Summer 8-11-2017

Attenuating the Antibody Reactivity of the Shrimp Major Allergen (Tropomyosin) using Food Processing Methods

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ATTENUATING THE ANTIBODY REACTIVITY OF THE SHRIMP MAJOR ALLERGEN (TROPOMYOSIN) USING FOOD PROCESSING METHODS

By

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A DISSERTATION

Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy (in Food and Nutrition Sciences)

The Graduate School
The University of Maine

August 2017

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Shrimp is one of the major causes of adverse allergic reactions in both adults and children. Consumers with shrimp allergy have an immune system that is hypersensitive towards certain proteins in shrimp. The immune system of these consumers produces an antibody, immunoglobulin E (IgE), which binds to specific regions on these proteins and activates reactions leading to adverse symptoms. Most shrimp allergic consumers have IgE that binds to a shrimp myofibrillar protein known as tropomyosin. Other than avoidance of shrimp, structural modification of shrimp tropomyosin using food processing methods could influence the capacity of this allergenic protein to bind IgE. Thus, the objectives of this study were to evaluate the IgE binding capacity of tropomyosin extracted from whole shrimp following: 1) different methods of heat processing of shrimp, 2) exposure of shrimp muscle to high acid condition, 3) treatment with three microbial proteases, and 4) treatment with microbial and plant proteases.
In study 1, the IgE binding of tropomyosin remained unchanged following boiling, steaming, baking, microwave roasting, grilling and frying of whole shrimp in comparison with tropomyosin from raw shrimp. However, high pressure steaming significantly reduced IgE binding capacity especially at lower concentration of antigen. In study 2, treatment of whole shrimp with vinegar reduced the solubility of tropomyosin and thus the IgE binding capacity of tropomyosin in the soluble extract was reduced. However, immunochemical analysis of the insoluble protein fraction indicated retention of significant IgE binding by tropomyosin following exposure to vinegar. In study 3, after treatment of whole shrimp with three microbial proteases, an alkaline protease significantly reduced (> 80%) the IgE reactivity of tropomyosin. Treatment of whole shrimp with alkaline protease and two plant proteases in study 4 revealed that, the plant proteases did not significantly reduce the IgE reactivity of tropomyosin.

In summary, treatment of whole shrimp with enzymes that can cleave the IgE binding sites of tropomyosin could be the first step in the development of a hypoallergenic shrimp product. However, an in vivo assay like the cell mediator release assay is necessary to confirm the reduced immunoreactivity of tropomyosin following treatment with alkaline protease.
DEDICATION

I dedicate this dissertation to my parents and siblings for their love, support, encouragement and prayers.
ACKNOWLEDGEMENTS

First, I want to give thanks to God, the creator of all things, for the gift of life without which this accomplishment would not have been possible. I also want to thank my advisor, Dr. Balunkeswar Nayak for giving me this opportunity in 2013 to pursue a doctoral degree at the University of Maine and for his support throughout the period of this program. I would like to appreciate members of my advisory committee Dr. Dorothy Klimis-Zacas, Dr. Denise Skonberg, Dr. Eric Gallandt, Dr. Soheila Maleki and Dr. Vivian Wu for their guidance, moral support and commitment to making sure that I understand all the relevant aspects of my research. Thank you all for the knowledge imparted. I am grateful to the School of Food and Agriculture for providing me with the financial aid for my doctoral program as well as travel funds to attend IFT conferences. Thank you very much for your investment and I would go from here to live a life worthy of that investment. I would also like to thank USDA – National Institute of Food and Agriculture and the University of Maine Graduate Student Government for providing funding that was used for different aspects of my research. Not forgetting, all the instructors of the classes that I took here at the University of Maine, thank you all for the knowledge imparted.

A special appreciation goes to Ms. Katherine Davis Dentici. She is one of first people I met here at the University of Maine and throughout these past four years she has selflessly helped me in my work in the lab in so many more ways than I can count. Thank you so much Kath. Not forgetting my current and past lab members especially Tamanna Ramesh and Amma Amponsah, thank you so much for helping me to believe in myself at those moments of self-doubt and for modelling hard work, perseverance and optimism.
Bouhee Kang, Xue Du, Praveen Kumar Sappati, Dhriti Nayyar, Bhargavi Rane, Hanjuan Cao, Angela Silke, Dhafer and Weeam Alshaibani, thank you guys for your encouragement and friendship. I also want to appreciate members of Dr. Maleki’s lab for providing us with polyclonal antibodies which was used in the last phase of my study.

All these would not have been possible without the prayers, love and sacrifice of my parents Dr. and Mrs. Lasekan. I am grateful for your endless support, guidance and encouragement and for imparting in me the value of education which you always describe as an asset that no one can take away from me. I also want to thank my siblings Dr. Olusiji Lasekan, Gbemisola Lasekan and the recent addition to our family, Elizabeth (my niece), thank you all for being a source of joy and inspiration.
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CHAPTER 1

INTRODUCTION

1.1. Background

Food allergies have been noted as one of the major food safety concerns especially in developed countries (Seiki et al., 2007; Sicherer, 2011). Food allergy is characterized by an immune-mediated hypersensitive reaction by an individual to dietary proteins in foods (Sicherer & Sampson, 2010). This means that the allergic individuals produce specific antibodies known as immunoglobulin E (IgE) against these harmless food proteins (Sicherer, 2011). The binding of this IgE with the allergenic proteins is responsible for the series of reactions that culminate in the elicitation of food allergy symptoms (Sicherer, 2011). These immune-mediated reactions could adversely affect the health of consumers as shown by common allergic symptoms such as urticaria, angioedema, asthma, rhinitis, vomiting, swollen tongue, abdominal cramp, eczema or rash, and life threatening anaphylaxis (Sicherer, 2011). Food allergies could also limit consumer freedom of choice as regard the type of food that should be consumed as well as general quality of life and psychological welfare (Sicherer & Sampson, 2014).

The factors that predispose certain consumers to allergic reaction to a food are not clearly understood and there is currently no cure for this condition. Hence, avoidance of the food causing the allergic reaction is the only safe approach for sensitized individuals (Sicherer & Sampson, 2014). The need to protect allergic consumers from accidental consumption of allergen-containing foods has been recognized by food processors, regulators and policy makers in different countries. For instance, in the United States, in line with the Food Allergen Labeling and Consumer Protection Act (FALCPA) of 2004
(effective on January 1, 2006), the government made it mandatory for products containing eight foods (milk, egg, fish, shellfish, peanut, tree nuts, soybean and wheat, also known as the “big 8”) that are known to be responsible for over 90% of food allergies to be declared on food label (US Code, 2004; Gendel, 2012; Thompson, Kane & Hager, 2006). This was aimed at assisting sensitized patients to avoid foods that could lead to serious or fatal reaction. In addition, food processors producing multiple food products (including those with allergens) on the same production line are also advised to inform the consumers about the possibility of cross-contamination of their product with allergenic residues (warning labels) (Remington, Baumert, Marx & Taylor, 2013). Furthermore, to address the problem of unintended contamination of food with allergenic residues (cross-contact), a recent provision in the Food Safety Modernization Act mandates food manufacturers and processors to treat allergens as hazard that are reasonably likely to occur. Hence, they are required to have preventive control measures to ensure that packaged foods do not have unintended allergens (FDA, 2011).

Apart from regulations to prevent accidental exposure to allergens, another potential food allergy mitigation strategy would be to reduce or abolish the capacity of allergens to cause adverse reactions (Chung & Reed, 2014). This can be accomplished by blocking or altering the first stage of the allergic reaction mechanism which is the binding of the protein with IgE which will prevent the release of chemical compounds that cause symptoms of allergy by immune cells. Allergenic proteins have highly conserved linear (primary structure) and conformational (secondary structure) regions or epitopes which must be recognized or crosslinked by antibodies such as immunoglobulin E (IgE) and immunoglobulin G (IgG) before the symptoms of food allergy can occur (Meno, 2011).
Consequently, the modification of allergenic protein conformation and cleaving of amino acid sequences that constitute an epitope in a food (protein denaturation) via thermal and non-thermal processes could alter their ability to bind IgE and elicit an allergic reaction (Achouri & Boye, 2013; Mondoulet et al., 2005; Sathe & Sharma, 2009). Moreover, almost all the “big 8” allergenic foods are subjected to one form of processing or another prior to consumption. Hence, there has been an interest to understand how the resultant denaturation of allergenic proteins due to these processing methods affects their capacity to bind IgE (Rahaman, Vasiljevic & Ramchandran, 2016; Verhoeckx et al., 2015).

However, food processing methods and conditions could increase, decrease or stabilize the allergenic capacity (IgE binding capacity) of food allergens through protein unfolding, protein hydrolysis, protein aggregation or via covalent interaction with other food components thereby masking or exposing allergenic epitopes (Mondoulet et al., 2005; Sathe, Teuber & Roux, 2005; Thomas et al., 2007; Verhoeckx et al., 2015). Factors that determine the effect of processing on allergenic capacity include the nature of the processing technique (moist or dry heat and thermal or non-thermal processing), properties of the allergenic protein, conditions of processing and nature of interaction of allergens with other components of the food matrix (Mondoulet et al., 2005).

Consequently, the effects of processing methods that are applied to foods either to improve their palatability or ensure microbial safety on immunoreactivity of allergens have been studied. The effect of common thermal processing methods including boiling, microwave heating, extrusion, blanching, roasting on immunoreactivity of allergens has been demonstrated in different foods (Achouri & Boye, 2013; Cabanillas et al., 2012; Kamath, Abdel Rahman, Komoda & Lopata, 2013; Venkatachalam, Teuber, Roux &
Sathe, 2002; Zellal, Kaddouri, Grar, Belarbi, Kheroua & Saidi, 2011). In addition, the influence of some novel food processing techniques such as high hydrostatic pressure, ultrasound, gamma irradiation and pulsed ultraviolet light on allergenic proteins had also been demonstrated (Dhakal, Liu, Zhang, Roux, Sathe & Balasubramaniam, 2014; Huang, Hsu, Yang & Wang, 2014; Li, Lin, Cao & Jameel, 2005; Shriver, Yang, Chung & Percival, 2011). It should be noted that some of these processing methods can modify the allergenic capacity of food proteins by altering their conformation while leaving the linear epitopes intact. Thus, these proteins could still retain substantial amount of their allergenic capacity following exposure to food processing conditions.

Shrimp is one of the major crustacean shellfish products that are widely consumed in different parts of the world. According to World Wild Life, 55% of global shrimp production occurs under aquaculture setting and the main producers are China, Bangladesh, Thailand, Indonesia, India, Brazil and Ecuador. The United States imports most of its seafood supplies and according to the National Oceanic and Atmospheric Administration (NOAA), shrimp is one of the most consumed seafood by the population.

Among the over 300 species of shrimp, the penaeid species are of high commercial value since they form the bulk of global production (Shiau, 1998). Some of the wild caught species in the penaeid group include *Penaeus setiferus* and *P. aztecus* while those that are commonly farmed include *P. monodon* and *P. vannamei*. Crustaceans in general are good sources of proteins, health benefiting highly unsaturated fatty acids (DHA and EPA) and minerals including calcium, magnesium and iron (Sriket, Benjakul, Visessanguan & Kijroongrojana, 2007).
Increased prevalence of allergic reactions to shrimp and other crustaceans has been observed among consumers especially in developed countries and in coastal areas (Lopata, O’Hehir & Lehrer, 2010). It has been estimated that 2% of the adult population and 0.1% of children in the United States are allergic to shellfish products (Sicherer & Sampson, 2010). From the standpoint of food scientists, the identification of allergenic components in foods as well as understanding how food processing methods affect the immunoreactivity of these allergenic components has been the focus of many researches. Consequently, shrimp allergy like other food allergies has been attributed to the presence of certain proteins (antigens) having specific linear and conformational epitopes that are recognized by IgE (Ayuso et al., 2010). However, over 80% of sensitized individuals are found to produce IgE against the muscle protein tropomyosin (Daul, Slattery, Reese & Lehrer, 1994). As a result, tropomyosin has been recognized as the major allergen of shrimp and other shellfish (Nakamura, Sasaki, Watanabe, Ojima, Ahn & Saeki, 2006; Shanti, Martin, Nagpal, Metcalfe & Rao, 1993). Tropomyosin is a myofibrillar protein with molecular weight range 34-38 kDa which plays a role in the contraction of muscle (Ayuso, Reese, Leong-Kee, Plante & Lehrer, 2002; Lehrer, Ayuso & Reese, 2003). The potency of this allergen has been attributed to its high thermal stability and resistance to proteolytic digestion (Usui et al., 2013).

