to cleave

peptide bond next to phenylalanine or tyrosine residues or leucine bond, while pancreatin was more likely to cleave at tryptophan, arginine, tyrosine, leucine, phenylalanine, and lysine bonds (Tapal & Tiku, 2019). During such two-stage enzymatic hydrolysis, an ample variety of smaller peptide and free amino acids were generated. The size, level, and compositional changes of these small peptides and amino acids can affect the antioxidant activity of samples (Kou et al., 2013). Peptide content was measured to assess the generated or hydrolyzed peptide during the ultrasonic process. Different from the IVPD of samples, there was no significant change of peptide content in treated samples compared with the untreated sample after two-stage hydrolysis (Figure 4.3, Table 4.4). However, a sharp increase of peptide amount was observed after partial hydrolysis of proteins by pepsin, further accompanied by a continuing generation of peptides after pancreatin hydrolysis. In the first stage, the peptide contents rapidly increased from 9.97 -10.22 mM to 15.64 -18.02 mM. However, in the stage of pancreatin digestion, proteins were digested slower compared with the first stage, which finally reached the highest value of peptide amounts at 19.65 -20.05 mM. The final peptide amount in each treatment was approximately two times the initial peptides.

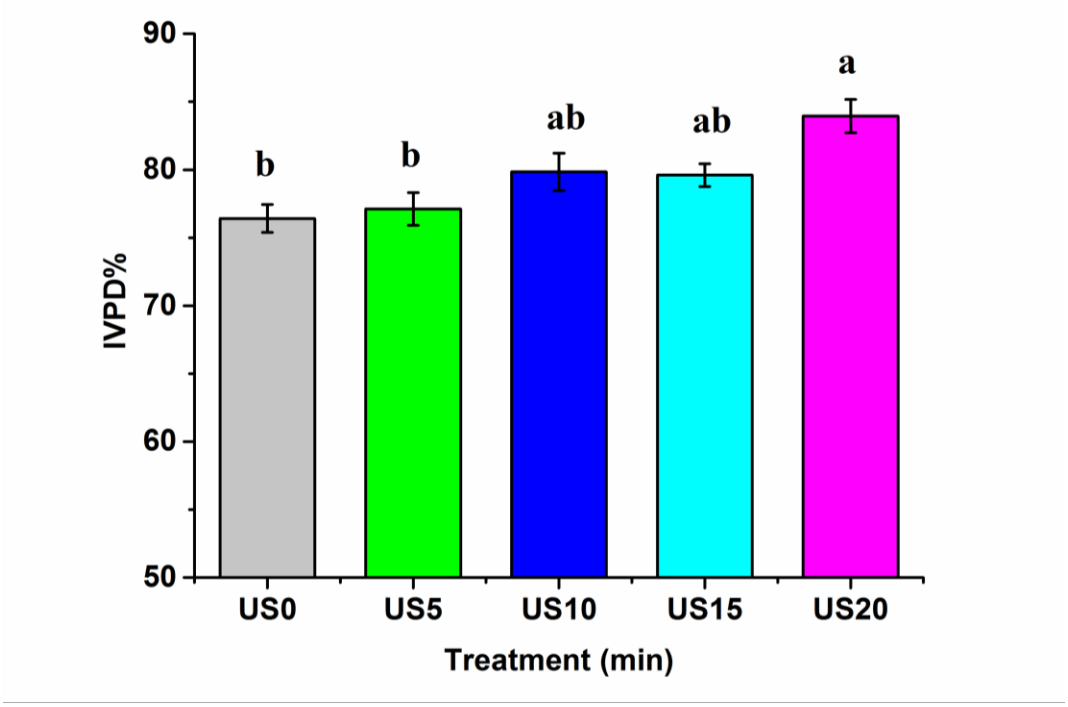

Figure 4.2. Effect of ultrasonic processing on protein digestibility (IVPD %) of shrimp meat protein

In egg white protein, similar results were described by Zhu, Vanga, Wang, and Raghavan (2018). The study reported that the *in-vitro* digestibility of egg white proteins was significantly increased from 73% to 79% after 16-min ultrasound processing at the frequency of 20 kHz, 400 W. Su, Li, and Jin (2006) also reported the digestibility of egg white proteins could be improved by 13-35% under 40-kHz ultrasound treatment for 2 hours. In shrimp *(Exopalaemon modestus)* tropomyosin, the significant enhancement of digestibility was observed in SDS-PAGE when treated by ultrasound treatment (100–800 W, 15 min) (Zhang, et al., 2018). The reason was probably because high-intensity ultrasound induced conformational changes of proteins and protein aggregates, leading to the exposure of enzyme cleavage sites, further resulting in enhanced digestibility (Zhang et al., 2018). However, an opposite result was obtained in faba bean protein as described by Martínez-Velasco, Lobato-Calleros, Hernández-Rodríguez, Román-Guerrero, Alvarez-Ramirez, and Vernon-Carter (2018). The *in-vitro* digestibility decreased by 3.6% due to the structural changes caused by 17.92-min ultrasonication (Martínez-Velasco, et al., 2018). Such an opposite result might be due to the different structures of food proteins.

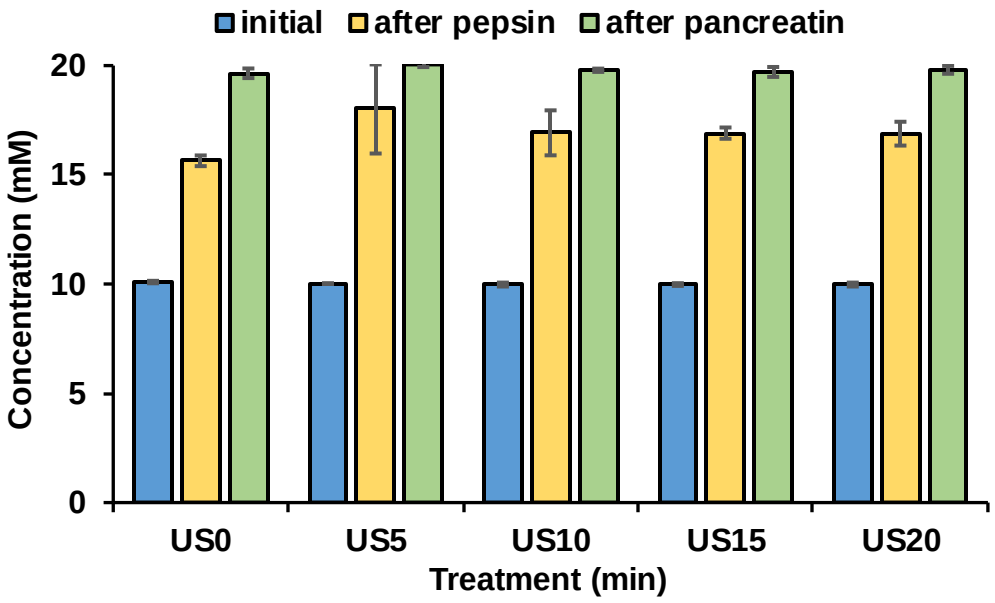

Figure 4.3. Effect of ultrasonic processing on the changes of peptide amounts of shrimp proteins

Table 4.4. Effect of ultrasonic processing on peptide generation of shrimp proteins

Treatment	Initial peptide (mM)	After pepsin (mM)	After pancreatin (mM)
US0	10.09±0.06 ^{b}	15.64±0.24 ^{b}	19.65±0.22 ^{b}
US5	10.02±0.01 ^{b}	18.02±2.04 ^{a}	20.05±0.12 ^{a}
US10	9.97±0.09 ^{b}	16.92±1.03 ^{ab}	19.79±0.07 ^{ab}
US15	10.22±0.06 ^{a}	16.91±0.26 ^{ab}	19.71±0.23 ^{b}
US20	10.04±0.09 ^{b}	16.88±0.55 ^{ab}	19.81±0.18 ^{ab}

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

With ultrasound treatment, the increased peptide yield was also observed by Wang et al. (2016). The study reported the peptides in rapeseeds protein were increased by 40.88% after dual-frequency ultrasound treatment. Jia et al. (2010) revealed the release of peptides from defatted wheat germ protein during enzymatic hydrolysis was promoted up to 42.9% by ultrasonic treatment at 20 kHz, 1500 W for 20 min. Therefore, the ultrasonication could significantly contribute to the reaction of enzymolysis and result in the significant increase of the peptides from rapeseed proteins. This was due to the intense energy generated by ultrasound led to the alteration of the protein structure. As known, general proteins were fold and curl tightly, so enzymes have no access to fully react with them in such circumstances. Whereas ultrasonication could unfold and uncurl to the secondary and tertiary structure to the active sites of proteins, which further caused the rise of infinities between proteins and enzymes (Wang et al., 2016). In the study, the secondary conformation changes of the shrimp protein were investigated by e FTIR analysis.

4.4.5 FTIR analysis of secondary structural changes in shrimp protein

FTIR analysis was utilized to investigate the effects of ultrasound processing on the secondary structures of shrimp proteins. In FTIR spectra, the amide I (1700-1600 cm^{-1}) region is one of

the most valuable and sensitive areas which is primarily used to study the secondary structure deviations in proteins (Vanga et al., 2016). It includes overlapping bands of various secondary structures, including β-sheets, α-helices, turns, and randomly coiled conformations (Y. Wang, Yang, Ding, Xie, & Liu, 2018). The typical FTIR spectra of ultrasound treated and untreated shrimp samples were shown in Figure 4.4. For the amide I region, the correlations between the amide I frequencies and secondary structures of β-sheets, α-helices, turns, and unordered were summarized in Table 4.5.

Table 4.5. Amide I band frequencies and assignment to protein secondary structure

Secondary structure	Frequency range (cm^{-1})
β-sheet	1613–1637; 1682–1696
Unordered	1637–1645
α-helix	1645–1662
Turns	1662–1682; 1630

The changes of secondary structures in shrimp proteins under ultrasound processing were represented in Figure 4.5. It was noticed that β-sheets were the major secondary structure present in shrimp, representing about 46.48-63.28% of the total secondary structures. Unordered structures were found to be dominant, accounting for 8.22-12.42% of the total protein secondary structures, which were converted from the helices present in the untreated sample. In comparison to the untreated sample, a noticeable increase in the α-helices was observed after ultrasound processing, while a remarkable decline in the turn structures was presented in proteins. These results suggested ultrasound treatment led to the unfolding of the turns, simultaneously promoting the formation of β-sheets, α-helices, and decreasing interaction between amino acids that were in a random coiled region.

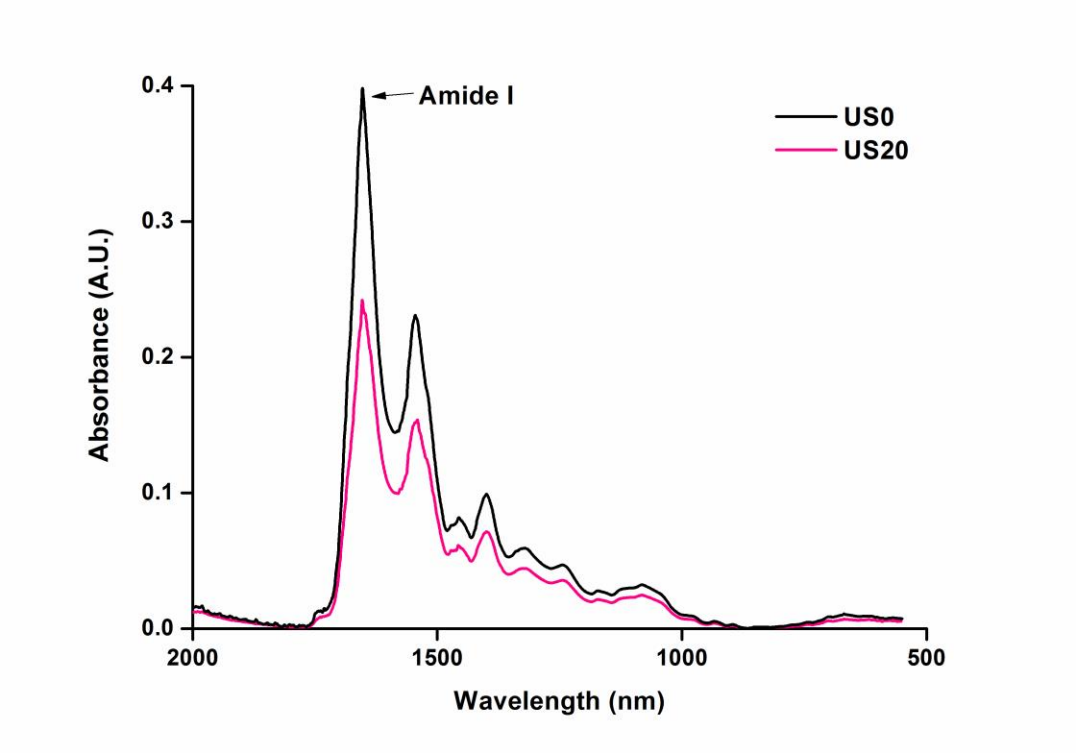

Figure 4.4. FTIR spectrum of shrimp samples with and without ultrasound processing

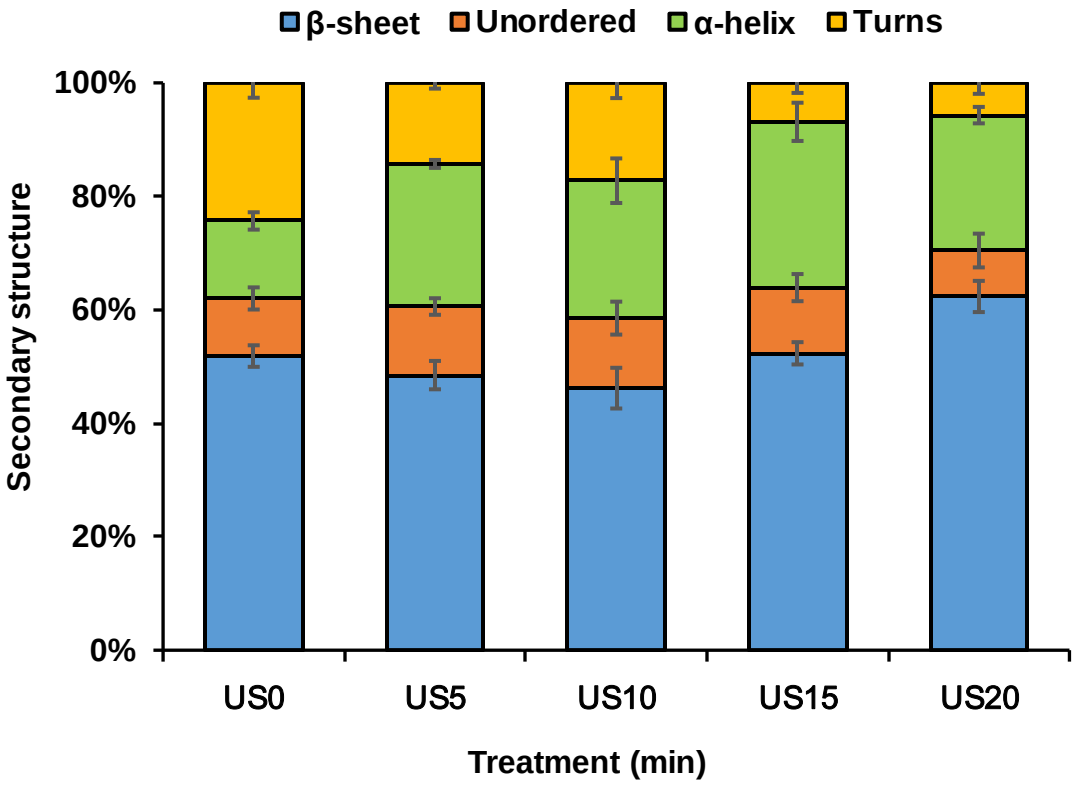

Figure 4.5. Secondary structures in shrimp samples under different ultrasonic processing time

These findings agree with the results obtained by Yang et al. (2017). They treated rice proteins with 300-W ultrasound at various frequencies (20/35/50 kHz). The FTIR analysis results showed that all the ultrasound treatments caused a reduction in the turns and increased β-sheet structures present in rice proteins. Besides, Jin et al. (2016) investigated the effect of dual-frequency power ultrasound pretreatment on corn gluten meal hydrolysates; they found ultrasound processing to have decreased the turn structure content by 27.2% while increasing α-helixes and β-sheets by 3.8% and 16.5%, respectively. The difference in secondary structure content might be due to the shear forces of ultrasound mechanical action, which disrupted the interactions between the protein molecules and influenced the protein molecule internal structures (Yang et al., 2017). Baltacıoğlu, Bayındırlı, and Severcan (2017) utilized thermosonication to pretreat mushroom polyphenol oxidase, and the results of FTIR showed the treatment to have decreased the α-helix and β-sheet contents while increasing the content of turns and random coil contents. Thus, the structural changes under ultrasound processing are different in various proteins. Thus, further studies are still in need in the future.

4.4.6 Allergenicity

A sandwich ELISA was used to determine whether there was an overall reduction in the allergenicity of shrimp samples after being treated with high-intensity ultrasound for the different time duration. As shown in Figure 4.6, the tropomyosin inhibition decreased gradually with the rise of processing time. Compared with US0 (1.50 ng/mg), the maximum decrease of 76% was observed in US20 (0.36 ng/mg), followed by US15 (0.38 ng/mg), US10 (0.88 ng/mg), US5 (1.24 ng/mg). The tropomyosin content in US10 had a significant decrease ($P < 0.5$) in comparison to that in control. Similarly, tropomyosin contents in US15 and US20 were also significantly lower ($P < 0.5$) than the tropomyosin content in US10, whereas no significant decrease was observed between US15 and US20.

As shown in SDS-PAGE (Figure 4.7), the band corresponding to 36-kDa tropomyosin exhibited a minor decrease in density or level after ultrasound treatment. It was observed that the density of the band became more and more weaker with the increasing time. The lightest

band corresponding to the lowest density of tropomyosin was observed in US20, which was consistent with the results in the ELISA test.

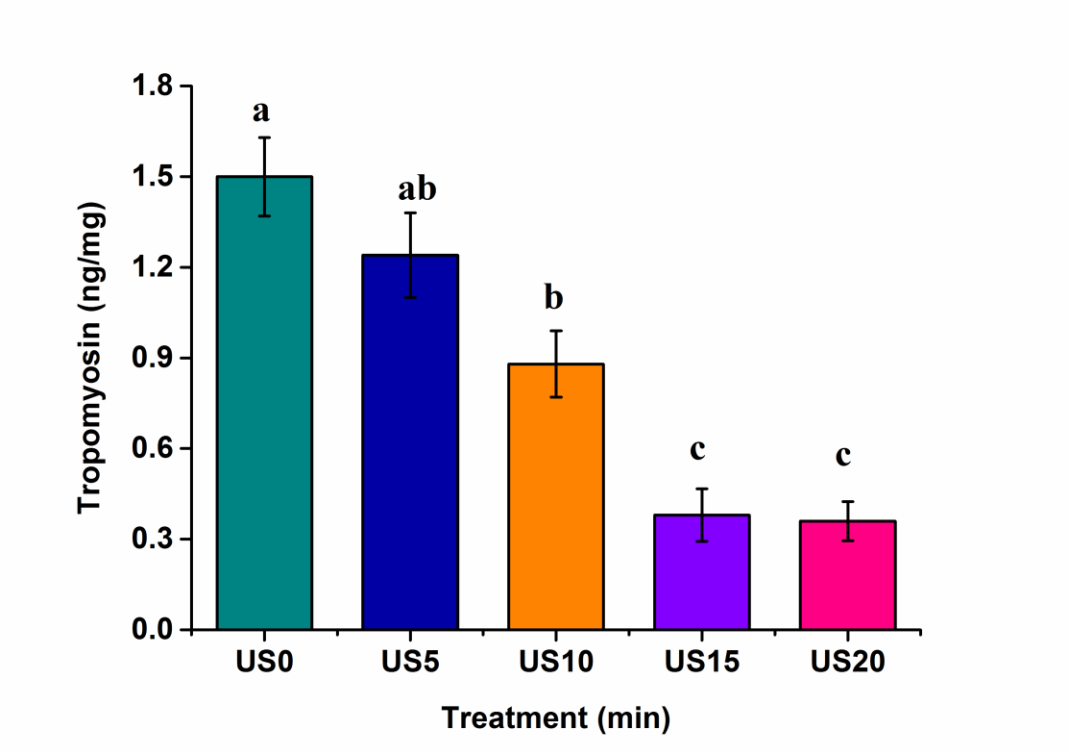

Figure 4.6. the tropomyosin contents of shrimp samples under ultrasound treatment

Besides, the markedly decreased allergenicity of 61% in β-lactoglobulin was measured by high-resolution mass spectrometry after 400-W ultrasound treatment due to the improvement of the glycation extent of some glycation sites with ultrasound (Liu et al., 2018). The allergenicity of tropomyosin from *Exopalaemon modestus* treated by ultrasound (100–800 W, 15 min) was similarly lower according to the results of the ELISA test and SDS-PAGE (Zhang et al., 2018). The degradation of tropomyosin might be due to the cleavage of bonds in conformational structures by ultrasound-induced free radicals attack (Zhang et al., 2018). In detail, hydroperoxyl radical (HO_2) is the main species of free radicals with highly-reactive and non-selective characteristics. It generally reacts with molecules in atom abstraction or additional reactions driven by bond strength energetics and further disrupts or cleavages the primary structure of tropomyosin molecules (Pera-Titus, Garcı´a-Molina, Baños, Giménez, & Esplugas, 2004). Thus, the lower tropomyosin content after ultrasound treatment suggested ultrasonic

processing was able to decrease the immunology of major shrimp allergens and consequently contribute to the elimination of allergenicity.

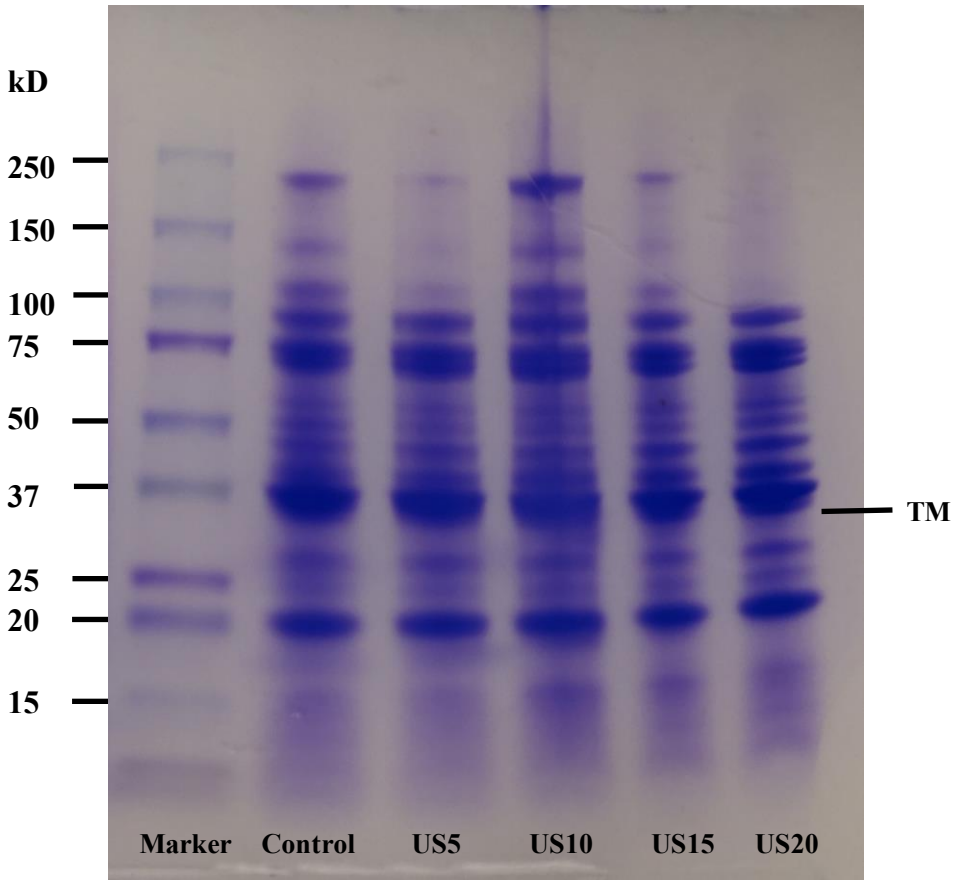

Figure 4.7. SDS-PAGE of ultrasound-treated and untreated proteins in shrimp sample

4.4.7 Microstructure observation

Food microstructure is the arrangement of structural elements within the food and the forces that bind them together (Aguilera, Stanley, & Baker, 2000). Various food components are assembled into structures determining the overall properties of foods (Morris & Groves, 2013). Microstructure and its degradation in food are generally used to assess food quality intuitively. The microstructure of shrimp samples was observed by a scanning electron microscopy (SEM) and an optical microscope.