Like other allergens, avoidance of shrimp and adherence to labeling regulations are the major strategy for protecting allergic consumers. In addition to these however, the capacity of different processing methods to reduce the allergenic capacity of shrimp have been attempted. The effects of boiling on the IgE reactivity of tropomyosin has been reported by different researchers (Liu, Cheng, Nesbit, Su, Cao & Maleki, 2010; Yu, Cao,
Cai, Weng, Su & Liu, 2011). These studies have shown that boiling does enhance the IgE binding with tropomyosin. Processing methods such as gamma irradiation (Byun, Kim, Lee, Park, Hong & Kang, 2000; Sinanoglou et al., 2007), ultrasound treatment (Li, Lin, Cao & Jameel, 2006) and pulsed ultraviolet light (Shriver, Yang, Chung & Percival, 2011), on the other hand, have been shown to reduce the reactivity of tropomyosin with IgE from the sera of patients that are allergic to shellfish. However, some of these studies have likely underestimated the binding capacity of tropomyosin following food processing due to a limitation in their study design. This limitation involves strong reliance on soluble protein fraction without considering the capacity of the processing methods to reduce the solubility of the target allergenic protein.

Immunochemical method like enzyme-linked immunosorbent assay (ELISA) is frequently used to detect the binding capacity of an allergenic protein with IgE following a food processing condition. This method relies on the effective extraction of the allergenic proteins from the processed food into the extraction buffer. However, significant reduction in protein solubility from a processed food matrix is one of the common causes of underestimation of IgE binding capacity. In this scenario, the reduction in IgE binding that is observed may be due to denaturation of tropomyosin which consequently reduced its concentration in the supernatant that is subjected to the ELISA assay. Moreover, certain simpler methods (in comparison with the non-thermal technologies) that could modify the structure of tropomyosin and influence its IgE binding capacity have not been attempted. These methods include other moist and dry heating techniques (frying, and microwaving), acidification and enzyme treatments.
Identifying a processing method that could help reduce or abolish the immunoreactivity of shrimp tropomyosin could be the first step in the development of hypoallergenic shrimp product that could be used for de-sensitization procedure such as oral immunotherapy. In addition, this could also help processors to become aware of processes that either increase or decrease allergenic capacity of crustacean shellfish.

1.2. Objectives

Thus, the main objective of this research was to identify a method of processing or treating whole shrimp that will result in significant reduction in the ability of tropomyosin to bind specific anti-tropomyosin antibody. The specific objectives were to:
1) evaluate the effects of common shrimp cooking methods such as boiling, steaming, baking, grilling, frying, high pressure steaming and microwave roasting on the IgE binding properties of tropomyosin; 2) determine the impact of low pH on tropomyosin IgE reactivity; 3) investigate the IgE reactivity of tropomyosin following treatment of whole shrimp with microbial proteases; 4) compare the IgE reactivity of tropomyosin of shrimp treated with a microbial protease with those treated with plant proteases.
CHAPTER 2

LITERATURE REVIEW

2.1. Prevalence of Food Allergies

Food allergy has been described as the erroneous response of the human immune system to certain food components leading to the development of serious or severe hypersensitive reactions in the sensitized individual (Sampson, 2004; Sicherer, 2002). Incidences of food allergies have become a major food safety and public health concern in many countries and it is increasing in prevalence (Sicherer & Sampson, 2010). It was estimated that 1% to 2% but less than 10% of the population displays hypersensitivity to certain foods (Chafen et al., 2010). In the United States, about 4% (12 million people) of the population are allergic to one food or another (Muñoz-Furlong & Weiss, 2009). However, in a recent report on the prevalence of self-reported food allergy carried out by The National Health and Nutrition Examination Survey between 2007 and 2010, overall prevalence of food allergy in the United States was put at 8.96% (McGowan & Keet, 2013). The proportion of children and adults in this figure was 6.53% and 9.72%, respectively and milk, peanut and shellfish were the most self-reported allergies in both groups (McGowan & Keet, 2013). However, shellfish allergy seems to predominate in adults compared to children as shown by the data from the survey, 2.04% and 0.87%, respectively. This agrees with a previous report on the prevalence of seafood (fish and shellfish) allergy in the United States where 5.9% of the 5529 households (almost 15,000 individuals) that were sampled reported fish or shellfish allergy (Sicherer, Muñoz-Furlong & Sampson, 2004). The proportion of individuals with shellfish and fish allergy
was put at around 2\% and 0.4\%, respectively and seafood allergy predominates in adults compared with children, 2.8\% and 0.6\%, respectively (Sicherer et al., 2004).

In order to arrive at a better estimate of the prevalence of childhood food allergy in the United States, Gupta et al. (2011) carried out an electronic survey comprising almost 40,000 households with children between June 2009 and February 2010. A report from this survey indicated that prevalence of food allergy is around 8\% (95\% CI) and almost 39\% of children had history of severe reactions while about 30\% had multiple food allergies (Gupta et al., 2011). Looking at specific foods, prevalence was highest for peanut followed by milk and shellfish (25.2\%, 21.1\% and 17.2\%, respectively). In a recent study that utilized a cross-sectional cohort of 330,200 children, the prevalence of food allergy was around 6.7\% and the most allergenic foods and their prevalence were peanut (2.6\%), milk (2.2\%), egg (1.8\%), shellfish (1.5\%), and soy (0.7\%) (Hill, Grundmeier, Ram & Spergel, 2016). Another research group in Canada estimated the prevalence of food allergy among Canadians to be around 6.7\% and the proportion of sensitized individuals was found to be higher in children than adults, 7.1\% and 6.6\%, respectively (Soller et al., 2012). Interestingly, they also showed that cow’s milk, peanut and tree nuts were the major sources of allergic reaction among children while shellfish, fruits and vegetables allergies were most common among adults (Soller et al., 2012). Furthermore, data from a ten-year period (1997-2007) revealed that incidences of food allergy among children (< 18 years old) increased by 18\% during this period (Branum & Lukacs, 2009). The report also showed that visits to emergency departments or physician due to food allergy related cases increased from 116,000 to 317,000 in 1993-1997 to
2003-2006, respectively. Hospitalization on the other hand was close to 10,000 cases in 2004-2006 (Branum & Lukacs, 2009).

Using data from The National Electronic Injury and Surveillance System (NEISS) obtained from 34 hospital emergency departments (EDs) between August to September 2003, over 20,000 hospital ED visits were attributed to allergy related cases with over 2,000 cases of anaphylaxis and 520 hospitalizations (Ross, Ferguson, Street, Klontz, Schroeder & Luccioli, 2008). In a study that asked the caregivers (1,643) of children with food allergy questions relating to the cost of providing care for their children, the annual cost of food allergy in the United States was estimated at $24.8 billion which correspond to $4,184 per year per child (Gupta, Holdford, Bilaver, Dyer, Holl & Meltzer, 2013). Based on this study, direct medical cost was around $4.3 billion while the cost borne by family was about $20.5 billion per year. An increase in the number of hospitalization and fatalities also attests to the increase in the prevalence of food allergy. For instance, Rudders, Banerji, Vassallo, Clark & Camargo Jr., (2010) reported cases of children taken for emergency department visits due to food induced anaphylaxis increased from 160 between 2001-2002 to 531 between 2005-2006. In addition, it was also reported that food induced anaphylaxis was responsible for 6.7% of the 2,458 anaphylaxes-related death between 1999 to 2010 (Jerschow, Lin, Scaperotti & McGinn, 2014).

All these data confirmed that food allergy is a major food safety issue in the United States and Canada. Understanding the natural history of food allergy and the foods responsible for most allergic reactions is key to the success of any intervention strategy to reduce its prevalence and impact. Consequently, the next section will explore the biological mechanism of the development of allergic reaction.
2.2. Mechanism of Immune-Mediated Allergic Reaction

2.2.1. The Gastrointestinal Tract as an Immune Organ

The major part of the human system that is exposed to materials from the outside world (other than the skin) is the gastrointestinal (GI) tract which comprise the mouth, oesophagus, stomach, intestines (small and large), rectum and anus (DeMeo, Mutlu, Keshavarzian & Tobin, 2002). Thus, a large proportion of the human immune system made up of tissues and cells that work together to protect the body from harmful materials and for fighting infections are concentrated in the GI (Pelaseyed et al., 2014). The small intestine is particularly important in the GI because it is the site at which the body can absorb nutrients from external materials like foods and it can also be permeable to pathogenic microbes and toxic chemicals (MacDonald, 2003). Consequently, this region of the gut has well-developed immune components commonly referred to as the gut-associated lymphoid tissue, GALT (Acheson & Luccioli, 2004; McGhee & Fujihashi, 2012) which has the task of distinguishing between what is harmful and harmless to the body (Forchielli & Walker, 2005). Some of the components of GALT include the epithelium, lamina propria, Peyer’s patches and mesenteric lymph nodes (Forchielli & Walker, 2005). These lymphoid tissues also contain effector cells, regulatory cells and chemical secretions such as cytokines and chemokines which work together to mount responses to inactivate or destroy harmful agents while tolerating innocuous antigens and microbes (Sanz & De Palma, 2009). The gut microflora inhabiting the distal part of the small intestine and large intestine are also critical in the development and normal functioning of the components of GALT (Forchielli & Walker, 2005; Sanz & De Palma, 2009). However, the overreaction of the components of GALT to dietary antigens,
helpful microbes and self-antigens are the main causes of inflammatory conditions or autoimmunity diseases such as food allergy and inflammatory bowel disease (IBD) (Forchielli & Walker, 2005).

A close examination of the surface of organs of the GI tract shows that they are made up of four distinct layers namely the mucosa, submucosa, muscularis propria and serosa (Reinus & Simon, 2014). These layers (apart from the mucosa) are similar in all the GI organs. In addition, these layers (especially the mucosa and submucosa) play a critical role in the protection of the body against harmful materials as indicated by the concentration of the gut-associated lymphoid tissues in these layers (Figure 2.1).

Figure 2.1. The immune cells of the GI mucosa (Adapted from McGhee & Fujihashi, 2012)

The mucosa is the layer of the small intestine that is in direct contact with components of the lumen including diverse antigens, macromolecules and microbes. It is made of three sections namely the epithelium, the laminar propria and the muscularis mucosae (McGhee & Fujihashi, 2012). The mucosa is consequently one of the first lines of
defense of the body against harmful materials (Sanz & De Palma, 2009). This it does by utilizing the barrier property of the epithelium (which also contributes to the process of digestion and nutrient absorption) which is linked together by tight junction. In addition, a mucus layer produced by goblet cells helps trap microorganisms and particulates on the surface of the epithelium while the antimicrobial peptides such as lysozyme and defensins that are produced by the Paneth cells also help inactivate pathogenic microbes (Bischoff & Crowe, 2005; McGhee & Fujihashi, 2012; Sanz & De Palma, 2009; Sicherer & Sampson, 2010). Thus, the epithelial layer serves as a selective barrier that separates luminal contents from the immune cells that are underneath this layer. A disruption in the barrier integrity of the epithelium could expose the underlying immune cells to large amount of luminal contents which would cause these cells to develop hyperactive responses to these luminal materials. This condition known as “leaky gut syndrome” is also partly responsible for immune mediated and non-immune disorders including food allergy, eczema, inflammatory bowel disease, celiac enteropathy, type 1 diabetes and asthma (Liu, Li & Neu, 2005). As mentioned earlier, commensal microorganisms in the gut play key roles in the protective and barrier property of the epithelium. For instance, these microbes regulate the expression of the mucin gene in goblet cells which influences their capacity to trap particulates (Sanz & De Palma, 2009). They also influence the production of antimicrobial peptides by Paneth cells and regulate the alteration in the permeability of the epithelium in response to infection, stress and inflammation (Sanz & De Palma, 2009). On the surface of the epithelium are another group of specialized cells known as M cells which help transport certain luminal materials to the sub epithelial region (transcytosis) where appropriate responses are mounted against them (McGhee &
Fujihashi, 2012). Hence, M cells could be categorized as those cells that have the function of presenting antigens to other immune cells (Neutra et al., 1996).

Another major component of the mucosa is the lamina propria which lies beneath the epithelium and it is the body’s second line defense against harmful materials. The lamina propria serve as a reservoir for immune cells such as lymphocytes, plasma cells, dendritic cells, macrophages and eosinophils. These cells are arranged in aggregated form called the Peyer’s patches (MacDonald, 2003) in the laminar propria where they undergo division and differentiation. In addition, the Peyer’s patches are also the site of humoral cells (B cells and T cells) activation and secretion of antibody by plasma cells in response to the presence of foreign antigen (MacDonald, 2003; Sicherer & Sampson, 2010).

The muscularis mucosae is a muscle fiber that divides the mucosa and the submucosa and the base of the crypts rest on it. The submucosa is a layer of connective tissue that supports the mucosa. It is also made up of lymphocytes, plasma cells, macrophages, some mast cells as well as eosinophils (Reinus & Simon, 2014). Both muscularis propria and serosa are mainly composed of connective tissue (Reinus & Simon, 2014).

2.2.2. GALT Effector Cells and their Function

The defensive role of the immune system is made possible by myriad of cells that performs different roles aimed at protecting every tissue and organ against harmful microbes and substances. These cells are involved in the determination of what is harmful and harmless and the subsequent response of the immune system (MacDonald, 2003). Since, food allergy is a condition that is mediated by the immune system, it is therefore important to understand the roles of these cells in the immune system.
Cellular components of the immune system also known as immunocytes are mainly the white blood cells generated by the bone marrow. These cells can be categorized into three groups namely the granulocytes, myeloid cells and lymphocytes (Luster, Alon & von Andrian, 2005). Granulocytes comprise eosinophils, neutrophils, basophil and mast cells (Luster et al., 2005). The myeloid cells are made up of dendritic cells, monocytes and macrophages while the lymphocytes group comprise B and T cells and the natural killer cells (Acheson & Luccioli, 2004; Luster et al., 2005).

2.2.2.1 Granulocytes

The granulocytes that play a major role in allergic reaction are mast cells and basophils. Mast cells are produced in the bone marrow by pluripotent hematopoietic stem cells and are found in tissues that are exposed to the outside environment (Kumar, Verma, Das & Dwivedi, 2012). They are also characterized by the presence of high affinity immunoglobulin E receptors (FcεRI) which are critical in the elicitation of allergic reaction (Figure 2.2). Basophils are like mast cells except that they are found in the blood rather than tissues (Kumar et al., 2012). In general, the granulocytes elicit immune response that is characterized by the release of their granular contents (Figure 2.2) to inactivate or destroy a foreign antigen (Amin, 2012). This degranulation process is preceded by the binding of the foreign antigen with an antibody followed by the attachment of the complex to the antibody receptor (also known as the high affinity receptor, FcεRI) on the surface of the granulocytes including mast cells and basophils (Amin, 2012). The FcεRI on the surface of mast cells and basophils is a tetramer comprising an IgE binding region (α chain) and signaling region (β-chain and two γ-chains) (Kumar et al., 2012). This crosslinking process activates the granulocytes to
release different pro-inflammatory mediators such as histamine, prostaglandins and cytokines such as interleukin-4 (IL-4) and interleukin-5 (IL-5) as well as proteases like tryptase and chymase (Amin, 2012).

Figure 2.2. Allergen binding with effector cells and degranulation process (Adapted from Amin, 2012).

In addition to the cytokines produced by other granulocytes (mast cell, basophils and eosinophils), the degranulation products of neutrophils also include antimicrobial peptides including cathepsin, lactoferrin, defensin as well as other pro-inflammatory cytokines such as IL-12, IFN-γ and TNF (Sampson, 2000). However, only eosinophils and neutrophils have the capacity to undergo phagocytosis among the granulocytes during which the foreign antigen is engulfed prior to further processing by the cellular components.
**2.2.2.2. Monocytes**

Monocytes are generated from the myeloid group of the hematopoietic stem cells present in the bone marrow. In certain tissues, these monocytes are differentiated into macrophages and dendritic cells (Luster et al., 2005). Macrophages are specialized cells that are adapted to engulf foreign substance including toxins, apoptotic cells, macromolecules and microbes (Warrington, Watson, Kim & Antonetti, 2011). Just like the granulocytes, the phagocytic ability of macrophages is only activated following recognition and binding of antibodies to the harmful materials or antigen (opsonization) (Warrington et al., 2011). Macrophages also participate in a process known as antigen presentation, hence they are called antigen presenting cells, APCs (Warrington et al., 2011). During this process, the protein components of the foreign body are digested into peptides. These peptides are then attached to a carrier molecule on macrophages known as major histocompatibility complex (MHC) (Warrington et al., 2011). MHC are divided into two namely MHC class 1 which are found on all nucleated cells where they serve as carrier for endogenous peptides and MHC class 11 which are found solely among immune cells like macrophages, dendritic cells and B cells where they serve as carrier for exogenous peptides (Warrington et al., 2011). Consequently, MHC class 11 is the major carrier of allergenic proteins. T cells, via their receptors can recognize these peptides-MHC complex and then initiate further immune response against the foreign substance (Warrington et al., 2011).

Myeloid dendritic cells on the other hand are characterized by long protrusions known as dendrites and they are abundant in the mucosa layer of the intestine especially in the laminar propria and Peyer’s Patches. Like the macrophages, they are actively
involved in antigen presentation followed by activation of immune response by T cells with the help of MHC class 11 (Luster et al., 2005). In addition, dendritic cells probe the small intestine for the presence of foreign macromolecules like proteins via pattern recognition receptors (PPRs) on their surface (Burks, Laubach & Jones, 2008; Luster et al., 2005). Thus, these cells must be able to discriminate between commensal microflora in the gut and pathogens as well as innocuous macromolecules. They also induce other immune cells to secrete immunosuppressive substances thus preventing adverse immune response to harmless antigens. Failure of these cells to correctly recognize harmless antigens and stimulate immunosuppressive molecules could lead to inflammatory conditions like food allergy, ulcerative colitis and inflammatory bowel disease.

2.2.2.3. Lymphocytes

The lymphocytes comprise the group of cells that either ensure the tolerance of the immune system to harmless external materials or mount aggressive response to harmful substances (Luster et al., 2005). One of the main lymphocytes of the immune system is the CD4+ T helper (Th) cell. These cells are part of the hematopoietic stem cell in the bone marrow but which become fully mature in the thymus (Warrington et al., 2011). Th cells are characterized by the presence of a CD4 glycoprotein molecule on their surface acting as a co-receptor (Warrington et al., 2011). The response of the Th cell to foreign antigens is based on the recognition of antigenic peptides on MHC class 11 present in an APC such as macrophages and dendritic cells (Warrington et al., 2011). CD4+ T helper (Th) cells respond to antigens by activating other immune cells like CD8+ T cells to produce cytotoxic substances, B cells to produce antibody and macrophages to carry out phagocytosis (Warrington et al., 2011). The activation of Th
cells is governed by the release of specific cytokines which also forms the basis for the classification of Th cells into Th1 and Th2 cells (Warrington et al., 2011). For instance, the Th1 response is characterized by the release of interferon-gamma (IFN-γ) responsible for the activation of the bactericidal properties of macrophages and production of opsonizing and neutralizing antibodies by B cells (Warrington et al., 2011). The Th2 cells on the other hand secrete several interleukins including IL-4, IL-5, IL-10 and IL-13 which activate B cells to produce immunoglobulin E (IgE) antibodies (Warrington et al., 2011). Therefore, production of the antibody, immunoglobulin E (IgE) by the B cells due to the bias towards Th2 cell activation is responsible for the development of allergic reaction among a certain population subset.

Interestingly, another group of T cells known as the regulatory T cells (Tregs) are known to have immunosuppressive capacity (Forchielli & Walker, 2005). This class of T cells help prevent immune response to self or harmless antigens and also downregulate the response of other effector T cells by inhibiting the production of pro-inflammatory mediators. They carry out this function with the aid of certain cytokines including IL-10 and TGF-β (Forchielli & Walker, 2005). Thus, Tregs are important in the prevention of autoimmune disease and maintaining a state of anergy in the GI.

B cells are lymphocytes that are also derived from bone marrow having unique antigen binding receptor on the cell surface (Warrington et al., 2011). They are responsible for the production of antibody or immunoglobulin which are either bound to the B cell surface as receptor or exist in soluble form capable of binding to a specific antigen. Although B cells have receptors that can bind antigens, they still depend on the
signals obtained from CD4+ Th cells for activation. Cytokines released by Th cells activate B cells to differentiate into antibody-producing plasma cells (Warrington et al., 2011).

Antibodies or immunoglobulins are soluble glycoproteins (3-13% glycoproteins) that play a critical role in immunological response to a foreign substance (Delves & Roitt, 2000). However, their antigen recognition process is different from that of Th cell receptors. For instance, the processing of an antigen to short peptides is not a prerequisite for recognition by antibodies. Rather, they can recognize certain domains known as epitopes within an intact protein. After the binding of an antibody to an epitope on an antigen, several immunological responses can be initiated when this antigen-antibody complex attaches to an effector cell via surface receptor (Fc receptor) on the latter.

Structurally, the antibody is composed of two regions namely the variable region where antigen binding occurs and the constant region where ligation to the Fc receptor on effector cells occurs (Delves & Roitt, 2000). An antibody is also made up of a pair of identical heavy chains and another pair of light chains. All parts of the light chain and a section of the heavy chain take part in antigen binding. However, the identical heavy chains in the constant region determine the effector binding and response (Delves & Roitt, 2000). Consequently, the characteristics of this heavy chain constant region have been used to differentiate antibody isotypes namely IgA (1 – 2), IgD, IgG (1 – 4), IgE and IgM (Delves & Roitt, 2000, Warrington et al., 2011). For instance, the binding of an IgE to an effector cell induce mast cell and eosinophil degranulation releasing products that are responsible for the symptoms of allergic reaction.
B cells are induced by cytokines released by Th cell to differentiate into plasma cells producing specific antibody via a process known as class switching (Gould et al., 2003). For example, secretion of IFN-γ from Th cells activates B cells to switch from IgM to IgG production. IL-4 and TGF-β activate production of IgA while IL-4 and IL-5 induce class switching of B cells to IgE production, which is critical for the elicitation of allergic response. Among the antibody isotypes, the IgE isotype has the least concentration in the serum of some non-atopic individuals. However, its concentration could increase 10 times in the serum of individuals with high risk of developing allergic reaction. Thus, quantification of IgE is often used in the diagnosis of food allergy.

IgA is an antibody that is secreted across the epithelium where it can form a large complex with several antigens thus preventing their entry into the sub-epithelial layer and occurrence of inflammatory reactions (Bischoff & Crowe, 2005). Hence, this antibody is critical in preventing the overreaction of the immune system. Interestingly, the generation of IgA has also been shown to depend on the presence of gut microflora because this antibody is almost absent in germ-free animals (Hexham et al., 1996).

2.2.3. Mechanism of IgE-Mediated Allergic Reaction

The immune system is well equipped to defend the body against pathogenic microbes and harmful substances. This it does by utilizing the barrier property of the epithelium, proper functioning of the antigen sampling and presentation cells and elicitation of the right signals in the form of different cytokines or antibodies to directly or indirectly combat the foreign antigen. In addition, the immune system has mechanisms in place to downregulate response against commensal microbial flora in the gut as well as innocuous antigens, especially from food components (Bischoff & Crowe, 2005).
However, a disruption in the normal functioning of the immune system can lead to allergies, autoimmune and immunodeficiency diseases.

Food allergy occurs when the immune system is activated to mount a response against a harmless food antigen (allergen) resulting in the generation of signals that cause certain immune cells to release pro-inflammatory mediators which can cause damage to tissues and adverse health condition for the individual (Yu, Freeland & Nadeau, 2016). It is basically the breakdown of the mechanism of oral tolerance through which the immune system is conditioned to suppress any immunological response against food antigens (Burks, Laubach & Jones, 2008). Consequently, the immune system of allergic individuals has been shown to have lower amount of immunosuppressive cytokines like IL-10 and TGF-β and higher levels of pro-inflammatory cytokines such as IL-4, IL-13 and IFN-γ (Bischoff & Crowe, 2005, Sicherer & Sampson, 2010). Also, low levels of regulatory T cells (Tregs) which are important for the downregulation of immune response are also observed among allergic individuals. In addition, any condition that disrupts the barrier property of the epithelium (for instance, breaking of the tight junction) can also ensure easy access of luminal contents to the immune cells in the submucosa.

Food allergies can be characterized as Type I to Type IV (Mine & Zhang, 2002). Type I allergies are IgE-mediated reactions also known as immediate hypersensitivity because symptoms can appear in less than 1 minute to a few hours after ingestion of the food (Mine & Zhang, 2002). Type II allergy is mediated by IgG and IgM antibodies and is referred to as cytotoxic hypersensitivity because they ensure the phagocytoses of cell-bound antigens. Type III is mainly mediated by IgG antibodies and is characterized by
formation of a large antigen-antibody complex which results in tissue damage especially at the joints and kidney (Mine & Zhang, 2002). Type IV hypersensitivity on the other hand does not involve antibodies but lymphocytes and macrophages and is characterized by late elicitation of clinical responses (Mine & Zhang, 2002).