The results showed that the degradation of microstructures in shrimp samples significantly increased with the rise of processing time (Figure 4.8, Figure 4.9). Obviously, the cells in US0 exhibited smooth and intact edges, whereas large cell fragments started to appear at 5 min

compared with the untreated sample. After 10-min ultrasound treatment, there were more flaky fragments generated in the shrimp samples. In US15, part of flaky fragments conversed into strips. Shrimp samples showed the most significant degradation in cell structures when the samples were treated for 20 min. In comparison to the control and other treatments, cells were disrupted to smaller pieces and irregular holes accrued on the cell surface after 20 min treatment.

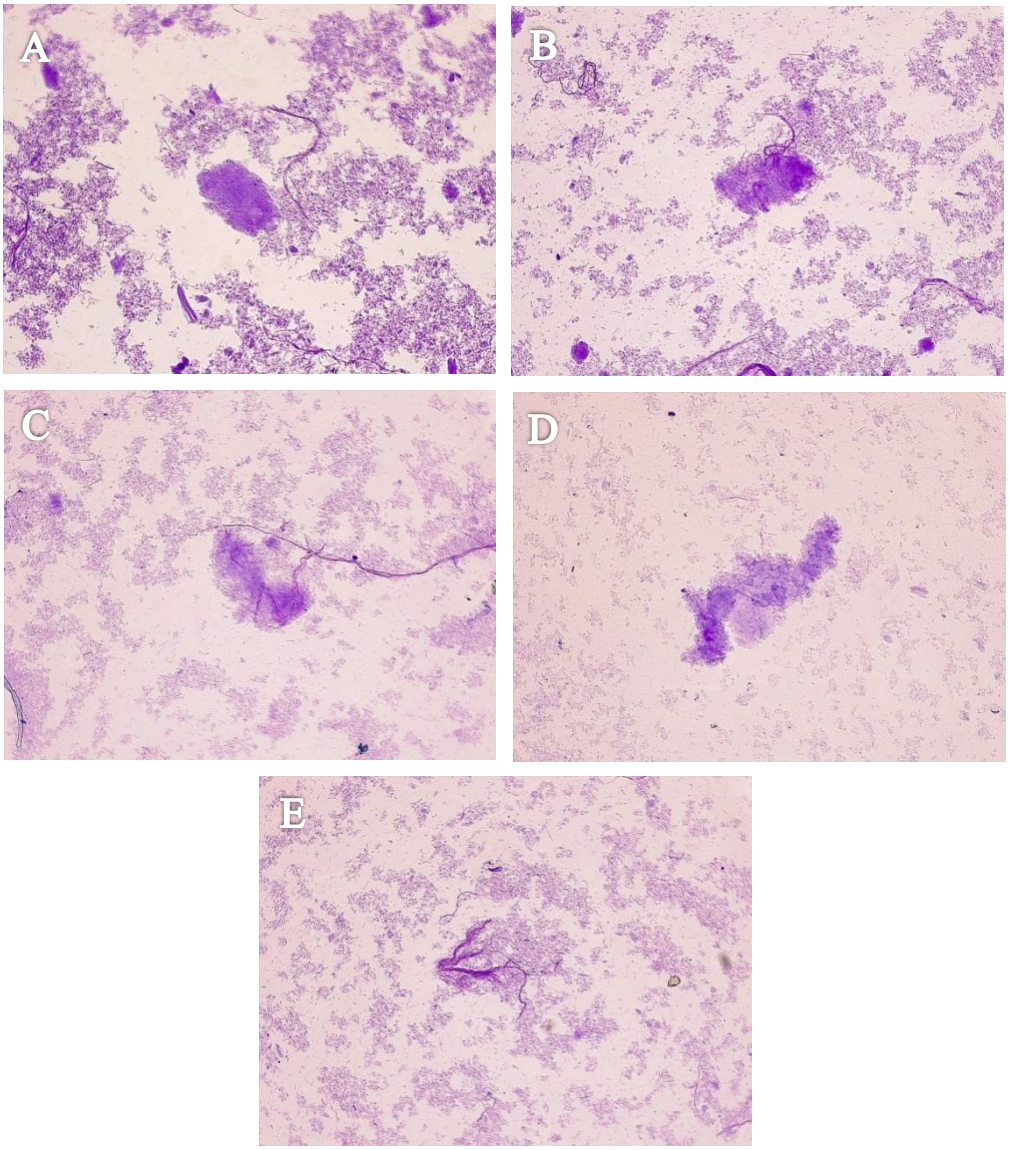

Figure 4.8. Photographs by microscopy of shrimp samples before freeze dried (A) US0 (untreated sample), (B) US-5, (C) US-10, (D) US-15, and (E) US-20

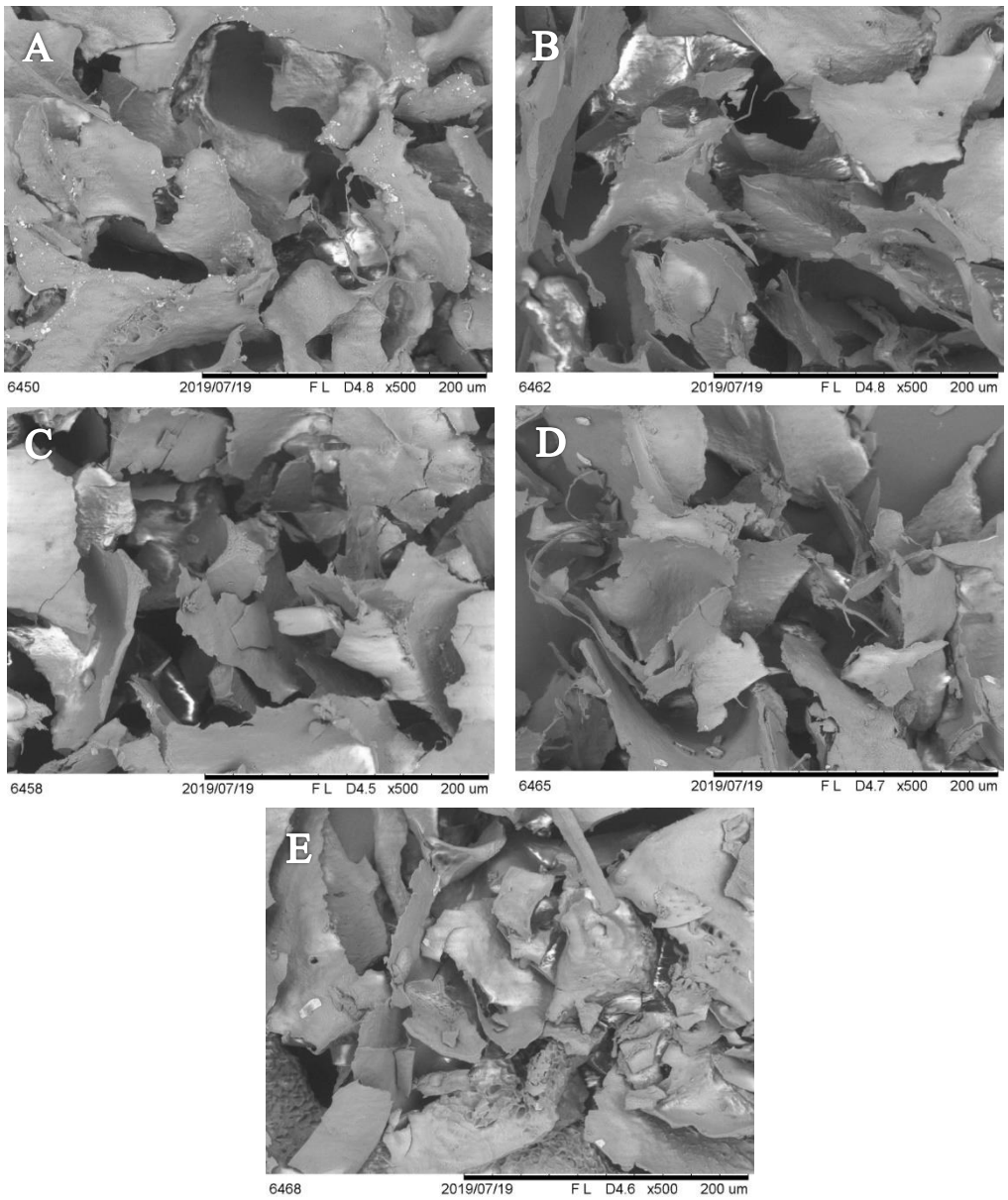

Figure 4.9. SEM photomicrographs of freeze dried shrimps (A) US0 (untreated sample), (B) US-5, (C) US-10, (D) US-15, and (E) US-20 (magnification at 500×)

Similar observations regarding microstructure changes have been reported in kiwifruit juice (Jin Wang et al., 2019b), apple and onion (Rajewska & Mierzwa, 2017), carrot (Nowacka & Wedzik, 2016), peach juice (Rojas, Leite, Cristianini, Alvim, & Augusto, 2016). They found with the further rise of processing time; ultrasound led to the irreversible enhancing changes in structure (growth of porosity, loss of tissue coherence) and cell composition (destruction of cell components). These phenomena result from shear stresses, cavitation, destruction of the internal cell components, and further leading to biochemical reactions (Rajewska et al., 2017).

Therefore, the modified conformations of ultrasound-processed foods can induce the decrease of allergenicity by unfolding and aggregation of allergenic proteins, which contributes to the descend of the total soluble protein, enhancement of peptides, and digestibility.

4.5 Conclusion

In our study, we evaluated the influence of high-intensity ultrasound treatment on the allergenicity, color attributes, antioxidant capability, microstructures, secondary structures, *in-vitro* protein digestibility of shrimp. Total soluble protein content decreased gradually with the increase of time and reached the lowest value at 20 min, simultaneously accompanying the production of peptides, which also related to the enhancement of total antioxidant capacity compared to the untreated sample. The *in-vitro* protein digestibility and peptide production showed an increment with the increase of time. More and more fragments and strips were observed by microscopy. Four kinds of secondary structures transferred with the increasing time, specifically showing the upward trend of β-sheets and α-helices and the downward trend of turns and unordered. These results demonstrated that ultrasound treatment has the potential to promote the elimination process of shrimp allergenicity with minimum destruction of shrimp original physiochemical properties. However, longer ultrasound processing time still needs to be studied in further work.

CONNECTING TEXT

In Chapter IV, we evaluated the influence of high-intensity ultrasound treatment on the allergenicity, color attributes, antioxidant capability, microstructures, secondary structures, in vitro protein digestibility of whiteleg shrimp (*Litopenaeus vannamei*). In the next chapter, we will investigate the effects of the microwave on the similar parameters of shrimp samples.

CHAPTER V

Effects of Microwave Processing on Shrimp Properties: Allergenicity, Secondary Structure, Antioxidant Capacity and Digestibility

5.1 Abstract

This study investigates the effects of microwave processing technique on the secondary structures, *in-vitro* protein digestibility, microstructural characteristics, and allergenicity of tropomyosin from *Litopenaeus vannamei*. Microwave treatment was performed at 75°C, 100°C, and 125°C for 5, 10, and 15 min at 2.45 GHz, 400W. The results revealed that microwave treatment significantly reduced the tropomyosin allergenicity by 46.15%-75% determined by the ELISA test. Same as the tropomyosin contents obtained by SDS-PAGE, the band intensities were weaker with the increase of temperatures and time, and the lowest allergenicity was observed in 125-15. Due to the protein degradation under high temperatures, total soluble protein decreased by 49.76%-74.73%. They hydrolyzed into peptides, which effectively increased the antioxidant capacity of treated samples over two times that of the control. The *in-vitro* protein digestibility overall reduced from 80.46% to 64.19%, whereas showing an upward trend at 125 °C despite still lower than the value in control. For the secondary structure as evaluated by FTIR analysis, β-sheet and unordered structures were increased, with turns increase and almost no effect to α-helix. These secondary structural alterations were also corresponding to the microstructural results observed by SEM and microscope. More microscopic holes, fragments, strips were captured after treatment, especially in the last four treated samples. Therefore, high-intensity microwave treatment could lead to a desirable provoking method to alleviate the allergenicity of shrimp while retaining a satisfying sensory quality and marketing value.