Most food allergies are categorized as a Type I (immediate) immunoglobulin E (IgE) hypersensitivity of the immune system to food proteins and it occurs in two phases (Bischoff & Crowe, 2005). The first phase (Figure 2.3) is the initial exposure of the immune system to the dietary allergenic protein known as sensitization (Yu, Freeland & Nadeau, 2016). Intact or partially hydrolyzed proteins are taken up by dendritic cells of the laminar propria that samples the surface of the lumen (Bischoff & Crowe, 2005). These antigens can also pass through the epithelium with the help of M cells and be transported to the Peyer’s patches where they are released to the antigen presentation cells (APCs), dendritic cells and macrophages. The antigens are hydrolyzed to peptide fragments in these immune cells and are then carried by the MHC class II molecules to the naïve CD4+ T cells (Kumar et al., 2012; Mine & Zhang, 2002). After recognition by the naïve Th0 cells via specific T cell receptors, the Th2 type of the CD4+ T cell is activated to release the interleukins IL-4 and IL-13 which cause the B cells to differentiate into IgE-producing plasma cells through a process of class switching (Bischoff & Crowe, 2005; Kumar et al., 2012; Mine & Zhang, 2002). In addition, during the initial exposure to the food allergen, no adverse reaction occurs. However, the release of antibodies that are specific to the allergen primes the immune system to respond upon subsequent exposure. The IgE produced during the sensitization stage attaches to the
surface of mast cells and basophils through the high affinity IgE receptors (IgεRI) on the surface of these immune cells (Bischoff & Crowe, 2005; Mine & Zhang, 2002).

After a re-exposure of the immune system to the allergen, the IgE on the surface of mast cells and basophils (connected via the IgεRI) binds to certain portions of the allergenic protein known as epitopes (Gould et al., 2003). This is then followed by a degranulation of these effector cells resulting in the release of inflammatory mediators including preformed histamine, tryptase, leukotrienes and prostaglandins which are responsible for the symptoms of food allergies among sensitized individuals (Figure 2.3) (Bischoff & Crowe, 2005; Gould et al., 2003; Yu et al., 2016). Adverse reactions may occur within seconds and minutes of exposure and this may be followed by a late phase reaction occurring 2-24 hours after contact caused by migration of granulocytes and lymphocytes to tissues leading to inflammation of these tissues (Bischoff & Crowe, 2005; Gould et al., 2003).

Figure 2.3. Sensitization and elicitation mechanism of allergic reaction (Adapted from Mine & Yang, 2008)
For instance, histamine causes the vasodilation of blood vessels which leads to the rashes observed on the skin as well as a drop in the blood pressure (Kumar et al., 2012). In the respiratory tract (which has abundant mast cells) on the other hand, histamine causes the contraction of the bronchioles that are connected to the lungs which can result in asphyxiation (Kumar et al., 2012).

Recognition of dietary antigens by T cells and IgE is one of the steps in the series of events leading to the development of allergic reaction. Both T cells and IgE antibody do not recognize the whole structure of the allergenic proteins. Rather, they bind to specific sites or regions on the antigen known as epitopes (Mine & Yang, 2008). These can either be sequential epitopes which are certain amino acid residues on the protein or conformational epitopes based on the secondary and tertiary structure of the protein (Mine & Yang, 2008). Identification of these epitopes helps unravel the part of the protein that contributes to allergic reaction and may also assist in the development of techniques aimed at preventing the binding of IgE with allergenic protein.

2.3. The Major Food Allergens

2.3.1. Peanut and Tree nuts

Peanut (*Arachis hypogea*) is a protein dense agricultural product consumed in different parts of the world and used as ingredients in the production of varieties of packaged products including peanut butter. However, peanut is also the source of one of the most common, permanent and severe food allergies in developed countries (Sicherer & Sampson, 2007; Sáiz, Montealegre, Marina & García-Ruiz, 2013). In most developed countries, peanut allergy is a major health concern because of the increasing number of
children that have this type of allergy. Reports in both United Kingdom and United States, the prevalence of peanut allergy among children is > 1% (Grundy, Matthews, Bateman, Dean & Arshad, 2002; Sicherer et al., 2003). In addition, the prevalence of this allergy seems to be increasing especially among the children population. For instance, when Sicherer et al. (2010) compared the prevalence of peanut allergy among children in 2008 with previous data from 1997 and 2002, the proportion of children with allergy was 1.1%, 0.5% and 0.2%, respectively. A similar survey conducted in Canada between 2005-2007 and 2000-2002 puts the prevalence of peanut allergy among children at 1.62% and 1.34%, respectively (Ben-Shoshan et al., 2009). Interestingly, the occurrences of peanut and tree nut allergy in children have been found to be relatively lower in Asian countries than western countries (Shek et al., 2010).

Eleven allergenic proteins have been identified in peanuts denoted as Ara h1-11 according to the guideline of the International Union of Immunological Societies Nomenclature Subcommittee (Sicherer & Sampson, 2007; Sáiz et al., 2013). However, some of the major peanut allergens are members of the seed storage proteins including vicilin (Ara h1, 63.5-64 KDa), conglutin (Ara h2, 16.7-18 KDa) and glycinin (Ara h3, 60 KDa). The symptoms that characterize peanut allergy include swollen face, urticaria, abdominal cramps and anaphylaxis (Sáiz et al., 2013).

Tree nuts also account for a significant amount of reported cases of food allergy in the United States, United Kingdom and European countries. Common tree nuts with allergenic potential include hazelnut, walnut, pistachios, Brazil nut, almond, cashew nut, macadamia and pecan (McWilliam, Koplin, Lodge, Tang, Dharmage & Allen, 2015). They are consumed as raw whole nuts or roasted and are found in different commercial
products such as chocolates, energy bars and bakery products (Vanga & Raghavan, 2016). One of the key drivers for the increased consumption of tree nuts is their nutritional and health benefits due to the presence of macro and micro nutrients including proteins, fats, minerals, vitamins, phenolics and flavonoids (Vanga & Raghavan, 2016). More so, they also form one of the main diets for consumers on weight loss programs and this has helped increase their popularity. Like peanut, tree nut allergy is also characterized by mild to severe symptoms including anaphylaxis which can be fatal. Based on the published literature from Europe, UK and USA between 1996 to 2014, the prevalence of confirmed cases of tree nut allergy was found to be less than 2% while the probable cases range from 0.05 to 4.9% (McWilliam et al., 2015). Tree nut allergy also seems to be increasing among children as shown by a self-reported survey which showed the prevalence of tree nut allergy to be 1.1%, 0.5% and 0.2% in 2008, 2002 and 1997, respectively (Sicherer et al., 2010). According to another report, the prevalence of tree nut allergy was found to be lower than that of milk, peanut and egg among the children population (Sicherer & Sampson, 2010b). However, tree nut allergy could constitute a higher burden because it does not get resolved with time unlike milk and egg. In a recent review, it was shown that there were 54 fatalities due to tree nuts in the United States between 1986-2011 making it one of the highest among the big 8 (Sathe, Liu & Zaffran, 2016). Several proteins in tree nuts that have been linked to the elicitation of allergic reaction include the 2S albumin protein family, 7S vicilin, 11S legumin, non-specific lipid transfer proteins, profilin amongst others (Sathe et al., 2016).
2.3.2. Milk

Cow milk is widely consumed by children and adults in most regions of the world because of its nutritional and health benefits. It is also one of the first foods in the diet of infants because it is rich in proteins, calcium and vitamins like A and B6 (Do, Williams & Toomer, 2016). Consequently, cow milk allergy is one of the most common among allergies among infants and young children although most tend to outgrow this allergy (Skripak, Matsui, Mudd & Wood, 2007). Adults could also have some reactions to cow milk but this may be due to lactose intolerance rather than an immune-mediated allergic reaction. Based on the published reports compiled by Sicherer (2011), the prevalence of milk allergy among the population in the United States is about 0.4% while the prevalence among age 1 and 1-5 years was 3.8% and 1.8%, respectively. When a self-reported study was used to estimate the prevalence of food allergy in Canada, allergic reaction to milk was the highest reported allergy among children (2.2%) and the proportion of the entire population with this allergy was also close to 2% (Soller et al., 2012). Immediate and late symptoms of cow milk allergy that have been reported include respiratory symptoms like rhinitis and wheezing, cutaneous symptoms including atopic eczema and dermatitis, gastrointestinal symptoms like vomiting and constipation as well as systemic anaphylactic symptoms (El-Agamy, 2007).

More than 20 proteins in cow milk can cause allergic reaction and these proteins can be found in both the casein and whey protein fractions (El-Agamy, 2007; Fritsché, 2003). However, caseins, α-lactalbumin and β-lactoglobulin components of whey fraction are considered as the major cow milk allergens (El-Agamy, 2007, Sharma, Kumar, Betzel & Singh, 2001). In a study involving 80 individuals with confirmed serum IgE for milk
proteins and history of milk allergy, specific IgE for caseins were detected in all the subjects via western blot while only 10 and 5 subjects had specific IgE for β-lactoglobulin and α-lactalbumin, respectively (Docena, Fernandez, Chirdo & Fossati, 1996). The casein fraction of milk proteins is divided into four types namely α-s1, α-s2, β, and κ and some studies have shown that majority of individuals with cow milk allergy specifically develop IgE mediated reactivity towards the β-casein fraction (Shek, Bardina, Castro, Sampson & Beyer, 2005). Management of cow milk allergy involves total exclusion from diet, utilization of extensively hydrolyzed whey or casein and introduction of an alternative protein source such as soy (Solinas, Corpino, Maccioni & Pelosi, 2010).

2.3.3. Egg

Hen’s egg is commonly used in diverse food products because of the high biological value of the proteins and their different functional properties including emulsifying, gelling and foaming properties (Jiménez-Saiz, López-Expósito, Molina & López-Fandiño, 2013). Just like cow milk allergy, immune-mediated reaction to eggs is also common among children affecting up to 2% of young children (Mine & Yang, 2008; Savage, Matsui, Skripak & Wood, 2007). This allergy has been shown to develop within the first two years of life and usually becomes resolved before the children attain school age (Mine & Yang, 2008). Eggæsbø, Botten, Halvorsen & Magnus (2001) found the prevalence of egg allergy (using diagnostic procedures such as skin prick tests and double-blind, placebo-controlled food challenges) among children aged 2½ years to be around 1.6% which represents two-thirds of the parentally perceived egg allergy from a population of 2,721 children. Symptoms of egg allergy could range from skin reactions
like atopic dermatitis and urticaria to respiratory (asthma), gastrointestinal (vomiting and diarrhea) symptoms as well as serious systemic anaphylaxis (Mine & Yang, 2008). These reactions could be triggered by 70 mg of egg protein corresponding to a bite of cooked egg (Burks et al., 2012). Consequently, strict avoidance of egg is employed by parents of children with egg allergy to prevent reaction. However, avoidance could be a challenge because of the numerous food products that are formulated with eggs (Mine & Yang, 2008) coupled with the potential nutritional deficiency caused by egg substitutes that may not have the same nutritional quality of hen’s egg (Burks et al., 2012, Mine & Yang, 2008).

Hen’s egg can be divided into egg white and egg yolk. A majority of the proteins that are responsible for allergic reaction have been shown to be concentrated in the egg white and to a lesser extent in the egg yolk (Mine & Zhang, 2002). Major allergens in the egg albumen (egg white) include 28 KDa ovomucoid (Gal d 1), 45 KDa ovalbumin (Gal d 2), 77 KDa ovotransferrin (Gal d 3) and 14.3 KDa lysozyme (Gal d 4) (De Silva, Dhanapala, Doran, Tang & Suphioglu, 2016). The allergenic proteins in the egg yolk include α-livetin (Gal d 5) and YGP42 (Gal d 6) (De Silva et al., 2016). It was shown that ovotransferrin and ovomucoid were the most IgE reactive allergens in the serum of anaphylaxis individuals whereas IgE from atopic patients bind with ovalbumin and ovomucoid (Mine & Zhang, 2002). With respect to the IgE binding sites of these proteins, most egg allergen epitopes are sequential rather than conformational (Mine & Rupa, 2003).
2.3.4. Fish

Fish is a major food in the diet of consumers in most parts of the world and most especially in coastal areas (Sharp & Lopata, 2014). Key drivers of the increased consumption of fish include its utilization as a source of dietary proteins and healthful lipids especially the polyunsaturated fatty acids. Despite these nutritional and health benefits, fish could also cause immune-mediated reactions in a subset of the consumer population including children and adults (Van Do, Elsayed, Florvaag, Hordvik & Endresen, 2005). Unlike milk and egg allergy, children with fish allergy usually do not outgrow their allergic predisposition (Sharp & Lopata, 2014). In a random telephone survey conducted in the United States, 5.9% of 14,948 consumers reported allergic reaction to seafood (fish and shellfish) with 2% and 0.4% reporting allergic reaction to shellfish and fish, respectively (Sicherer et al., 2004). A meta-analysis of published studies from 2000 to 2012 puts the prevalence of self-reported and diagnosed fish allergy in Europe at 2.2% and 0.1%, respectively (Nwaru, Hickstein, Panesar, Roberts, Muraro & Sheikh, 2014). Fish allergy is notably common in Asian countries especially among children and this may be due to early introduction of fish to infants from these countries (Sharp & Lopata, 2014). In a population based study among 11,434 Filipino, 6,498 Singaporean, and 2,034 Thai, 2.29, 0.26 and 0.29 % of the children were found to have hypersensitive reactions to fish (Connett et al., 2012).

In the United States, the major fish species that commonly cause allergic reaction include salmon, tuna, catfish, cod, flounder, halibut, trout, and bass (Sharp & Lopata, 2014b). In the study conducted by Connett et al. (2012) in three Southeast Asian countries, anchovy and mackerel scad were found to be responsible for most allergic
reactions to fish. Other fish species with potential allergenic capacity include Alaska pollock, Atlantic cod, European hake, Atlantic herring, Japanese sardine, whiff, Beluga sturgeon, carp and Rose fish (Sharp & Lopata, 2014).

Individuals with fish allergy often react to different species of fish because of cross-reactivity of the major allergenic protein among the different species. Although several fish proteins ranging from 12-250 KDa have IgE reactivity, the major allergenic protein has been shown to be the monomeric (12 KDa) and oligomeric form of parvalbumin (Sharp & Lopata, 2014). Parvalbumin also known as Gad c 1 is a sarcoplasmic protein that has the physiological function of regulating calcium switching in muscle cells during relaxation (Lim et al., 2008). It has also been shown to possess both linear and conformational epitopes and to be resistant to thermal and enzymatic degradation (Wild & Lehrer, 2005). Other minor allergens include glyceraldehyde-3-phosphate dehydrogenase (van der Ventel et al., 2011) and tropomyosin from the fish parasite Anisakis (Asturias, Eraso, Moneo & Martinez, 2000). Like other food allergies, symptoms of fish allergy also cut across dermal reactions, gastrointestinal symptoms and systemic reaction or anaphylaxis (Van Do, Elsayed, Florvaag, Hordvik & Endresen, 2005). These reactions can occur via ingestion, inhalation of aerosolized allergens or vapors from cooking as well as skin contact during filleting and other industrial processing operations (Van Do et al., 2005).

2.3.5. Wheat

Wheat (*Triticum aestivum*) is one of the most widely cultivated cereals in the world in addition to maize, rice, barley, oats, rye, sorghum and millet (Scherf, Koehler & Wieser, 2016). It is also a major ingredient of common products like bread, pasta,
pastries, cookies and crackers. Wheat is another common source of immune-mediated hypersensitivity in both adults and children. Individuals with this allergy could experience both immediate and delayed skin, gastrointestinal and respiratory reactions (Palosuo et al., 2001). In a population based study, it was shown that the prevalence of wheat allergy could be as high as 3.6% (Zuidmeer et al., 2008). Bakers' asthma and rhinitis are common reactions to inhaled wheat flour especially among individuals in wheat processing facilities (Baur & Posch, 1998). In addition, wheat-dependent, exercise-induced anaphylaxis is a common reaction among adults where ingestion of wheat-containing product followed by exercise could cause anaphylaxis (Palosuo et al., 1999).

Wheat proteins can be divided into water-soluble albumins, salt-soluble globulins, dilute alcohol-soluble gliadins, acid and alkali-soluble glutenins (James, Sixbey, Helm, Bannon & Burks, 1997; Scherf, Koehler & Wieser, 2016) and allergenic proteins have been detected in all these fractions. These allergenic proteins include ω-5 gliadin (Tri a 19) which is the major allergen, α-amylase inhibitor, acyl-CoA oxidase, fructose-bisphosphatase aldolase, and peroxidase (James et al., 1997; Palosuo et al., 2001). For instance, when the IgE in the sera of seven wheat allergic children were probed with wheat protein extracts, 3 out of the seven subjects showed antibody reactivity against certain salt soluble proteins with molecular weights of 16, 35-67 and 94 KDa (Takizawa, Arakawa, Tokuyama & Morikawa, 2001). In another study, both radioallergosorbent test and immunoblotting confirmed the presence of IgE in the sera of 28 individuals that are reactive towards albumin, globulin, gliadin and glutenin protein fractions of wheat (Battais et al., 2003). Like other allergenic foods, avoidance of wheat is the major strategy of preventing adverse reactions. However, some wheat allergic individuals may
include other cereals including rye, barley and oats in their diets (Pietzak, 2012). A gluten free diet on the other hand (for individuals with celiac disease) must exclude all gluten containing cereals from the diet. Another major difference between wheat allergy and celiac disease is that the former may be outgrown by children while the latter is usually lifelong (Pietzak, 2012).

2.3.6. Soybean

Soybean (Glycine max) is a widely cultivated leguminous crop which is utilized in diverse food applications especially as source of protein as well as oil. It is also used as a meat substitute by consumers seeking vegetarian option and as an infant formula for babies with milk allergy (Sicherer, Sampson & Burks, 2000). However, soybean is another source of frequent hypersensitive reaction among children (Savage, Kaeding, Matsui & Wood, 2010) although a large proportion of these children will outgrow the allergy by the time they reach preschool age (Sampson & Scanlon, 1989). Using information from published reports, Sicherer (2011) showed that the prevalence of soy allergy among children aged 1 years old in the US is about 1.4%. Typical symptoms include adverse skin, gastrointestinal and respiratory reactions as well as anaphylaxis (Holzhauser et al., 2009). The allergenic proteins in soybean that have been identified include soybean Kunitz trypsin inhibitor, thiol-protease Gly m Bd 30k which is the major allergen and also storage proteins including β-conglycinin and glycinin (Holzhauser et al., 2009). However, the proteins that have been officially recognized and registered by the IUIS Allergen Nomenclature Sub-committee include the soy hydrophobic protein (Gly m 1), defensin (Gly m 2), profilin (Gly m 3), PR-10 protein (Gly m 4), β-
conglycinin (Gly m 5, vicilin, 7S globulin), glycinin (Gly m 6, legumin, 11S globulin) and seed biotinylated protein (Gly m 7) (Sung, Ahn, Lim & Oh, 2014).

2.3.7. Shellfish

Shellfish are widely consumed in different parts of the world because of their nutritional and health benefits (Khora, 2016). They are generally classified into crustaceans which include shrimp, lobster, crabs, krill and crayfish, and mollusks such as snails, abalone, clams, squid, scallops, oysters and octopus (Ayuso, 2011, Khora, 2016). The crustacean shellfish is recognized as one of the “big eight allergens” because they are responsible for most of the IgE-mediated, type 1 allergic reactions to seafood in developed countries as well as in coastal areas (Ayuso, 2011, Khora, 2016). More so, among the crustacean shellfish, shrimp, lobster and crab have been shown to contribute significantly to the occurrence of food allergy and sensitized individuals can develop reactions via contact, ingestion and inhalation (Khora, 2016). Among the mollusks on the other hand, oyster, clam and mussel are the common causes of allergic reaction (Ayuso, 2011).

In general, about 0.5-2.5% of the population have hypersensitive reaction towards shellfish (Khora, 2016). It has been estimated that 2% of the adult population and 0.1% of children in the United States are allergic to shellfish products (Sicherer & Sampson, 2010). The prevalence of shellfish allergy is higher in some Asian regions where consumption of shellfish is high (Lopata, O’hehir & Lehrer, 2010). For instance, in a study that reported shellfish allergy among 14-16 years old in Singapore and the Philippines, the proportion of this subset of the population with this allergy was put at 5.2% and 5.1%, respectively (Shek et al., 2010b).
Just like other food allergens, adverse reaction to shellfish is caused by the activation of the immune system by certain proteins that are naturally present in these foods. Activation of the immune system leads to the release of compounds which are responsible for the symptoms of shellfish allergy which could range from mild itching to swelling in the oral cavity, gastrointestinal symptoms like vomiting and diarrhea, trouble breathing as well as life threatening systemic reactions such as anaphylaxis (Khora, 2016; Lopata, O'hehir & Lehrer, 2010). Unlike some of the other big 8 allergens, shellfish allergy is usually lifelong (Ayuso, 2011).

The black tiger shrimp (*Penaeus monodon*) is a major aquacultured crustacean shellfish species in different parts of the world although the white leg Pacific shrimp (*Litopenaeus vannamei*) is also gaining ground (Ayuso, 2011). The allergenic proteins that have been identified in crustacean shellfish like shrimp include myosin light chain (17-20 kDa), calcium binding sarcoplasmic protein (20-25 kDa), actin (31-42 kDa), arginine kinase (40-45 kDa), troponin C (20 kDa), triosephosphate isomerase (28 kDa) and myosin heavy chain (227 kDa) (Abdel Rahman, Kamath, Gagné, Lopata & Helleur, 2013; Faber et al., 2016). However, different studies have shown that the major allergen of shellfish is the 34-39 KDa muscle protein known as tropomyosin because over 80% of sensitized individuals produced IgE that are reactive towards this protein (Ayuso et al., 2002; Shanti et al., 1993; Lopata, O'hehir & Lehrer, 2010).

Tropomyosin is a component of myofibrillar protein found in both vertebrates and invertebrates where it helps in muscle contraction in association with actin and troponin (Emoto, Ishizaki & Shiomi, 2009). However, only tropomyosin from invertebrates such as shellfish, dust mite and cockroach produces allergic reaction (Reese, Ayuso & Lehrer,
It has been described as 41nm long coiled-coil α-helical dimeric strands (Figure 2.4) (Ayuso et al., 2002, Kamath et al., 2014). The amino acid sequence of tropomyosin (281 amino acid residues) is characterized by repeating heptads in which the first and fourth residues are nonpolar (Shanti et al., 1993). It has also been shown that over 90% of the regions within its sequence contain mainly hydrophilic residues (Zheng, Lin, Pawar, Li & Li, 2011). Its molecular mass ranges from 35-38 kDa depending on the species and its isoelectric point is around 4.5 (Lopata, O'Hehir & Lehrer, 2010). Amino acid analysis also indicated that this protein is rich in alanine, aspartic acid, glutamic acid, lysine, leucine and arginine.

Figure 2.4. Molecular model of shrimp tropomyosin and its linear epitopes (Adapted from Reese et al., 2005)

Both the secondary and primary structures of allergenic proteins play a role in their binding with antibodies. The potency of an allergen is partly due to its highly conserved primary structure as well as hydrophilicity which facilitate improved interaction with antibody molecules leading to allergic reaction (Zheng et al., 2011). Therefore, information about the linear epitopes of allergenic proteins could help predict the cross-reactivity of different allergens and the allergenic potential of novel proteins. For instance, studies have shown that antibodies in the sera of shrimp allergic patients also bind with tropomyosin from other crustacean like lobster and crab (Shanti et al., 1999).
Towards unravelling the linear epitopes that contributes to shrimp allergy, Shanti et al. (1993) subjected tropomyosin from *Penaeus indicus* to partial tryptic digestion and identified two peptide fragments as the major allergenic epitopes since they were able to achieve 50% inhibition of tropomyosin. These peptide fragments have 9 (F L A E E A D R K) and 17 (M Q Q L E N D L D Q V Q E S L L K) amino acid residues, respectively. However, only the latter was found to be peculiar to shrimp tropomyosin as it has very low homology with tropomyosin from species including *Drosophila melanogaster*, rat, chick and human. Ayuso et al. (2002) on the other hand isolated five peptides fragments representing the major IgE binding linear epitopes of shrimp tropomyosin Pen a 1. In another study making use of bioinformatic tools to predict allergenic sequence of *Penaeus monodon* tropomyosin, 10 potential allergenic epitopes were identified out of which only 8 reacted with IgE in the sera of over 50% sensitized patients that were studied (Zheng et al., 2011). However, among the 8 peptide fragments only two were able to bind all the sera and thus considered critical for shrimp tropomyosin IgE binding (Zheng et al., 2011). Furthermore, the eight allergenic epitopes were found to be abundant in tyrosine, arginine, serine, glutamic acid and phenylalanine (Zheng et al., 2011).

### 2.4. Strategies for Mitigating Food allergy

#### 2.4.1. Avoidance and Labeling

The prevalence of food allergy and the adverse health effects experienced by allergic individuals makes it necessary to identify prevention or treatment options for food induced hypersensitivity. Unfortunately, there is currently no known cure for food allergy although certain food allergies including egg and milk allergies are resolved with
time while others including peanut, tree nuts and seafood allergies are lifelong (Tang & Hsiao, 2016). Consequently, strict avoidance of the offending food as well as availability of an epinephrine auto-injector is the only way allergic consumers can protect themselves from accidental exposure to allergens (Choi, Ju & Chang, 2015; Jackson et al., 2008, Muraro et al., 2014). However, the avoidance strategy is not without challenges. Although, accidental exposure to allergens can be minimized by avoiding the allergenic foods, this benefit is usually at the expense of good quality of life for the allergic sufferer (Kobernick & Burks, 2016; Muraro et al., 2014; Primeau et al., 2000). Allergic individuals are saddled with the responsibility of knowing what food to avoid and must be effective at reading food labels before making a purchase (Muraro et al., 2014). In addition, parents with allergic children often live in constant fear of exposure of their children to an allergen and they often need to adjust their lifestyle to safeguard the health of their children (Kobernick & Burks, 2016; Primeau et al., 2000; Tang & Hsiao, 2016). More so, the avoidance strategy may also carry a nutritional risk since allergic individuals need to source for alternative foods that are non-allergenic but also provide them with adequate nutrients (Muraro et al., 2014).