Keywords: Microwave, *Litopenaeus vannamei*, Allergenicity, Digestibility, Antioxidant capacity, Structure

5.2 Introduction

Shrimp, a kind of common table food, is beneficial to human health due to its quite rich nutrients. It contains all the essential nutrients such as vitamins, minerals, omega-3 fatty acids and astaxanthin antioxidants (Bowen, Harris, & Kris-Etherton, 2016; Fassett & Coombes, 2011; Van Blarigan et al., 2017). Among them, selenium is the most significant amount of mineral in shrimp and helpful in maintaining appropriate conditions of the immune system (Rayman, 2000). Astaxanthin can not only protect human brains and hearts to reduce the risk of mental fatigue and heart attacks but beneficial to male fertility (Capelli & Ding, 2019). Similarly, the benefits of omega-3 fatty acids to the cardiovascular system are also confirmed (Bäck & Hansson, 2019).

Shrimps are highly accessible and consumed around the world; however, an increasing number of cases related to shrimp allergy have been reported (A. J. Lee, Thalayasingam, & Lee, 2013). The common symptoms induced by shrimp allergy were skin symptoms including urticaria or angioedema, respiratory symptoms including rhinitis or wheezing, or gastroenterological symptoms such as abdominal pain, nausea, emesis, diarrhea (Gleich, Sebastian, Firszt, & Wagner, 2016). Tropomyosin has been considered as the major allergen in shellfish, which is one of the "big eight" allergenic foods in the human diet (Faber et al., 2017). Tropomyosin was first identified by Hoffman, Day, and Miller (1981) in shrimps, and later tropomyosin was found in brown shrimp (*Penaeus aztecus*) reacting with 82% of shrimp-allergic patients as reported by Daul, Slattery, Reese, and Lehrer (1994). Its molecular mass is at 35 to 38 kDa, consisting of two identically coiled subunits with heat-stability and water-solubility (Rolland et al., 2018). Besides, minor allergens in shrimp were also identified, such as arginin kinase (AK), myosin light chain (MLC), sarcoplasmic calcium-binding protein (SCP) (C.-H. Lee et al., 2018).

Microwave is an electromagnetic wave with the frequency range of 0.3-300 GHz (Singh & Heldman, 2001). Microwave heating (2450 and 915 MHz) belonging to dielectric treatment is applied to the food industry (Alfaifi et al., 2013). The Institute of Electrical and Electronics Engineers (IEEE) reports proteins and peptides have higher dielectric constant, which indicates

the microwave may have a significant impact on their activity and structure (Plagemann, von Langermann, & Kragl, 2014). In recent years, microwave treatment as a novel processing technique has been applied to many allergenic foods, such as eggs (Zhu, Vanga, Wang, & Raghavan, 2018), almonds (Su, Venkatachalam, Teuber, Roux, & Sathe, 2004), whey proteins (El Mecherfi et al., 2019), fishes (Ketnawa & Liceaga, 2017), and milk (Saxena, Vanga, & Raghavan, 2019). However, few studies applied microwave treatment to the modification and elimination of shrimp allergens. Kim et al. (2006) treated shrimp samples by 2450 MHz microwave at 16-17 °C for 1, 5, 10, 20 min; the competitive indirect ELISA revealed there was little change of shrimp allergenicity, which was probably due to the heat-stable property of tropomyosin. In this study, high-temperature microwave treatment (75, 100, 125 °C for 5, 10, 15 min) was performed. The reduction of shrimp allergenicity was analyzed by the ELISA test. Meanwhile, physiochemical characteristics of shrimp such as secondary structure, microstructure, antioxidant capacity, *in-vitro* protein digestibility were also investigated.

5.3 Materials and methods

5.3.1 Sample preparation

Frozen whiteleg shrimps (*Litopenaeus vannamei*) were bought from a local supermarket (Liantai Chinese Market, Montreal, Canada) in June 2019 (Figure 5.1, A). They were delivered immediately after purchase and stored in a freezer at -20ºC until use. The mean length and mean weight of whiteleg shrimps were 8.42 ± 0.35 cm and 31.75 ± 0.94 g, respectively. The shrimps were thawed, beheaded, and deshelled. The experiment samples were prepared by mincing edible portions with double distilled water by a ratio of 1:3 (per gram/per milliliter) in a juicer (Figure 5.1).

5.3.2 Microwave treatment

Microwave processing of shrimp was conducted in a Mini WAVE Digestion Module (SCP Science, 115 V–60 Hz, 15 A, 1000 W, Canada) that operates at a frequency of 2.45 GHz. Ten grams of shrimp were mixed with 20 grams of distilled water in a cylindrical quartz reactor vessel by using a vortex mixer to avoid aggregation after processing. Each experiment was

done in triplicate. Shrimp samples were treated using a microwave reaction system with the following conditions: (1) 75°C for 5 min, 75-5; (2) 75°C for 10 min, 75-10; (3) 75°C for 15 min, 75-15; (4) 100°C for 5 min, 100-5; (5) 100°C for 10 min, 100-10; (6) 100°C for 15 min, 100-15; (7) 125°C for 5 min, 125-5; (8) 125°C for 10 min, 125-10; (9) 125°C for 15 min, 125-15. IR sensors located on the sidewalls of the chamber that monitor the sample temperatures. The heating time was set for 1 min to reach the processing temperature 75°C, 100°C, and 125°C and then was held at the set temperature for processing 5, 10, 15 min. Once the treatment was completed, the shrimp solution was removed, and the temperature was measured. The reported temperature was within an error of ±2 °C. After the treatment, certain samples were stored at 4°C for color and microstructure analysis. All of the other processed samples were dried by a freeze drier (7420020, Labconco Corporation, Kansas City, USA) for 48 h and then frozen at −20°C for further analysis. All treatments and analyses were performed in triplicates.

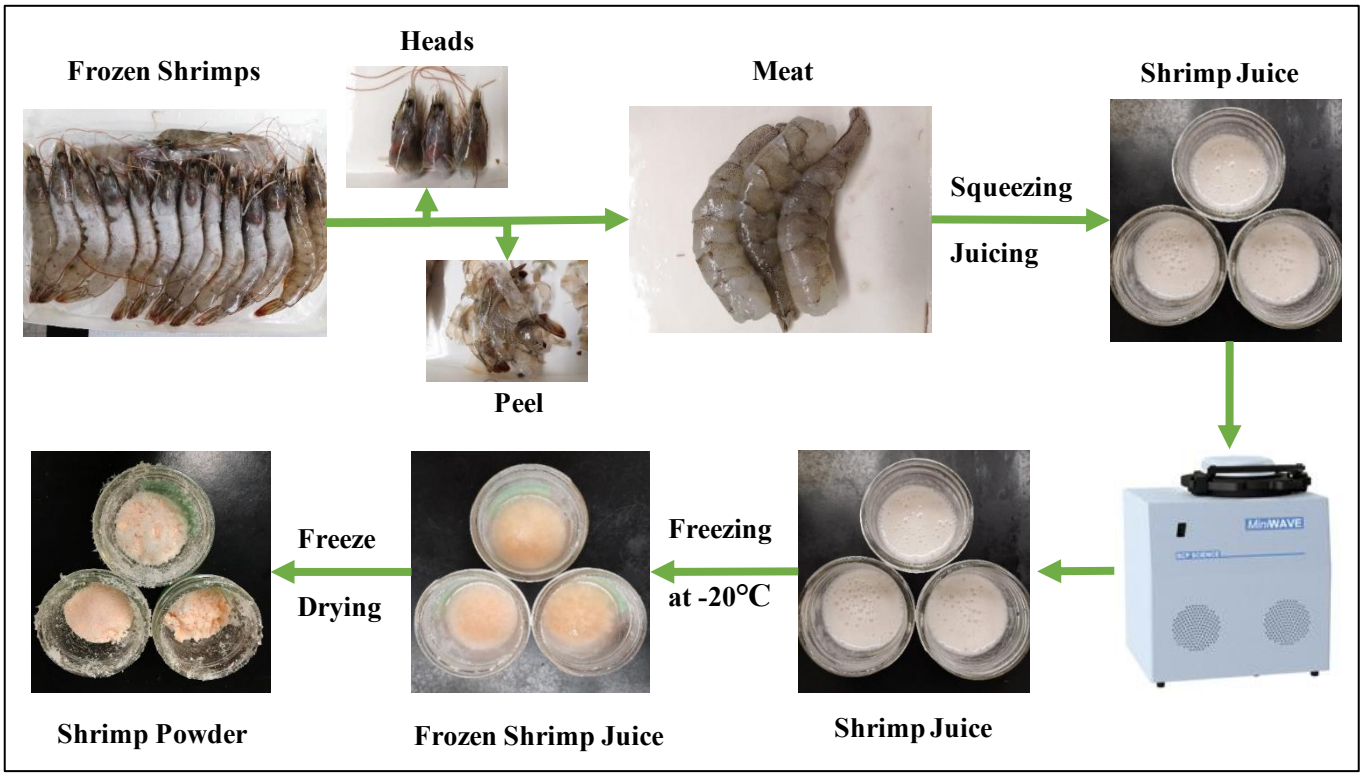

Figure 5.1. Schematic overview of the pretreatment of shrimp samples

5.3.3 Total soluble protein content

The total soluble protein content of shrimp samples was determined by a Pierce BCA protein assay kit (purchased from Thermo Fisher Scientific, Canada) according to the protocol of the protein assay kit (Jin Wang, Sai Kranthi Vanga, & Vijaya Raghavan, 2019).

5.3.4 SDS-PAGE

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was performed according to Laemmli (1970) with some modifications. After each electrophoresis, gels were stained using 0.1% Coomassie Brilliant Blue R-250 (Sigma-Aldrich Chemical, St. Louis, MO, USA). Protein molecular weight markers were used to estimate shrimp protein molecules (SDS-6H, Sigma-Aldrich).

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

Sandwich ELISA (Shrimp Tropomyosin ELISA 2.0, Indoor Biotechnologies, USA) was used to measure the tropomyosin content as described by Faisal, Buckow, Vasiljevic, and Donkor (2019) with some modifications. Each sample was tested in replicate. Microwells on the plate were pre-coated by anti-tropomyosin antibodies. The standards and samples were added to each well and then incubated for 1 hour at room temperature away from direct sunlight. The microwells were washed three times, with 150 µL wash buffer to remove unbound proteins. Each well was added 100 µL detection antibody/peroxidase-conjugate mix and incubated for 1 hour at room temperature away from direct sunlight. After subsequent washing with 150 uL wash buffer, 100 µL of TMB was added to all wells. As the blue color develops, add a 50uL stop solution to all wells. Finally, read the absorbance within 10 min using ELISA plate reader at 450 nm.

5.3.6 Secondary structure

Fourier transform infrared spectroscopy (FTIR) technique was applid to study the secondary structures of shrimp proteins. The sample powder was palced at the diamond crystal under normal air. Windows-based OMNIC software (Version 8, Thermo Nicolet Co., Madison, WI)

was used for data analysis, which was connected to the FTIR spectrometer (Nicolet Magna 158,750 FTIR, Nicolet Instrument Corp., Madison, WI). There were 32 scan spectra at a spectral resolution of 4 cm^{-1} recorded and averaged in the mid-infrared region (4000–500 cm^{-1}). A background spectrum with no sample was collected under the same conditions to avoid the impact of air.

OriginPro (Version 9, Origin Lab Corporation, Northampton, MA, USA) was used for curve-fitting procedure to quantify the conformational changes of shrimp protein in the amide I band. The component bands were measured by using the second derivative spectrum. The results were analyzed according to the peak areas at the wavelength ranges.

5.3.7 Analysis of total antioxidant capacity

In the study, Ferric-reducing/antioxidant power (FRAP) assay, as described by Wang et al. (2019), was performed to test the total antioxidant activity of shrimp samples. FRAP working solution was prepared by mixing ferric chloride solution (20 mM), acetic acid buffer (300 mM), and 2,4,6-tripyridyl-S-triazine (TPTZ) (10 mM) in the ratio of 1:1:10 (v/v/v), respectively, at room temperature. The extraction of antioxidants from shrimp samples was done by mixing samples with methanol. 200 mL of FRAP solution and 67 μL of the extract was added to the microwells of plates, respectively. The plate was incubated with a slight shake for 5 min at 37 °C in the dark. A spectrophotometer was used to determine the color change at a 593 nm. Standard curve was performed by preparing stand solutions from a 1000 μM ferrous sulphate solution. The antioxidant activity of the shrimp sample was presented as μmol Fe (II)/100 mg of samples.