However, food allergy has been recognized has a major food safety concern in several countries. Hence, certain legislation has been put in place to protect members of the population that have food allergy from accidental ingestion of food containing allergens. These legislations usually involve the identification of major allergens responsible for most of the allergic reaction among the population followed by the mandatory declaration of these allergens on packaged product label. For instance, in the United States, the Food Allergen Labeling and Consumer Protection Act (FALCPA) of
2004 makes it mandatory for food processors to declare the presence of any of the “big 8” allergens namely soy, wheat, milk, egg, shellfish, fish, peanut and tree nuts or their proteins on their product package (US Code, 2004). Similar laws have also been passed in other countries including China where buckwheat is added to other “big 8” foods that must be declared on product label. In Canada, the list includes mustard, sesame and sulfites whereas in the European Union, celery, lupin, molluscan shellfish, mustard, sesame and sulfites must be declared (Allen et al., 2014).

Despite these regulations, the number of reported cases of allergen exposure leading to emergency visit or hospitalization has not reduced. This is due to the fact that, most food allergen labeling regulations focused solely on allergens that are intentionally added to foods (Allen et al., 2014) and not the unintended contamination of foods (cross – contact) with allergenic residues which can occur at any point in the food chain. For instance, most food processors utilize the same processing line or equipment for products containing allergenic residues and those that are non-allergenic (Allen et al., 2014). In addition, food manufacturers source ingredients or materials from countries that may not have a stringent allergen prevention policy and thus increase the risk of consumer exposure. Consequently, even though policies like FALCPA are in effect, a significant number of food recalls have been attributed to the presence of undeclared allergens in food products (Bucchini, Guzzon, Poms & Senyuva, 2016). For instance, in the United States, Gendel & Zhu (2013) showed that the number of allergen-related food recalls by the FDA increased from 78 recalls in 2007 to 189 in 2012. The study also found that milk was the most undeclared food allergen while bakery foods were the most frequently recalled food for allergen labeling violation (Gendel & Zhu, 2013). Another study that
reported food allergen recalls between 2012 and 2014 in the EU, UK, USA, Australia, Hong Kong and New Zealand found also found that milk as well as cereals, soy and eggs were the main undeclared allergens in recalled products (Bucchini et al., 2016).

To address this shortcoming, the United States Food and Drug Administration recently mandated food processors to treat food allergens as a hazard reasonably likely to occur and to develop and document control measures to prevent the hazard. This regulation forms part of the FDA Food Safety Modernization Act (FSMA) final rule for Preventive Controls for Human Foods (US FDA, 2016). Hence, food manufacturers must take steps to prevent cross contact such as separating raw materials and finished products containing allergens from those that are free of allergens. They must have proof that the ingredient or raw materials supplier has allergen control plan and must be able to verify the efficiency of their sanitation procedure to effectively remove allergenic residues from food contact surfaces amongst others. Although food manufacturers can place a precautionary warning statement on their food product label to inform consumers about the likelihood of the presence of an allergen, this should not be used as a substitute for good manufacturing practices to eliminate or prevent allergen cross contact (Allen et al., 2014). Moreover, some studies have shown that consumers may ignore the precautionary warning on product labels due to the different and sometimes vague statements used by manufacturers for precautionary labeling. Also, these types of labels may limit the food choices available to allergic consumers since some of the foods may not contain any allergenic residues.
2.4.2. Immunotherapy

As mentioned in the previous section, some food allergies get resolved with time while others are lifelong. The former case suggests that there is a natural process in place where an allergic individual can develop tolerance to the offending food over time. Understanding of the mechanism of this food allergy resolution could assist in the development of alternative treatment methods aimed at reducing sensitivity and inducing tolerance. In addition, understanding the factors that predispose certain individuals to become sensitized to a dietary protein could also aid the development of a curative method. The immune response of allergic individuals is characterized by a T cell response that is skewed towards Th2 rather than Th1, high production of allergen-specific IgE and minimal production of IgG, regulatory T cells (Tregs) and TGFβ + lymphocytes (Tang & Hsiao, 2016). Consequently, a curative procedure must be one that can alter these responses without inducing any adverse health effects.

Immunotherapy methods involving administration of increasing doses of an allergen to an allergic individual over a period of time have been shown to have the potential for desensitization and the eventual development of tolerance (Wood, 2016). Desensitization describes a state of non-reactivity to the ingestion of a certain dose (which is specific for each individual) of the allergen (above an initial baseline) which is sustained by continuous consumption of the offending food (Chinthrajah, Hernandez, Boyd, Galli & Nadeau, 2016). Tolerance on the other hand describes the prolonged retention of that increased threshold level for the allergen by the patient after cessation of the immunotherapy (Kobernick & Burks, 2016). Although the mechanism by which immunotherapy reduces clinical reactivity to food allergens is still being studied, some
immune responses that have been observed include the downregulation the Th2 response and increased production of Tregs and IgG (Wood, 2016; Varshney et al., 2011).

Immunotherapy methods can be categorized based on the route of administration of the allergen and they include oral, epicutaneous and sublingual immunotherapy. During oral immunotherapy (OIT), the allergen is embedded within a food matrix and it is given to the allergic patient in increasing doses under the supervision of a clinician until the threshold level for the individual is determined (Kobernick & Burks, 2016; Wood, 2016). A continuous consumption of the allergen at the subthreshold level can then be recommended by the clinician which the patient must adhere to outside the hospital. The desensitization efficacy of the OIT procedure is determined by monitoring the increase in the threshold limit for the allergen. After a successful desensitization, a period of withdrawal is maintained in which the patients will not be exposed to the allergen for up to 8 weeks after which another oral food challenge will be conducted to determine whether sustained unresponsiveness is achievable (Kobernick & Burks, 2016; Wood, 2016). Sublingual immunotherapy (SLIT) and epicutaneous immunotherapy (EPIT) are also aimed at increasing allergen threshold via exposure of the allergen to the immune cells in the oral cavity (for about two minutes before swallowing) and underneath the skin, respectively. However, unlike OIT where the actual food allergen is consumed, an extract from the food allergen is administered during SLIT either in liquid form or as a tablet (Kobernick & Burks, 2016).

Currently, these immunotherapy methods for allergy management are still in the research phase (Kobernick & Burks, 2016). Also, these procedures often lead to the occurrence of adverse symptoms like abdominal pains and reactions requiring
epinephrine injection which could make patients to discontinue the procedure before they can achieve desensitization (Keet et al., 2012). Other immunotherapy approaches aimed at enhancing the safety of the procedure are currently in different stages of clinical trials and they include utilization of anti-IgE, tolerogenic peptides and reducing the IgE binding capacity of the allergen through food processing methods.

2.4.3. Effects of Thermal and Non-Thermal Food Processing Methods

Most allergenic foods are subjected to some processing conditions to ensure palatability, efficient digestion and microbial safety prior to consumption (Rahaman et al., 2016). Consequently, attempts have been made to capitalize on the protein denaturation properties of different food processing methods to reduce or eliminate the allergenic capacity of the proteins (Lepski & Brockmeyer, 2013; Rahaman et al., 2016). The goal of this allergy mitigating strategy is to reduce or prevent the binding of allergenic proteins with their specific antibodies. IgE antibodies that bind to mast cells and basophils can recognize some sequences of amino acids that are near or far from each other on allergenic proteins and are thus referred to as linear and conformational epitopes, respectively (McClain, 2017). Hypothetically, processing methods that can disrupt or eliminate these epitopes will help prevent their binding with IgE and consequently the release of chemical compounds responsible for the symptoms of allergic reaction. Specifically, the protein processing-induced structural changes that modulate IgE reactivity include aggregation, unfolding, cross-linking with other food components especially through the Maillard-type reaction as well as chemical modification including oxidation and glycosylation (Lepski & Brockmeyer, 2013; Rahaman et al., 2016).
Over the years, the effects of thermal processing methods including boiling, baking, roasting, extrusion and high pressure steaming as well as non-thermal processing techniques such as irradiation, high pressure processing and light based technologies on the allergenic capacity of several foods have been examined in different studies. Reports from these studies have shown that processing can increase, decrease or maintain the allergenic capacity of foods, and these responses will depend on the subject exposed to the allergen, allergenic food matrix, the biochemical properties of the target allergenic protein, the processing method and conditions (Rahaman et al., 2016).

For instance, the allergenic capacity of certain fruit allergens especially those that are cross-reactive to the Bet v 1 birch pollen allergens (for example Api g 1 in celery, Pru av 1 in cherry) can be reduced by heat treatment. Hence, individuals that are sensitized to these birch pollen-related allergens may be able to tolerate the heated form of these fruits. However, another set of proteins known as the non-specific lipid transfer proteins (nsLTP) have been recognized as heat-stable fruit allergens. Unlike the Bet v 1 homologues which result in oral allergy syndrome (OAS), nsLTP can cause sensitization (Pastorello et al., 1999) which may lead to severe reactions including anaphylaxis and they can maintain their immunoreactivity even after extensive heat treatment. The thermal stability of nsLTP has been demonstrated in apple (Mal d 3), cherry (Pru av 3) and peach (Pru p 3) (Gaier et al., 2008; Scheurer et al., 2004; Sancho et al., 2005). Rahaman et al. (2016) in their review on processing-induced conformational changes of allergenic proteins submitted that physical processing methods such as heat, irradiation, ultrasound and pressure-induced changes only alter the conformational epitopes of allergens while leaving the linear epitopes intact (Rahaman et al., 2016).
Interestingly, loss of conformational epitopes can either reduce IgE-binding capacity or cause an increase.

2.5. Effects of Processing Methods on the Allergenic Capacity of Shellfish Tropomyosin

2.5.1. Boiling

In general, tropomyosin has been shown to be able to withstand boiling temperature. In comparison with raw extract, tropomyosin from boiled extract usually has a more enhanced electrophoretic band. In a study showing the protein profile of heated extracts (boiling for 20 minutes) of 11 crustaceans and 7 molluscs, uniform as well as heat-stable 18 kDa and 37 kDa bands were observed in all extracts from the latter while the former only had a stable 37 kDa bands of varying intensities (Kamath et al., 2013). It was also reported that heating increased the tropomyosin binding (shown by immunoblot analysis) with a monoclonal antibody for all the crustacean extracts and some of the mollusc extracts although no binding was observed among the extracts from raw molluscs (Kamath et al., 2013). This shows that boiling does make tropomyosin assume a structure with more exposed IgE binding sites thus increasing its allergenic capacity. A similar result was reported in a study using the boiled extract of *Litopenaeus vannamei* where a prominent 38 kDa tropomyosin band that also binds with IgE from the sera of five shrimp allergic patients was observed (Liu, Cheng, Nesbit, Su, Cao & Maleki, 2010). However, an inhibition ELISA protocol revealed that raw shrimp extract has about 8-fold higher inhibition rate compared with extract obtained from boiled shrimp (Liu et al., 2010). Since the crude extract contains other proteins and biomolecules, boiling might facilitate interaction of tropomyosin with these compounds thus reducing the exposure of
the allergenic epitopes. When the IgE binding of the purified tropomyosin from boiled extract was assessed using dot-blot, a higher IgE binding was observed in comparison with purified tropomyosin from raw extract (Liu et al., 2010). In this case, protein unfolding as well as protein-protein interaction might be responsible for the improved exposure of allergenic epitopes.