5.3.8 Color measurement

The color parameters of shrimp samples ws determined by a colorimeter (CR-300 Chroma, Minolta, Japan) with 2° N skylight as the light source. In the calibration procedure, a specific hand shank was placed directly on a standard white tile (Y = 93.35; x = 0.3152; y = 0.3212) to ensure the value shown on the screen same as that on the tile. L* (light /dark), a* (red/green) and b*(yellow/blue) would be recorded in terms of the Commission Internationale de

l'Eclairage (CIE) Lab color parameters, with the range from 0 (black) to 100 (white), −60 (green) to +60 (red), and −60 (blue) to +60 (yellow), respectively (Pathare, Opara, & Al-Said, 2013; Xiao, Gao, Lin, & Yang, 2010). The following equations (1)-(5) were used for evaluating the total color difference (TCD), hue angle (h), chroma (C), yellow index (YI), and color index (CI) (Ordóñez-Santos, Martínez-Girón, & Arias-Jaramillo, 2017):

$$TCD = \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2} \quad (1)$$

$$h = \tan^{-1}\frac{b^*}{a^*} \quad (2)$$

$$C = \sqrt{a^{*2} + b^{*2}} \quad (3)$$

$$YI = \frac{142.86 b^*}{L^*} \quad (4)$$

$$CI = \frac{180 - h}{L^* - C} \quad (5)$$

Note: The parameters a_0^*, b_0^*, and L_0^* represent the samples without microwave treatment and were immediately determined after homogenization; a*, b*, and L* represent the samples with microwave treatment and were determined after every processing. Each sample was measured eight times to reduce bias.

5.3.9 Microstructure

A Scanning Electron Microscope (SEM) (TM3000, Hitachi High-Technologies Corporation., Tokyo, Japan) was applied to shrimp samples, which is aim to observe the microstructural alteration of each sample under different ultrasonic treatments according to Jin Wang, Sai Kranthi Vanga, and Vijaya Raghavan (2019). The shrimp homogenates obtained by mixing shrimps and water were applied to microwave treatment, and then were freeze-dried for 48 h in a freeze drier (7420020, Labconco Corporation, Kansas City, USA). The samples at powder status gained eventually and were stored at -20°C until analysis. Before the examination of microstructure, each sample should be at room temperature for at least 30 min and was randomly stuck a few pieces to measuring platform. The level of magnification was observed

at × 500.

Twenty μL of diluted shrimp samples were transferred to the glass slide, and 0.1% of toluidine blue solution was used for staining for 2 min (Stratakos et al., 2016). An optical microscope equipped with a digital camera (Leica DM500, Leica Microsystems Inc., Canada) was used in the observation procedure. An imaging software (Leica LAS EZ, Leica Microsystems Inc., Canada) was used to capture images at a 10× magnification.

5.3.10 Simulated vitro gastrointestinal digestibility

The *in-vitro* protein digestibility of shrimp proteins was conducted in two stages as described by Hejazi and Orsat (2016) and Vilela, Lands, Chan, Azadi, and Kubow (2006) with some modifications. In the first stage, shrimp powder was mixed with the double distilled water to a protein concentration of 3 mg/mL with the pH of 1.5 adjusted by using1.0 N HCl. The digestion was conducted in a water bath at 37°C, and started by adding fresh pepsin solution (5 mg pepsin/mL in 0.01 M HCl) to reach an enzyme to substrate ratio of 1: 100. After 30 min, 1.0 M NaOH solution was added to stop the reactions. A 1.0 M NaOH solution was added to adjust pH to 7.8. The second stage of digestion was started by adding fresh pancreatin stock solution (5 mg/mL in sodium phosphate buffer, pH 7.0) to reach an enzyme to substrate ratio of 1: 30 and incubated at 40ºC. The digestion was stopped by using a 150 mM Na_2CO_3 solution was used to stop after 60 min of digestion. The total protein contents of shrimp samples were determined using the BCA method (Beazley, 2017). The protein content of shrimp samples that were not treated with pepsin and pancreatin were also measured. The *in-vitro* protein digestibility was calculated using the following equation:

$$\text{IVPD}\% = \frac{\text{initial protein} - \text{final undigested protein}}{\text{initial protein}} \times 100 \qquad (6)$$

5.3.11 Determination of peptide content

The peptide content was determined according the methods suggested by Church, Porter, Catignani, & Swaisgood (1985). Fifty mL of OPA reagent was prepared by adding 25 mL of 100 mM sodium tetraborate in water, 2.5 mL of 20% (wt/wt) sodium dodecyl sulfate, 40 mg of

OPA dissolved in 1 mL of methanol, 100 μL of β-mercaptoethanol and ddH_2O. A 50 μL sample of digestion mixture was incubated with the 950 μL OPA reagent solution for 2 min. Absorbances were read at 340 nm by using a spectrophotometer (Thermo Fisher Scientific, Canada). Leucine-glycine was used as a standard peptide to obtain a standard curve.

5.3.12 Statistical analysis

The analysis of variance (ANOVA) of the SPSS software (SPSS Inc., Ver. 18, Chicago, IL, USA) was applied to analyze the experimental data. The means and significance ($p \leq 0.05$) were separated by using the Duncan multiple-range test.

5.4 Result and discussion

5.4.1 Changes of color attributes

Color of shrimps plays a significant role in food quality evaluation and marketing consumption. The eye-catching color of food products can effectively facilitate the acceptance and satisfaction level by customers. As shown in Table 5.1, the color parameters (L*, a*, b*, TCD, C, h, CI, and YI) of shrimp samples with and without microwave treatment are summarized. The lightness (L*) of samples increased significantly with the increase of temperature and time, which was probably because the shrimp meat was cooked under high temperature. Again, the results showed the red/green color (a*) was firstly developed and then decreased to lower values compared with the untreated sample. Development of redness on the exposure of shrimp meat to heat is due to the release of astaxanthin when carotenoproteins breakdown during protein denaturation (Akonor, Ofori, Dziedzoave, & Kortei, 2016). The tendency of later decrease may be due to an increase in the number of carotenoid degradation products, as observed by (Condurso et al., 2016; Martínez-Alvarez et al., 2020). Yellowness (b*) of shrimp meat also increased after treatment, probably due to the formation of yellow pigments from browning reactions (Akonor et al., 2016).

Table 5.1. The color parameters L*, a*, b*, TCD, C, h, CI, and YI of shrimp samples under MW treatment

	L	a	b	C	h	CI	YI	TCD
CK	48.37±0.05 [j]	2.63±0.06 [g]	5.28±0.07 [i]	5.9±0.04 [i]	1.11±0.01 [j]	4.21±0.01 [i]	15.58±0.22 [j]	
75-5	48.93±0.02 [i]	5.24±0.03 [b]	13.55±0.03 [a]	14.52±0.03 [b]	1.2±0 [g]	5.2±0.01 [a]	39.54±0.07 [a]	8.69±0.05 [b]
75-10	51±0.02 [f]	4.94±0.04 [c]	10.96±0.05 [f]	12.02±0.05 [d]	1.15±0 [h]	4.59±0.01 [d]	30.71±0.16 [f]	6.68±0.06 [d]
75-15	49.77±0.01 [h]	6.33±0.03 [a]	13.21±0.06 [b]	14.64±0.06 [a]	1.12±0 [i]	5.09±0.01 [b]	37.91±0.17 [b]	8.86±0.08 [a]
100-5	51.25±0.03 [e]	3.29±0.05 [e]	11.34±0.04 [d]	11.81±0.04 [e]	1.29±0 [e]	4.53±0 [e]	31.61±0.11 [d]	6.75±0.08 [d]
100-10	51.83±0.02 [b]	3.04±0.02 [f]	10.75±0.03 [g]	11.17±0.03 [g]	1.3±0 [d]	4.4±0 [h]	29.63±0.1 [h]	6.49±0.05 [f]
100-15	50.38±0.07 [g]	4.13±0.06 [d]	12.85±0.05 [c]	13.5±0.06 [c]	1.26±0 [f]	4.85±0.02 [c]	36.45±0.19 [c]	7.98±0.04 [c]
125-5	51.46±0.02 [d]	2.68±0.03 [g]	11.25±0.03 [e]	11.57±0.03 [f]	1.34±0 [c]	4.48±0 [f]	31.24±0.09 [e]	6.73±0.03 [d]
125-10	52.53±0.01 [a]	1.79±0.04 [h]	9.92±0.02 [h]	10.08±0.02 [h]	1.39±0 [b]	4.21±0 [i]	26.98±0.06 [i]	6.29±0.05 [g]
125-15	51.57±0.05 [c]	1.74±0.05 [i]	10.97±0.13 [f]	11.1±0.14 [g]	1.41±0 [a]	4.41±0.02 [g]	30.38±0.4 [g]	11.1±0.14 [e]

Note: Different letters in the same column reveal significant differences ($P < 0.05$).

According to Abers and Wrolstad (1979), the h value has the most significant correlation with visual scores, and the C value is a good indicator of the amount of color. As shown in Table 5.1, the h values and C values of the treated shrimp samples were significantly ($P < 0.05$) increased compared to those of the fresh samples. The CI values in treated samples significantly ($P < 0.05$) increased firstly and afterward decreased to the same value as the control. This indicated the microwave treatment could improve the color attributes of shrimps through increasing CI values and C values, whereas cannot avoid the h values increasing. Compared to the control, more than a twofold increase of treated samples was observed with a significant difference in the yellow index (YI) value in all microwave-treated samples, which may be a result of the formation of browning reactions. The most significant TCD values of samples

were measured at the last treatment, which may indicate that the increase of color range has a positive effect on the time duration and temperature increase.

5.4.2 Total protein and total antioxidant capacity (TAC)

As shown in Table 5.2, the results of total soluble protein and total antioxidant capacity (TAC) in shrimp samples are summarized. It was noticed that the total soluble protein content of microwave-treated samples significantly decreased by 49.76% to 74.73% compared with the control. There was an overall downward trend in the treatments at 75°C and 100°C, from 193.88 mg/g in control to 49.57-63.38 mg/g. Consequently, an upward trend was observed when the temperature went to 125°C with the processing time for 10 min (76.75 mg/g) and 15 min (97.41 mg/g). A similar reduction of protein content in rice was observed from 8.9% to 8.3% with the increase of microwave energy consumption (0-0.027 kw×h/kg) (Zhao, Xiong, Qiu, & Xu, 2007). However, the total protein content in chickpea (*Cicer arietinum L.*) seeds treated by microwave at a high level for 15 min had no significant difference with that of the untreated sample (Alajaji & El-Adawy, 2006). Similarly, Walde, Balaswamy, Velu, and Rao (2002) found the total protein content of wheat samples dried by microwave (700W, up to 150 s) remained the same as that of the control (9.9%). Campana, Sempe, and Filgueira (1993) reported that the total protein content in wheat was not affected even by microwave heating at 91 °C. These different results might be due to various food protein structures and treatment conditions. The reduction of total soluble protein content indicated that large amounts of proteins were degraded and hydrolyzed into peptides or amino acids. In contrast, the increase of total protein in the last two treatments could be due to the dehydration of samples under extremely high temperatures for a long time (Al-Haidary et al., 2012). Further, the unfolding protein and increasing peptide bonds may contribute to the good bioactivities such as the increasing of TAC (Ketnawa & Liceaga, 2017).

The comparison of the total antioxidant capacity of treated and untreated samples is shown in Table 5.2. There was a first deduction from 31.78 μmol/100 mL in control to 24.20 μmol/100 mL in 75-5, followed by an overall increment in the later treatments compared to the control.

Table 5.2. Changes of bioactive compounds in microwave-treated shrimp samples

Treatment	Total protein (mg/g)	Tropomyosin (ng/mg)	TAC (μmol/100 mL)
Control	193.88±19.18 ^{a}	1.248±0.14 ^{a}	31.78±3.22 ^{f}
75-5	56.40±10.52 ^{fg}	0.52±0.13 ^{bc}	24.20±6.79 ^{g}
75-10	49.57±3.67 ^{h}	0.64±0.12 ^{bc}	46.93±4.28 ^{e}
75-15	63.38±2.46 ^{d}	0.548±0.14 ^{bc}	59.5±16.55 ^{c}
100-5	62.24±1.22 ^{de}	0.672±0.19 ^{b}	47.13±3.43 ^{e}
100-10	53.42±8.85 ^{g}	0.368±0.134 ^{bc}	50.03±13.4 ^{e}
100-15	59.45±1.81 ^{ef}	0.352±0.16 ^{bc}	71.13±5.13 ^{a}
125-5	58.67±1.55 ^{f}	0.328±0.12 ^{bc}	59.55±1.06 ^{c}
125-10	76.75±1.05 ^{c}	0.344±0.12 ^{bc}	55.2±18.88 ^{d}
125-15	97.41±1.15 ^{b}	0.312±0.13 ^{c}	65.78±6.54 ^{b}

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

The highest TAC value of 71.13 μmol/100 mL was observed in 110-15. Similarly, an increase of antioxidant activity was observed in *Scomberomorus niphonius* under microwave treatment (400 W, 40°C) for 5 min by Huang, Ruan, Qin, Li, and Zheng (2017). Oppositely, the total antioxidant capacity of broccoli florets and stems declined from 60.5% to 21.0% and 62.8% to 21.7%, respectively, with the time increase (0-300s) in microwave processing due to a large number of antioxidants being dissolved in the cooking water (D. Zhang & Hamauzu, 2004). Ketnawa, Wickramathilaka, and Liceaga (2018) found the peptides hydrolyzed from protein can be a rich source of natural antioxidants. It is generally agreed upon that peptides possess substantially better antioxidant activities than their parent proteins; this is possibly due to increased accessibility of the functional side of the chain to the reactive species and the

electron-dense peptide bonds generated by enzymatic hydrolysis (Udenigwe & Aluko, 2011). Further, an increase in antioxidant activity is probably related to the molecular weight of peptide; specifically, higher antioxidant activity might be associated with the lower molecular-weight peptides (Sae-leaw & Benjakul, 2018). This might indicate that the lower molecular-weight peptides were produced more in the process.