2.5.2. **Gamma Irradiation**

Gamma radiation resulted in the unfolding of the heat stable allergen of brown shrimp exposed to radiation dose between 1-10 kGy as shown by the significant increase in the turbidity and surface hydrophobicity of the extracts (Byun, Kim, Lee, Park, Hong & Kang, 2000). A radiation dose of 3 kGy was enough to reduce the IgE binding of HSP by 50% while a dose of 10 kGy gave less than 20% IgE binding. The SDS-PAGE profile of the irradiated HSP showed that the main 36 kDa tropomyosin band was no longer visible at a dose of 5 kGy. This might be due to complete degradation of the allergen by irradiation or formation of aggregates with other molecules. More so, IgE binding was observed in both the sarcoplasmic and myofibrillar protein fractions of irradiated whole shrimp. However, this binding was below 30% at a radiation dose of 10 kGy (Byun et al., 2000). However, in another study where shrimp (*Penaeus vannamei*) was exposed to radiation doses between 1 -15 kGy, the electrophoretic pattern of the total protein extract and purified allergen seems to suggest that a radiation dose of 10 kGy only has minimal effect on tropomyosin (Zhenxing, Hong, Limin & Jamil, 2007). This is evident from the thick 36 kDa band which is like that of untreated and boiled shrimp extract. Immunoblotting analysis using sera from shrimp allergic patients however indicates that allergenic capacity decreased only in shrimp protein extracts and purified allergen...
exposed to increasing dosage of gamma radiation followed by boiling for 15 minutes (Zhenxing et al., 2007). Immunoreactivity of extracts exposed to radiation alone increased significantly up to 10 kGy before it declined. In addition, results of competitive inhibition ELISA showed up to 30-fold increase in the inhibition of protein extract treated with irradiation followed by boiling which is higher than that of extract exposed to only gamma radiation and from the untreated sample. The same trend was also observed in extracts containing the purified allergen (Zhenxing et al., 2007).

2.5.3. Ultrasound

The SDS-PAGE profiles of tropomyosin extract obtained from crab (Scylla paramamosain) that was treated with boiling (100 °C for 20 minutes), ultrasound treatment (200W at 30 °C for 60 minutes) followed by boiling for 20 minutes (CUB) and high pressure steaming (HPS) at 121 °C for 20 minutes, revealed that tropomyosin has varying stability under different processing conditions (Yu, Cao, Cai, Weng, Su & Liu, 2011). In this study, both boiling and combination of ultrasound treatment with boiling did not seem to affect the structure of tropomyosin as shown by the thick band of 38 kDa observed in both cases. However, the tropomyosin band was only faint in the sample treated with high pressure steaming. Immunoblotting and inhibition ELISA indicate that extracts obtained from HPS had significantly reduced allergenic capacity.

It has also been reported that ultrasound treatment and high temperature could have a synergistic effect in the reduction IgE binding of tropomyosin. It was shown that the IgE capacity of Penaeus vannamei extract treated with ultrasound (30 Hz, 800 W at 0 °C for 90 minutes) was similar to that of extract obtained from untreated sample (mean 505.1 IU/mL and 484.4 IU/mL, respectively). Extract from the sample subjected to
similar ultrasound treatment but at 50 °C showed about 2-fold reduction (206.6) in IgE binding capacity as measured by enzyme allergosorbent test (Li, Lin, Cao & Jameel, 2006).

2.5.4. Pulsed Ultraviolet Light

Pulsed ultraviolet (PUV) light has been applied to certain foods to inactivate microorganisms. PUV treatment can either be thermal or non-thermal depending on the duration of exposure to the food products. During PUV treatment, light energy in the form of ultraviolet, infrared and visible lights are released in pulses to the target products. This energy causes excitation of the molecules within the products with subsequent release of heat energy that could denature protein. This potential for protein denaturation has been applied to alter the conformational epitopes of certain allergenic proteins in foods such as almond, peanut and soybean. Shriver et al. (2011) demonstrated that Atlantic white shrimp (*Litopenaeus setiferus*) extract exposed to radiation energy at around 0.27 J/cm²/pulse from a pulse ultraviolet light source could reduce the intensity of the tropomyosin band on SDS-PAGE gel after 4 minutes. In addition, western blot, dot blot and inhibition ELISA all confirmed the reduction of IgE binding with the extract treated with pulsed ultraviolet light compared with untreated and boiled extracts. However, it is yet to be seen if this treatment will produce similar results if actual shrimp samples are used rather than extracts.

2.5.5. Acid Treatment

Very few studies are currently available on the modulation of allergenic capacity of shrimp tropomyosin after exposure to acidic condition. Perez-Macalalag, Sumpaico &
Agbayani (2007) reported that protein extracts obtained from boiled shrimps that were previously soaked in vinegar for up to eight hours showed smaller wheal diameter during skin prick test. However, the authors did not provide any explanation on the mechanism by which acid soaking reduced the allergenic capacity of tropomyosin. In a recent study, the effect of different pH (1.0-11.0) on the conformation and allergenic capacity of tropomyosin isolated from short-neck clam (Ruditapes philippinarum) was examined (Lin, Li, Lin, Song, Lv & Hao, 2015). The authors reported that significant modification of tropomyosin secondary structure took place in the acid region (pH 1.0-5.0) compared to the alkaline region. Consequently, the immunoreactivity of tropomyosin at the acidic pH regime was lower than at the basic pH as shown by indirect ELISA and dot blot assay (Lin et al., 2015). The effect of acid treatment on the conformation and allergenic capacity of other food proteins has been reported. For example, when peanut kernel was soaked in vinegar or acetic acid at different pH (1.0-5.0), some of the peanut allergens could not be resolved by SDS-PAGE indicating denaturation or degradation of the protein (Kim, Lee, Seo, Han, Ahn & Lee, 2012). The IgE binding intensities of the peanut allergens including Ara h1, Ara h2 and Ara h3 decreased after exposure to acetic acid at pH 1 and vinegar at its natural pH (2.3). Armentia, Dueñas-Laita, Pineda, Herrero & Martín (2010) also reported that exposure of chicken and lentil to vinegar reduced their allergenic capacity as shown by smaller wheal size.

2.5.6. Enzyme Treatment with Digestive and Non-Digestive Proteases

For an allergic reaction to occur, the IgE must bind to either the linear or conformational epitope of the dietary protein. Consequently, attempts have been made to utilize enzymatic hydrolysis to cleave the antigenic protein to disrupt both the linear
(which often remain intact during most processing condition) and conformational epitopes (Bahna, 2008; Cabanillas et al., 2012; Li, Yu, Goktepe & Ahmedna, 2016; Rahaman et al., 2016). One aspect of enzymatic hydrolysis in food allergy research involves the utilization of non-digestive proteases to cleave the antibody binding sites of the allergen prior to ingestion of enzyme treated food. The goal of the investigator in this case is to determine whether the hydrolysate form of the allergenic foods can be tolerated by sensitized subject. Another aspect involves the determination of the stability of food allergens to digestive proteases. In this case, the goal is often to improve the digestibility of the allergenic proteins prior to contact with the immune system.

Reports from previous studies that have utilized enzymatic hydrolysis to attenuate the IgE reactivity of food allergens have shown that different factors including the enzyme type, substrate type, time of hydrolysis and enzyme concentration are some of the factors that affect the outcome of this processing method (Li et al., 2016, Panda, Tetteh, Pramod & Goodman, 2015). Consequently, while some investigators have reported the capacity of enzyme treatment to reduce the IgE reactivity of some foods others have also shown that enzymatic hydrolysis could enhance the IgE reactivity of some proteins due to exposure of new epitopes (Panda et al., 2015, Shi et al., 2013).

With respect to shrimp, the applications of enzymatic treatment to modulate the allergenic capacity of tropomyosin have revolved around the utilization of digestive proteases. For instance, Toomer et al. (2015) identified pepsin and pancreatin-stable shrimp proteins (including a 36 KDa band) which were also allergenic using a simulated gastric and intestinal fluid digestion protocol. Also, Liu et al. (2011) compared the in vitro digestibility of tropomyosin from grass prawn and Pacific white shrimp and
concluded that tropomyosin from the latter is more stable to digestive protease and thus, more allergenic. Another study investigated the effects of boiling, combined ultrasound and boiling, and high pressure steaming on the in vitro digestibility of crab (*Scylla paramamosain*) tropomyosin (Yu et al., 2011). Their result showed that high pressure steaming (0.14 MPa at 121 °C for 20 minutes) of crab helped to accelerate the gastrointestinal digestion using simulated gastric and intestinal fluid. More so, western blot analysis of the digest showed significant reduction in IgE binding to tropomyosin from high the pressure steamed sample that was treated with trypsin for more than 120 minutes (Yu et al., 2011).

### 2.6. Conclusions

Food allergy is indeed a major food safety issue in different parts of the world. Shrimp is one of the most widely consumed seafood in the United States and a major cause of allergic reaction. Like other allergens, avoidance is the major strategy for protecting shrimp allergic consumers. However, attenuating the allergenic capacity of the shrimp major allergen (tropomyosin) is also a potential mitigating strategy. Development of a hypoallergenic shrimp product may be useful for the oral immunotherapy procedure because it will not induce a severe reaction which will enable the subjects to undergo the procedure to its completion stage. Although the effects of different shrimp processing methods on the IgE reactivity have been assessed, there are gaps that need to be filled. Hence, in this study which was aimed at evaluating the effects of different food processing methods on the IgE reactivity of shrimp tropomyosin, the IgE reactivity of tropomyosin in the whole shrimp rather than in shrimp protein extract would be determined. In addition, some of the previous studies only examined the IgE reactivity of
tropomyosin in the soluble protein fraction. However, in this study, the IgE reactivity of tropomyosin that is soluble in dilute salt solution as well as in the insoluble pellet would be investigated. This would help account for poor solubility of the allergen caused by food processing methods. Lastly, one of the main characteristics of the food processing techniques that would be used in this study is their simplicity. These simple processing techniques include shrimp cooking methods such as boiling, steaming, baking and frying among others. In addition, treatment of whole shrimp with food grade organic acid as well as non-digestive enzymes (rather than digestive enzymes which have been used in previous studies) would be attempted to evaluate their effects on tropomyosin reactivity.
CHAPTER 3

EFFECTS OF BUFFER ADDITIVES AND THERMAL PROCESSING METHODS ON THE SOLUBILITY OF SHRIMP (*PENAEUS MONODON*) PROTEINS AND THE IMMUNOREACTIVITY OF ITS MAJOR ALLERGEN

3.1. Introduction

Food allergies are abnormal response of the body’s immune system to certain food proteins leading to the occurrence of severe dermatological and physiological reactions in certain individuals (Kamath et al., 2013). Eight foods (soy, wheat, peanut, tree nuts, fish, shellfish, egg and milk) have been shown to be responsible for over 90% of all food allergies in the United States. Thus, the Food Allergen Labeling and Consumer Protection Act (FALCPA) of 2004 made it mandatory for food manufacturers in the United States to declare the presence of these foods or their components on the product label (US Code, 2004). Similar laws have been developed in Canada and some parts of Europe.

Shellfish has been noted as one of leading causes of allergic reactions especially in coastal areas (Lopata et al., 2010). Nearly 6.5 million people in the United States have been reported to have an allergic reaction to shellfish (Lopata et al., 2010). Symptoms of this reaction include urtacaria, angioedema, asthma and life threatening anaphylactic shock (Lopata et al., 2010). The major allergen of shellfish is a 34-38 kDa myofibrillar protein known as tropomyosin (Liu et al., 2010).

Rapid and effective detection of allergens in foods is crucial for the protection of allergic consumers from accidental exposure to allergens as well as ensure industry
compliance with food allergen regulatory policies such as labeling (Khuda, Jackson, Fu & Williams, 2015; Poms, Capelletti & Anklam, 2004). Although methods such as PCR assay and mass spectrometry have been successfully utilized for allergen detection in foods, an immunochemical method especially enzyme-linked immunosorbent assay (ELISA) is most commonly used (Poms et al., 2004). Most ELISA methods require effective solubilization of the allergenic proteins in an extraction buffer prior to detection (Albillos, Al-Taher & Maks, 2011; Poms et al., 2004; Schmitt, Nesbit, Hurlburt, Cheng & Maleki, 2010; Steinhoff, Fischer & Paschke-Kratzin, 2011). However, studies have shown that food processing methods could pose a challenge to allergen detection using immunochemical method (Downs & Taylor, 2010; Fu & Maks, 2013; Schmitt et al., 2010). This is due to denaturation of allergenic proteins or their interaction with other components (such as during Maillard reaction) which result in decreased solubility or formation of insoluble complexes (Downs & Taylor, 2010).

Although most foods are consumed in processed form, the extraction buffers supplied with some ELISA kits are only optimized to extract allergenic proteins from raw or unprocessed samples (Schmitt et al., 2010). Thus, underestimation of allergenic protein concentration in processed foods may occur during immunochemical detection of allergens due to reduced solubility of the proteins in the extraction buffer. This underestimation may be important where a threshold has been set for the concentration of allergen that will cause a reaction. Consequently, the efficiency of complex buffers such as carbonate buffer (pH 9.6) and high salt buffer (containing up to 1M NaCl) for the recovery of soluble proteins from processed foods has been researched (Cong, Lou, Xue, Li, Wang & Zhang, 2007; Khuda, Jackson, Fu & Williams, 2015). In addition, the
potential of buffers containing surfactants such as SDS and Tween 20 as well as reducing agents such as 2-mercaptoethanol and dithiothreitol (DTT) to improve the extraction of allergenic proteins from processed food samples have been tested (Khuda et al., 2015; Watanabe et al., 2005). Results from these studies showed that buffer composition and processing conditions affect the recovery of soluble proteins. Moreover, extraction buffers need to be formulated such that they ensure quantitative and reproducible recovery of major and minor allergenic proteins from raw and processed food matrices.