5.4.3 *In-vitro* protein digestibility and peptide determination

The effect of microwave processing technique on the *in-vitro* protein digestibility (IVPD) of shrimp samples is demonstrated in Figure 5.2. The digestibility of the untreated sample was 80.46%, while the digestibility of the treated samples was significantly decreased, with the lowest IVPD at 64.19% in 75-10. In a similar study, durum wheat semolina was heated at varying time periods (1, 1.5 and 2 min) in a microwave oven set at 900 W. The results suggested *in-vitro* protein digestibility to have decreased significantly from $71.35 \pm 0.05\%$ of the untreated sample to $53.64 \pm 0.04\%$ of the two min-heated samples. Kadam, Smithard, Eyre, and Armstrong (1987) also found animals feed in the microwave treated winged bean meal to have lower *in-vitro* protein digestibility (37.28%) as compared with the untreated meal (50.52%). This reduction in protein digestibility may be due to the alteration of protein molecules, blocking of active amino acid sides, and establishment of crosslinks and development of compounds responsible for digestible enzymes inhibition, inhibition of aminopeptidase through advanced Maillard derivatives of lysine (Mauron, 1990).

However, the IVPD value increased in the last two treatments compared with the other treated samples under lower temperatures and shorter time durations, although the raised values were still lower than the IVPD of the control. Similar results were observed by Vagadia et al. (2018). They subjected microwave processing (2450 MHz, 1000W) at different conditions of temperature (70, 85, and 100°C) and time (2, 5, and 8 min) to soymilk samples; they found the IVPD to have increased with increase in time and temperature during microwave processing. This might be due to the protein denaturation and the proceeding digestion as the increasing time and temperatures (Vagadia, Vanga, Singh, Gariepy, & Raghavan, 2018).

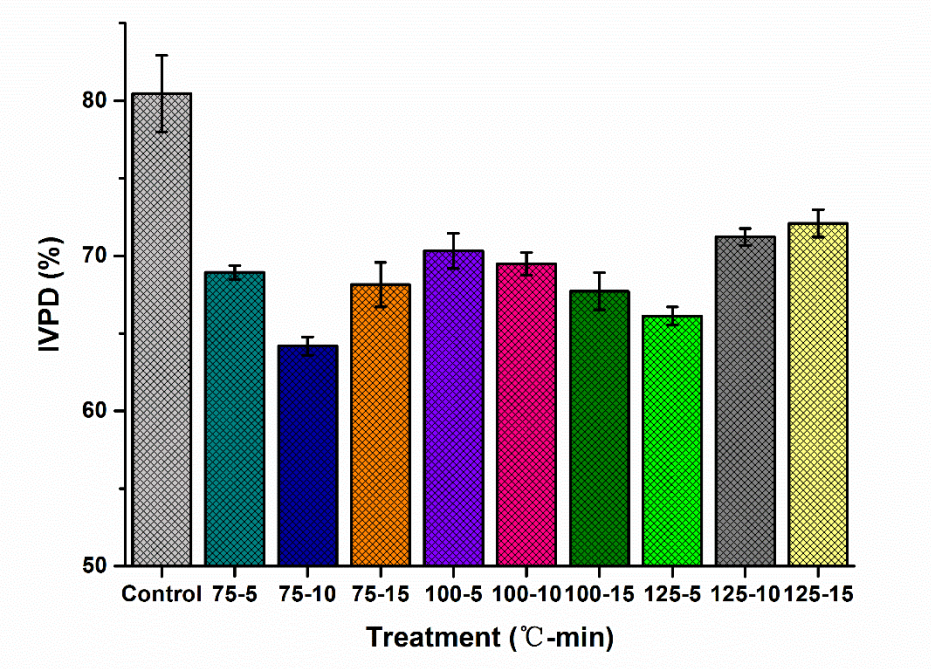

Figure 5.2. The *in-vitro* protein digestibility (IVPD) of shrimp samples under microwave processing treatment

In Figure 5.3, the peptides of treated samples overall decreased in comparison to that of the control. After two-stage digestion, peptides produced at 75 °C and 125 °C showed upward trends of 12.16-13.93 mM and 13.52-15.42 mM with the increase of processing time, respectively. The highest peptide concentration in treated samples was 15.42 mM in 125-15. At 100 °C, initial peptides (12.90 mM) first decrease to 11.42 mM and then increased to 11.87 mM at 15 min. Large amounts of peptides hydrolyzed from milk proteins pretreated by microwave at 800 W up to 8 min when five enzymes (Alcalase, Trypsin, Neutrase, Alkaline Protease, and Flavourzyme) were applied (Uluko et al., 2013). Moreover, microwave treatment (90 °C for 10 min) of milk proteins before hydrolysis increased peptides produced (Uluko et al., 2015). This can be explained that microwave reduced the affinity of the enzymes to the substrate and caused the formation of hydrophobic interactions. Microwave can facilitate the destruction of the peptide bond by a localized interaction with each polarizable amide group of the peptide chain (Margolis, Jassie, & Kingston, 1991). The reason for the decrease of peptides in treated samples compared with the control is that protein denaturation occurred after microwave treatment and led to the reduction of protein content, further resulting in the decrease of peptide generated. The increasing trend in treated samples can be explained that

protein denaturation occurred during the heating process by doing damage to native protein structures. The degraded protein fractions changed into peptides or amino acids, and visually showed increasing values of peptide contents.

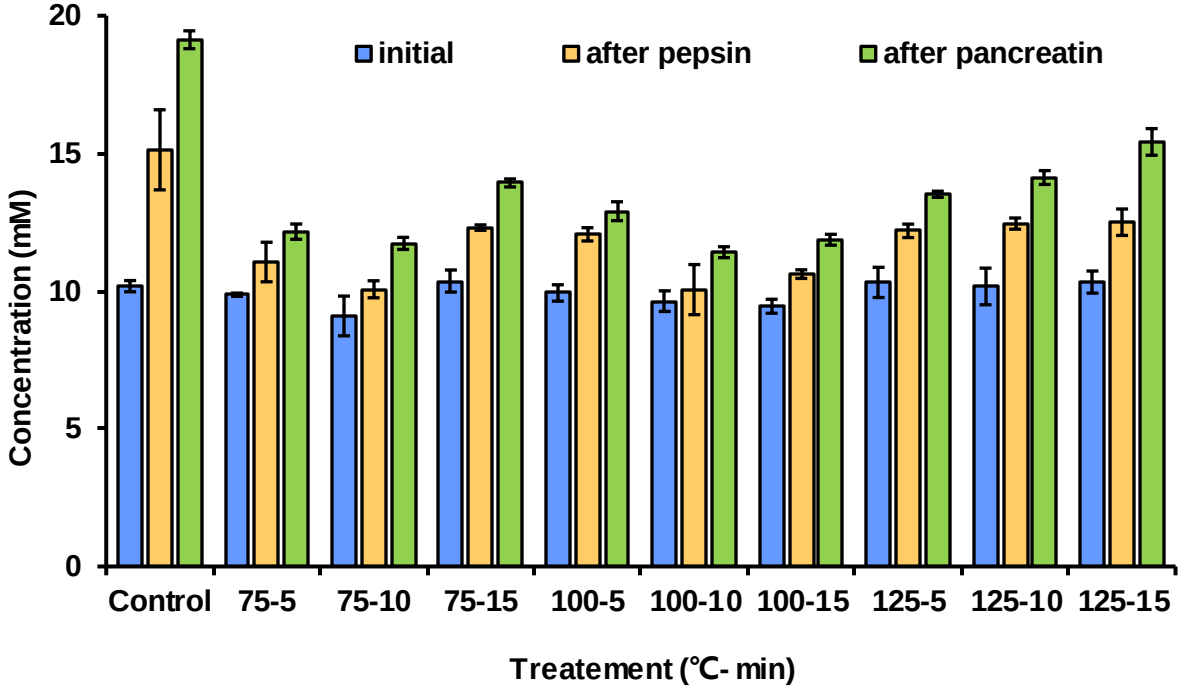

Figure 5.3. The peptides hydrolyzed of microwave treated and untreated shrimp samples

5.4.4 Changes of secondary structure

As proteins represent the vital components in shrimp, FTIR spectroscopy analysis is vital to be used to test the effects of microwave on the secondary structure of shrimp proteins. Figure 5.4 shows the FTIR spectra of shrimp samples subjected to various microwave treatments. Figure 5.5 shows the peak areas for microwave processing at 75°C, 100°C, and 125°C for 5, 10, and 15 min. The amide I frequency bands (1600-1700 cm^{-1}) of protein secondary structures is shown in Table 5.3. β-sheets, located at the lowest and highest frequency in the provided frequency range, representing the most significant proportion (52.22-67.80%) in protein structures as shown in Figure 5.5. In all microwave-treated samples with an exception of 100-10, a remarkable increase of β-sheet proportion was observed compared with the control. Although a slight decline of α-helix proportion was found in all treated samples except for the 75-5 in comparison to the control, α-helix still plays a second-dominant role in protein

structures with a percentage of 15.56 to 24.02. Turns account for 5.67-16.96% of protein structures after treatment, which all decreased compared with the percentage of the control (18.88%). The least structure proportion was unordered in most samples, with the largest value of 14.9 at 100-10 and the least value of 3.99 at 75-5. The percentage changes of each secondary structure might be attributed to the relocations from each other (Vanga et al., 2016).

Table 5.3. Amide I band frequencies and assignment to protein secondary structure (Vanga et al., 2016)

Secondary structure	Frequency range (cm^{-1})
β-sheet	1613–1637; 1682–1696
Unordered	1637–1645
α-helix	1645–1662
Turns	1662–1682; 1630

Gomaa, Nsonzi, Sedman, and Ismail (2016) reported that microwave might enhance unfolding pathways in protein structure due to its non-thermal effects. They evaluated the effects of microwave heating (over a temperature range of 40-90 °C) on bovine β-lactoglobulin (β-Lg) and found α-helical secondary structures to have decreased under microwave heating similar to the study results of Zhu et al. (2018). In their study, microwave treatment was performed at 60°C, 70°C, and 80°C for 1, 3, and 5 min. The results revealed that the secondary structure of the β-sheets increased at temperatures 60°C and 70°C as the processing time rose while became almost constant at 80°C. α-helices were found to decrease with time at each of the temperatures at which microwave was applied. Turns and unordered structures were stable. In peanut proteins heated by microwave (50, 75, and 100°C) for 5, 10, 15, and 20 min, increments of α-helices and unordered structures were observed with an increase in the treatment time and temperature Vanga et al. (2016). In soybean proteins after microwaving at 600 W for 3 min, the amount of α-helix showed no change while that of the β-sheet decreased. However, both the turns and unordered structures increased to different degrees. This indicated the protein

structure to have become loose due to the heating effect of the microwave treatment (Wang & Chi, 2012).

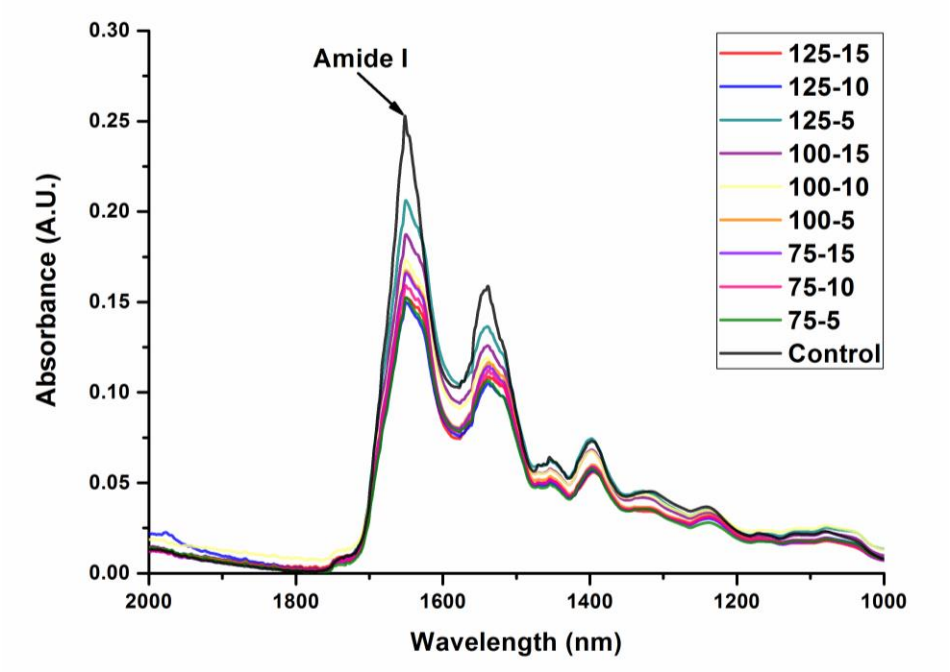

Figure 5.4. FTIR spectrum of raw and microwave processed shrimp samples

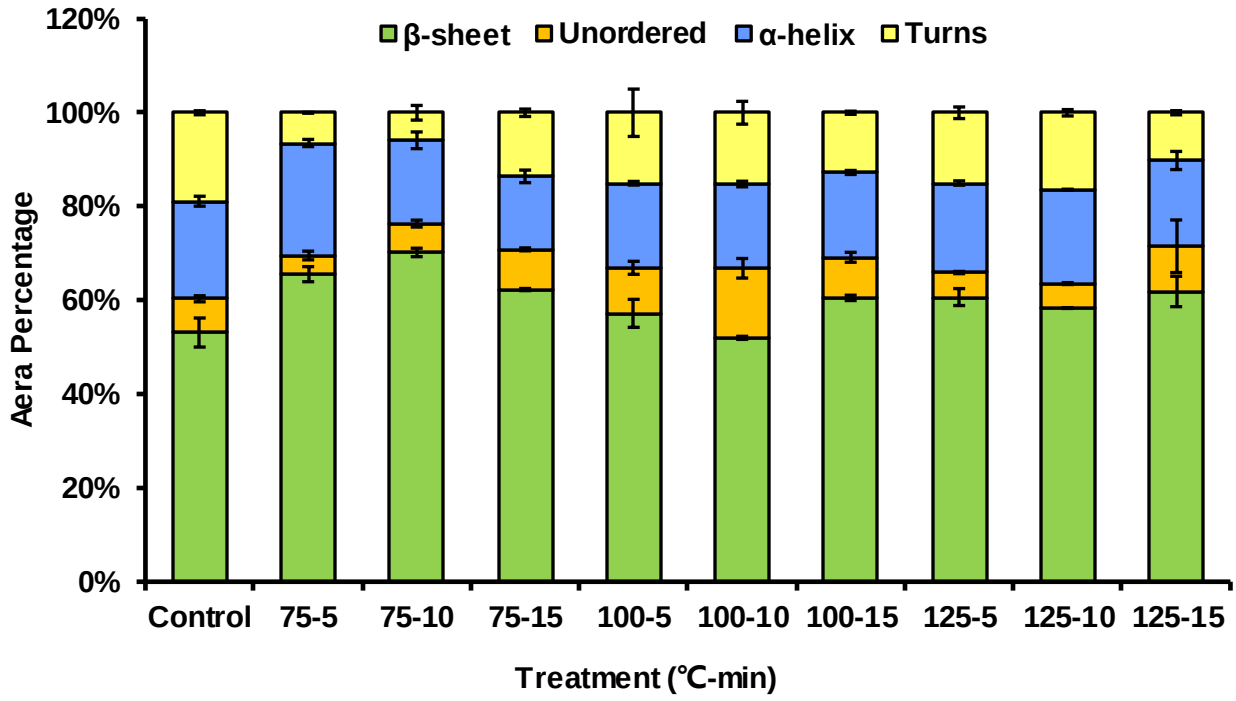

Figure 5.5. Variations in relative areas of the bands fitted to the normalized FTIR spectra of the Amide I region (1700–1600 cm^{-1}) of shrimp samples

5.4.5 Allergenicity

The tendency of allergenicity was implied by the content of shrimp major allergen tropomyosin detected by using a sandwich enzyme-linked immunosorbent assay (ELISA). As shown in Table 5.2, microwave-treated tropomyosin exhibited a gradually weak immune response trend as the increasing temperature and time, with the tropomyosin content decreasing from 1.248 ng/mg in control to 0.312 ng/mg in 125-15. The tropomyosin contents in the treated samples almost had no significant difference. During 75°C, there was a fluctuation of tropomyosin contents with a range of 48.71% to 58.33%. During 100 and 125°C, there was a downward trend with the increase of time; except for the 100-5 (0.672 ng/mg), other treated samples were reduced by 70.51% to 75%. The maximum reduction of allergenicity was 75% at 125°C for 15 min. The decreased tropomyosin might be due to the microwave destroying the conformational or linear epitopes of tropomyosin molecules.

Microwaves have the potential to alter the native structure of proteins and therefore might potentially have an impact on the ability of specific proteins to be recognized by IgE of sensitized subjects (Jiménez-Saiz, Benedé, Molina, & López-Expósito, 2015). As described by Zellal, et al. (2011), the allergenicity of β-lactoglobulin of whey proteins tested by ELISA markedly reduced after microwave irradiation at 300 or 700 W. However, Cabanillas and Novak (2019) reported microwave processing treatment to have little effect on food allergenicity. Certain studies also confirmed this issue. Su, Venkatachalam, Teuber, Roux, and Sathe (2004) evaluated the antigenicity changes of certain proteins in tree nuts such as almond, cashew or walnut; they found the allergenicity was stable after microwave heating at 500 W for 1 or 3 minutes. Sharma, Roux, and Sathe (2009) and Leszczynska, Łącka, Szemraj, Lukamowicz, and Zegota (2003) also obtained similar results in Brazil nut and wheat, respectively.

To better understand this phenomenon, protein bands of shrimp samples were separated by SDS–PAGE, as shown in Figure 6. Analysis of these fractions by SDS–PAGE showed that the microwave treatment caused a decrease in the number and intensity of bands. Our results also showed that in SDS-PAGE, the band corresponding to tropomyosin (molecular mass around

36 kDa) exhibited a trend to be weaker and thinner with the rise of temperature and time, which suggested a decrease of tropomyosin under microwave heating. This result is correlated to the values determined by the ELISA and confirmed that microwave treatment might be helpful to eliminate the allergenicity of tropomyosin in shrimp, despite different effects observed in other different foods.

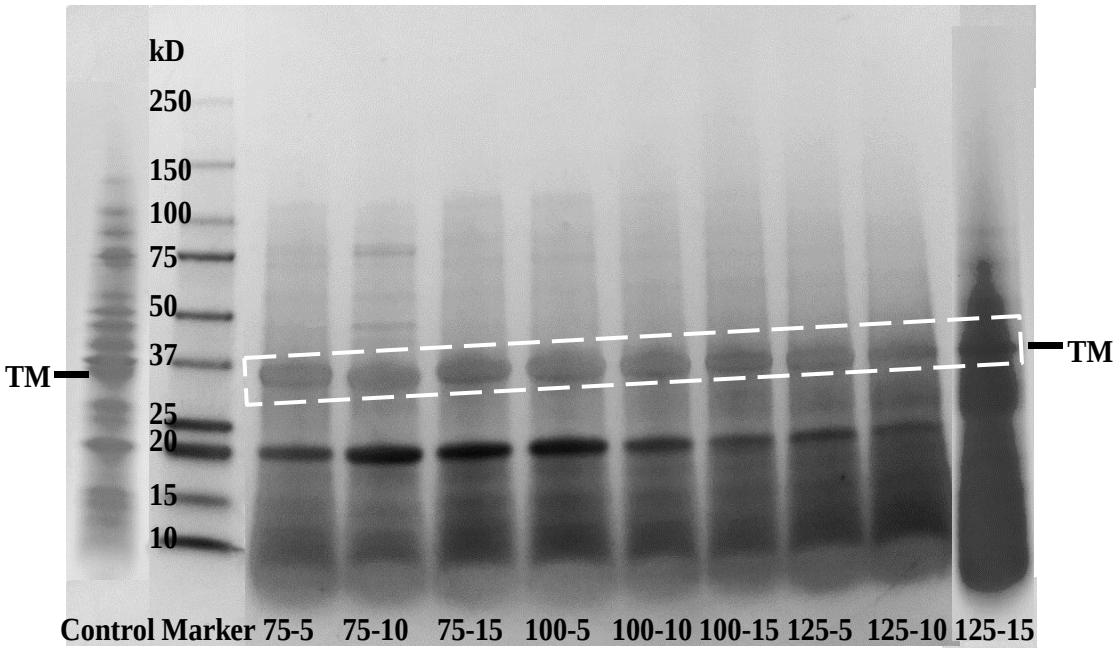

Figure 5.6. SDS-PAGE of microwave treated and untreated shrimp samples

5.4.6 Microstructure observation

As shown in Figure 5.7, microwave treatment has a significant effect on the microstructure destruction of shrimp samples. It was clear that the cells observed in the control were intact and dense, while a greatly structure degradation of samples occurred after treatment of 75°C for 5 min. With the longer time and higher temperature, less and less samples can be seen in the view gradually, and eventually, the cells totally vanished when treated by 125°C for 10 min and 15 min.

Figure 5.8 shows the SEM images of the freeze-dried shrimp samples after the different microwave treatments. Compared with the control, the samples treated at 75°C exhibiting destroyed structures with rough surfaces and more microstructural holes. At 100°C, more

alveolate holes on the surface were observed, with more fragments generated, especially in 100-15. At 125°C, the extremely degraded and collapsed structures were shown with more fragments and strips, whereas the differences in such three samples were not significant. Similar results were reported by Izli and Isik (2015). In their study, the microstructure changes of tomato samples were investigated after microwave treatment (90, 160, 350, and 500W); the results were revealed by the SEM images that a higher temperature and microwave power caused more considerable damage to the microstructure of the tomato samples. Yarmand, Nikmaram, Djomeh, and Homayouni (2013) indicated the microwave treatment (2450 MHz, 600 W for 3 min) can affect the microstructure of camel longissimus dorsi muscle, which could be attributed to the high loss of water and fat caused by protein denaturation and disintegration of the texture matrix with the microwave treatment. In beef semitendinosus muscle during microwave heating (50, 70, 90 °C), the higher the internal endpoint temperature, or the longer the heating led to the higher the insoluble collagen contents viewed by SEM due to the thermal denaturation and shrinkage of collagen (Chang et al., 2011). These were due to the higher internal endpoint temperature or, the longer the heating time of samples during the processing leading to its structural damage, destroying the external surfaces of the samples (Chang et al., 2011; Izli & Isik, 2015).

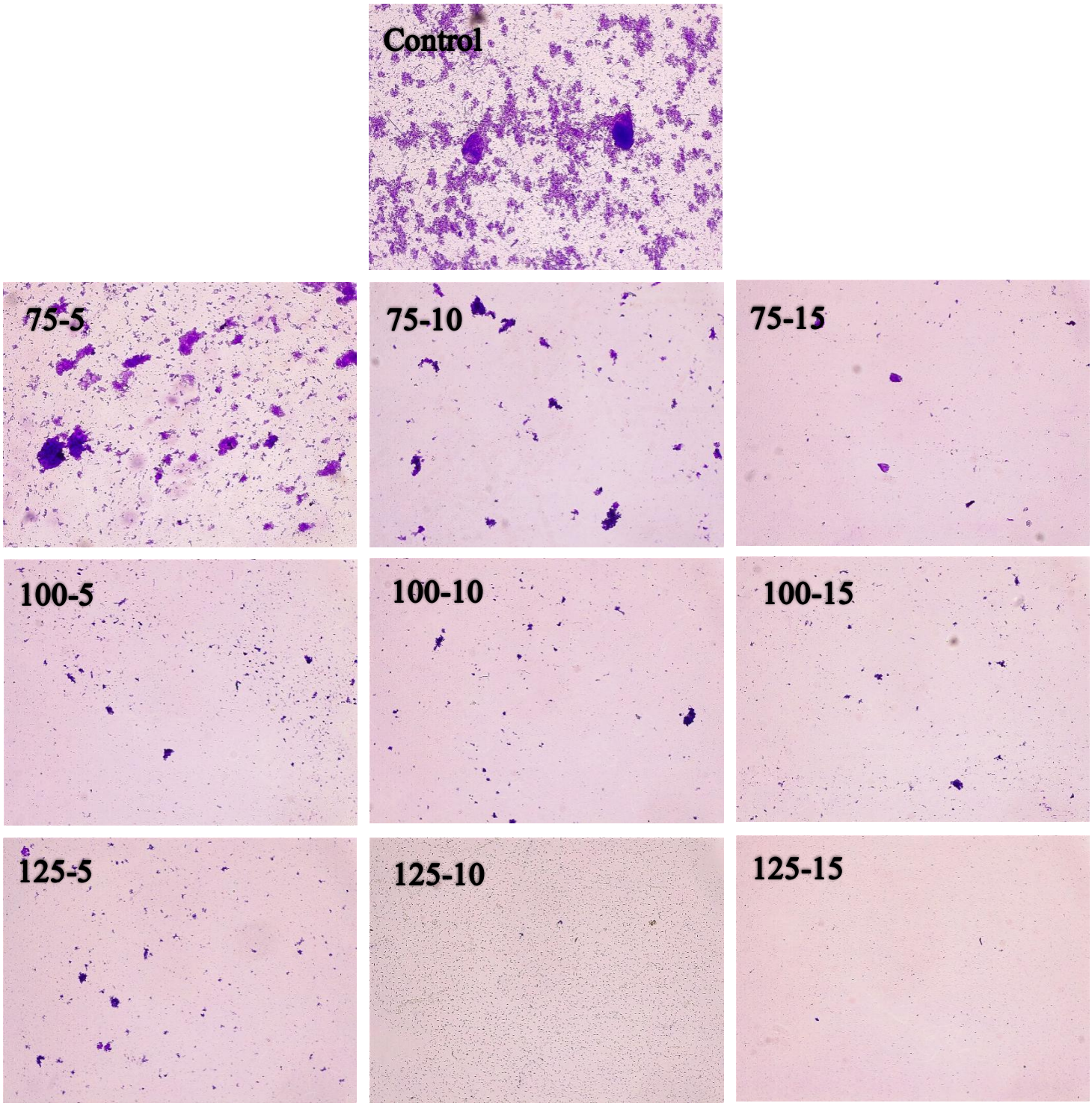

Figure 5.7. Photographs by microscopy of shrimp samples under microwave treatment (magnification at 10×)

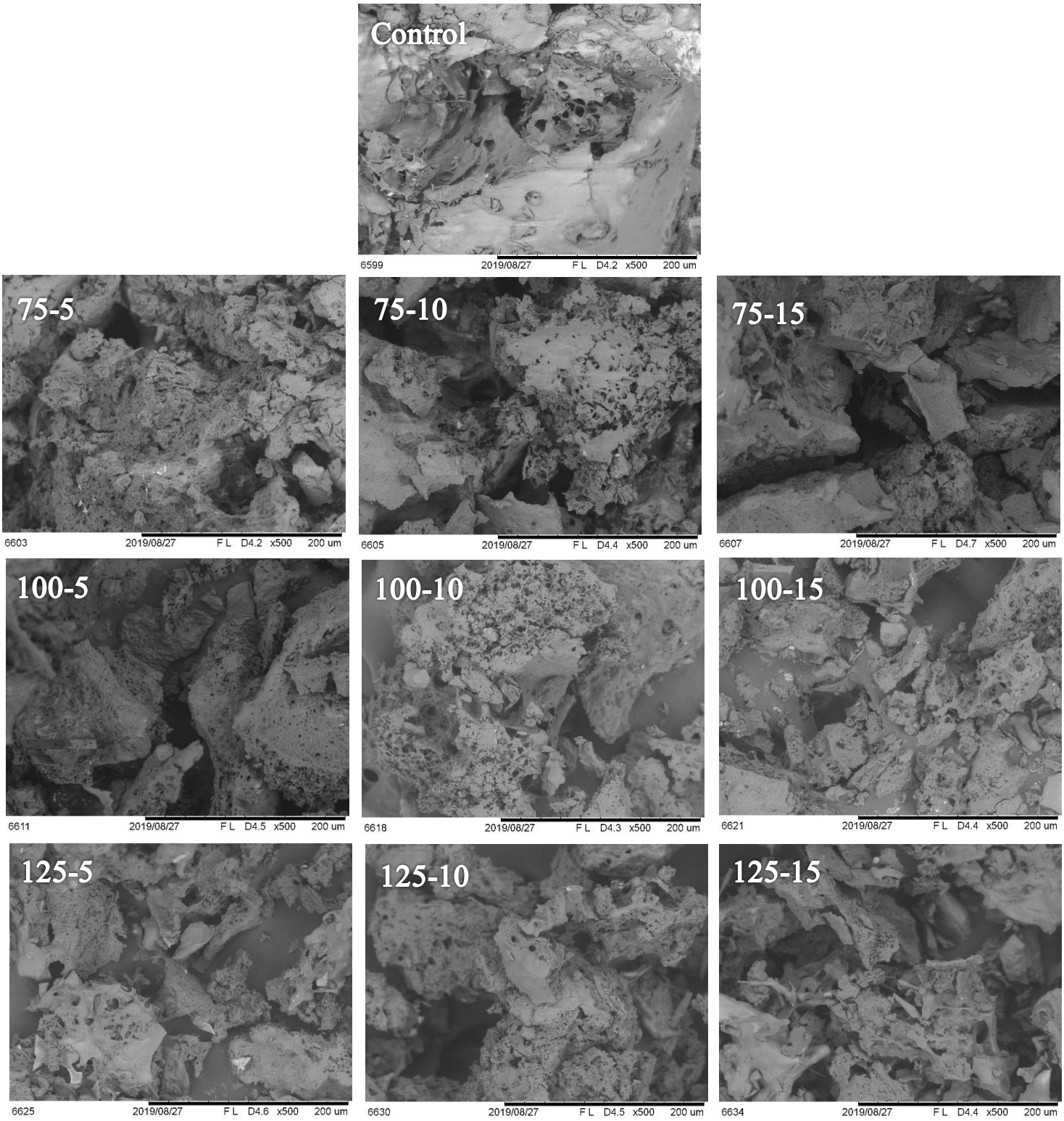

Figure 5.8. SEM photomicrographs of freeze dried shrimp samples treated by micrrowave with different time and temperature (magnification at 500×)

5.5 Conclusion

In the present study, we found microwave processing to have the capability to decrease the allergenicity of shrimp caused by major allergen tropomyosin, which would do great benefit to shrimp-sensitive patients. The percentage of reduced allergenicity (46.15%-75%) was positively related to the increase of microwave temperatures and time; the minimum allergenicity with a 75% reduction was observed in 125-15. Meanwhile, the total soluble protein content decreased by 49.76% to 74.73%, leading to an increase of peptide amounts and further improving the bioactive molecules and antioxidants capacity of shrimps. The total antioxidant capacities in 100-15 and 125-15 were over two times the values of the untreated sample. Meanwhile, shrimp protein degradation and denaturation may induce the increasing of β-sheet and unordered structures in most treated samples, and thus enhancing the destruction of the cell microstructures. Numerous microstructural holes and fragments were observed in microwave-treated samples, especially in 100-15, 125-5, 125-10, 125-15. The *in-vitro* protein digestibility overall reduced from 80.46% to 64.19%, whereas showing an upward trend at 125 °C despite still lower than the value in control. Further studies focusing on the microwave approach with higher temperatures and a longer time should be conducted to provide a better understanding of the shrimp allergenic-protein denaturation process.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Food allergy as a global health issue is expected to be addressed by using some innovative non-thermal processing techniques, such as high-pressure processing, pulsed light, cold plasma, ultrasound, fermentation, pulsed electric field, enzymatic hydrolysis treatment, and combination of them. As discussed above, these novel processing techniques showed potential applications in the reduction of food allergens to produce hypoallergenic foods for individuals living with food allergies. In comparison to every single treatment, the combination of processing has a better performance in most cases. Ultrasound contributes to making proteins more accessible to the action of other treatments, such as enzymatic hydrolysis or thermal processing, thus promoting the reduction of the immunoreactivity of some foods. Long-time fermentation is also recommended as an efficient processing approach reported by studies until now, but the optimal conditions are still needed to be investigated. The pulsed UV light, cold plasma, and enzymatic hydrolysis generally show polarized effects in various foods and might have the potential to be applied to specific food allergens. High-pressure processing and pulsed electric field have a less positive impact on the reduction of food immunoreactivity compared with other non-thermal treatments. Indeed, multiple food allergens typically can be reduced by altering sequential or conformational epitopes and even eliminating them at certain optimal conditions. Thus, to improve the efficiency in the reduction of food immunoreactivity, the optimization of processing conditions (e.g., duration, energy, frequency, and temperature) in each treatment is needed. Further, the immune responses of individuals with food allergy history to these processed or modified foods are unknown, and further clinical studies are still in need. To date, the best therapeutic strategy suggested by the doctors is to avoid exposure to allergenic foods or related food sources.

Until now, many studies focus on the processing impact on the reduction of shellfish allergens, which is one of the "big eight" allergens. Shrimp allergy belonging to shellfish allergy has an increasing prevalence worldwide and possibly leads to mild to severe anaphylaxis. The major allergic protein tropomyosin is heat-stable. Due to the limitations (i.e., not useful to heat-stable

allergens, reduction of nutrients, changes of original sensitive properties) of thermal techniques, more studies investigated the modification of secondary and tertiary protein structures by non-thermal techniques, but the efficiency of these processing methods need to be improved and well-studied. Among nonthermal processing techniques, ultrasound processing has been discussed more and has a satisfying potential in reducing shrimp allergenicity, whereas the investigations about optimal conditions are still needed. Microwave as a novel thermal technique has been applied in many allergenic foods, whereas few studies applied microwave treatment to the modification and elimination of shrimp allergens. Thus, in-depth research for reducing or eradicating shrimp allergens should be focused.

In our fisrt research, we evaluated the impacts of the high-intensity ultrasound processing (0, 5, 10, 15, 20 min) on the physiochemical and allergenic properties of shrimp samples (*Litopenaeus vannamei,* whiteleg), including the allergenicity, antioxidant capability, microstructures, secondary structures, *in-vitro* protein digestibility, and color. The results indicated the allergenicity decreased with the increasing time, and the best hypoallergenic effect showed at 20 min with 76% of tropomyosin reduction. At 20 min, the total soluble protein content decreased by 28.26%, while the *in-vitro* digestibility, peptide content, total antioxidant capacity strengthened by 7.53%, 0.81% and 71.29%, respectively. β-sheets and α-helices increased with time processing, accompanied by the reduction of turns and unordered conformations. More fragments, strips and holes were observed after 20 min, which indicated the physical essence of ultrasound treatment in improving the extraction of bioactive molecules. However, longer ultrasound processing time still needs to be studied in further work.

In our second research, similar parameters to shrimp samples were tested and analyzed after microwave treatment, which was performed at 75°C, 100°C, and 125°C for 5, 10, and 15 min at 2.45 GHz, 400W. It is found microwave processing to have the capability to decrease the allergenicity of shrimp caused by major allergen tropomyosin, which would do great benefit to shrimp-sensitive patients. The percentage of reduced allergenicity (46.15%-75%) was positively related to the increase of microwave temperatures and time; the minimum allergenicity with a 75% reduction was observed in 125-15. Meanwhile, the total soluble

protein content decreased by 49.76% to 74.73%, leading to an increase of peptide amounts and further improving the bioactive molecules and antioxidants capacity of shrimps. The total antioxidant capacities in 100-15 and 125-15 were over two times the values of the untreated sample. Meanwhile, shrimp protein degradation and denaturation may induce the increasing of β-sheet and unordered structures in most treated samples, and thus enhancing the destruction of the cell microstructures. Numerous microstructural holes and fragments were observed in microwave-treated samples, especially in 100-15, 125-5, 125-10, 125-15. The *in-vitro* protein digestibility overall reduced from 80.46% to 64.19%, whereas showing an upward trend at 125 °C despite still lower than the value in control. Further studies focusing on the microwave approach with higher temperatures and a longer time should be conducted, which perhaps could provide strategies for better management and development of shrimp hypoallergenic products and positive health of the population.

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