Food processing has also been found to either aggravate or mitigate allergic reactions via alteration of allergen binding with immunoglobulin E (IgE) (Dhakal et al., 2014; Jiménez-Saiz et al., 2014; Noorbakhsh et al., 2010). For instance, Liu et al. (2010) reported that the IgE binding of tropomyosin in the extract of shrimp that was boiled for 10 minutes was lower than that of untreated shrimp. This was attributed to a masking effect caused by protein-chemical cross-linking during the boiling process or epitope destruction which inhibits IgE binding with the allergen. In another study, boiling of different crustaceans and mollusks for 20 minutes was found to increase the reactivity of tropomyosin in these sample extracts towards a monoclonal antibody (Kamath et al., 2013). These studies indicate that structural modification of tropomyosin could occur under processing conditions which could alter its IgE binding and consequently affect the severity of allergic reaction experienced by allergic patients. Understanding the relationship between food processing and allergenic capacity could help food processors and regulators design a more effective allergen control plan that will help guarantee consumer safety. This understanding can also help identify a potential food processing
method which can be employed to develop a hypoallergenic shrimp product for clinical
desensitization of allergic patients (Maleki, Schmitt, Galeano & Hurlburt, 2014).

A search of the available literature showed that the effects of processing on the
allergenic capacity of shrimp tropomyosin have mainly focused on boiling, high intensity
ultrasound, gamma irradiation and pulsed ultraviolet light (Li et al., 2005; Liu et al.,
2010; Shriver et al., 2011; Zhenxing et al., 2007). However, a comparative study on the
effect of a variety of processing methods employed during home or retail processing of
shrimp on tropomyosin allergenic capacity has not been conducted to the best our
knowledge. These processing methods often involve heating at elevated temperatures
(>200°C), wet and dry heating, volumetric heating and pressure assisted thermal
treatments. These processing methods could influence the solubility of shrimp proteins
during extraction as well as the allergenic capacity of tropomyosin in the soluble extract.
Moreover, some studies have shown that addition of a mild surfactant like Tween-20 and
reducing agent like DTT could help minimize hydrophobic interactions and cleave
disulphide bonds, respectively, in denatured proteins thus increasing their solubility
(Albillos et al., 2011; Hildebrandt, Steinhart & Paschke, 2008; Seiki et al., 2007;
Steinhoff, Fischer & Paschke-Kratzin, 2011; Zhenxing et al., 2007, Wu et al., 2014).

Hence, the aims of this study were: (1) to evaluate the effect of two buffer
additives (Tween 20 and DTT) on the recovery of soluble proteins from shrimp subjected
to different processing methods including boiling, steaming, baking, frying, high pressure
steaming, microwave roasting and grilling, and (2) to determine the effect of these
processing methods on the immunoreactivity of tropomyosin.
3.2. Materials and Methods

3.2.1. Materials and Chemicals

One bag of frozen black tiger shrimp (*Penaeus monodon*), 16-20 counts/lb were purchased from a local seafood store (McLaughlin Seafood, Bangor, ME, USA). The shrimp were kept frozen until use.

Tween-20 and dithiothreitol (DTT) used as buffer additives, were from Sigma (St. Louis, MO, USA) and Promega (Madison, WI, USA), respectively. Ammonium sulfate was from Sigma (St. Louis, MO, USA), acetone and sodium chloride were from Fisher Scientific (Fair Lawn, NJ, USA). Protein standards and precast gels for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio Rad (Hercules, CA., USA). Bovine serum albumin was obtained from Thermo Scientific (Rockford, IL., USA). 3, 3’, 5, 5’-Tetramethylbenzidine (TMB) was obtained from Sigma Aldrich (St Louis, MO., USA). HRP-labelled goat anti-human IgE was from Life Technologies (Grand Island, NY., USA). Costar™ 96-well microtitre plates used for immunoassay were from Thermo Fisher (Waltham, MA., USA).

3.2.2. Extraction Buffers

Buffers used for protein extraction were as follows: Buffer 1 was 20 mM sodium phosphate + 1M NaCl, Buffer 2 was 20 mM sodium phosphate + 1M NaCl + 1% Tween 20, and Buffer 3 was 20 mM sodium phosphate + 1M NaCl + 1% Tween 20 + 0.5 mM DTT. All the buffers had their pH adjusted to 7.5 with 1M NaOH.
3.2.3. Human Serum

Sera of three patients (25274-CP, 22104-JC and 22515-JH) with confirmed IgE antibody to shrimp tropomyosin were obtained from PlasmaLab (Everett, MA., USA). These sera were pooled before analysis.

3.2.4. Thermal Processing of Shrimp

Shrimp were thawed, deshelled and deveined. They were subsequently subjected to different thermal treatment including steaming (3 minutes), baking in an oven (200 °C for 4 minutes), frying with canola oil (> 200 °C for 1 minute), high pressure steaming in an autoclave (0.14 MPa at 121 °C for 20 minutes), microwave roasting in a domestic microwave oven (General Electric Co, Louisville, KY) at power level 3 for 2 minutes, grilling (> 250 °C for 7 minutes) and boiling (for 5 minutes). Unprocessed shrimp were used as control. Samples were treated in triplicate for each processing method. The samples were analyzed immediately after processing or stored.

The internal temperatures of the slowest heating portion (second abdominal segment) of all the processed samples were monitored with a thermocouple during or immediately after processing (except samples subjected to high pressure steaming) and their values were found to be higher than that recommended (145 °F) by United States Food and Drug Administration (FDA) for safe cooking of seafood.

3.2.5. Preparation of Shrimp Soluble Protein Extracts

Shrimp were minced manually after each processing method. Five grams of the minced samples were added to 50 mL of each extraction buffer. Based on trials with different types of extraction buffers and pH (sodium phosphate buffer and Tris buffer, pH
7-8), a high salt buffer comprising 20 mM sodium phosphate buffer and 1 M sodium chloride was chosen as the base extraction solution. Two different additives (Tween 20 and DTT) alone or in combination were subsequently added to this base extraction solution. The final extraction buffers were 20 mM sodium phosphate + 1M NaCl, 20 mM sodium phosphate + 1M NaCl + 1% Tween 20 and 20 mM sodium phosphate + 1M NaCl + 1% Tween 20 + 0.5 mM DTT, respectively. Extraction was carried out overnight with stirring at 4 °C. The extracts were first centrifuged at 7,000 × g for 15 minutes and the resulting supernatant was aliquoted. The aliquots were further centrifuged at 21,000 × g for 10 minutes and the clear supernatant was designated total protein extract.

The target protein (tropomyosin) is heat-stable. Thus, to obtain an extract rich in tropomyosin and other heat-stable proteins, an aliquot of the total protein extract was heated inside a water bath at 100 °C for 10 min. The resulting extract designated heat – stable protein (HSP) extract was centrifuged as above and both extracts were stored at -20 °C.

3.2.6. Purification of Tropomyosin from Shrimp Muscle

Acetone powder of minced shrimp muscle was prepared according to the method of Huang & Ochiai (2005). Briefly, minced shrimp muscle (raw sample) was homogenized in 10-fold volume of 20 mM Tris-HCl containing 50 mM KCl. The homogenate was centrifuged at 7,000 × g for 15 minutes and the supernatant containing soluble sarcoplasmic proteins was discarded. Homogenization and centrifugation with this buffer was repeated four times. The residues were then washed three times with cold absolute acetone. After the final wash, the residues were spread on a filter paper and dried overnight room temperature.
Extraction of myofibrillar proteins from the dried acetone powder was carried out as described by Liu et al. (2011) and Liang et al. (2008). The dried powder was suspended in 20 mM Tris-HCl buffer (1:10 w/v), pH 7.5, containing 1 M KCl and 10 mM 2-mercaptoethanol. Extraction was carried out with stirring at 4 °C overnight. The mixture was then centrifuged at 7,000 × g for 20 minutes and the supernatant adjusted to pH 4.6 with 1 N HCl and the resulting precipitate was dissolved in 20 mM Tris-HCl (pH 7.5). The dissolved precipitate was fractionated by adding solid ammonium sulfate to 40-60% saturation and centrifuged at 7,000 × g for 20 minutes. The precipitate was re-dissolved in 20 mM Tris-HCl (pH 7.5) and was passed through 0.45 µm filter paper. Further purification was accomplished via dialysis (Pur-A-Lyzer™ Maxi dialysis kit, MWCO 12-14 kDa, Sigma Aldrich) in 1 mM NaHCO₃ for about 24 hours. The dialyzed solution was then heated in a water bath at boiling temperature for 10 minutes and centrifuged to obtain supernatant containing tropomyosin. The presence of tropomyosin in this extract was confirmed by gel electrophoresis. The supernatant was aliquoted and stored at -20 °C.

3.2.7. Protein Analysis

The concentration of soluble protein in the resulting extracts (total protein extract and the heat-stable protein extract) was quantified with the aid of a bicinchoninic acid assay (BCA) kit (Pierce Biotechnology Inc, Rockford, IL., USA). The extracts were serially diluted with buffer to get the range of concentrations that fit a standard curve. Bovine serum albumin (BSA) supplied with the assay kit was used as a protein standard. The standard was diluted to a range of concentration between 2000-125 µg/mL. Approximately 25 µL of the sample extracts and standards were added to a micro plate.
followed by 200 µL of reagents A and B supplied by the manufacturer. After incubation at 37 °C for 30 minutes, absorbance was read at 562 nm with a plate reader (BioTek Instruments, Inc). All analyses were performed in triplicate.

### 3.2.8. SDS-PAGE Analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to visualize the protein components of extracts obtained from thermally processed samples according to the method of Laemmli (1970). All the three replicates of protein extracts from each processing methods were pooled. The pooled protein extract was diluted 1:1 with Laemmli buffer (containing 5% 2-mercaptoethanol) and quickly heated at 100 °C for 5 minutes. Exactly 7.5 µg of protein was loaded onto each well of a 12% Mini-Protean TGX Precast gel (Bio Rad, Hercules, CA., USA). Electrophoretic separation was achieved at 170 V in a Mini-Protean Tetra Tank (Bio Rad, Hercules, CA, USA). Precision Plus Protein Dual Color Standard was used as protein marker. Separated protein bands on the gel were visualized by staining with GelCode Blue Stain reagent (Pierce Biotechnology Inc, Rockford, IL, USA).

### 3.2.9. Inhibition ELISA

The binding of tropomyosin in the protein extracts of the processed samples with anti-tropomyosin antibodies was determined with an inhibition ELISA. Purified tropomyosin in 50 mM carbonate-bicarbonate buffer pH 9.6 was coated onto a high (0.2 µg/well) binding 96-well microtiter plate overnight at 4 °C. After incubation, the plates were washed five times with phosphate buffered saline containing 0.05% Tween-20, pH 7.4 (PBST). Coated wells were then blocked with 200 µL of PBST containing 1% bovine
serum albumin (BSA) for 2 hours at 37 °C to reduce non-specific binding. After blocking, the plate was washed with PBST and 50 µL of a pre-incubated (1 hour at 37 °C) mixture of diluted pooled sera (diluted 1/20 with PBST containing 0.5% BSA) and different concentrations of sample extracts (diluted to 100, 10, 1, 0.1, 0.01 and 0.001 µg/mL with PBST containing 0.5% BSA) was added to the wells. After incubation for 1 hour at 37 °C, the plate was washed with PBST and HRP-conjugated goat anti-human IgE antibody (1/2000 dilution) was added and incubated for 2 hours at 37 °C. After final washing with PBST, bound peroxidase activity was determined by adding 50 µL TMB (3, 3′, 5, 5′-Tetramethylbenzidine) followed by addition of equal volume of stop solution (2 M H₂SO₄). Absorbance was read at 450 nm with the aid of a plate reader (BioTek Instruments, Inc). The percentage inhibition was reported as the average of duplicate analysis and was calculated as follows

\[
\%\text{\ Inhibition} = \left( \frac{OD_{\text{no inhibitor}} - OD_{\text{inhibitor}}}{OD_{\text{no inhibitor}}} \right) \times 100\%
\]

3.2.10. Statistical Analysis

One-way analysis of variance (ANOVA) of treatments was carried out using the JMP software, version11 (SAS Institute Inc, Cary, NC, USA). Tukey’s HSD post-hoc test was used to determine the significant differences among means. Mean differences were statistically significant when \( p \leq 0.05 \).
3.3. Results

3.3.1. Influence of Buffer Additives and Thermal Processing on Soluble Protein Concentration

A significantly higher concentration (p < 0.05) of total soluble proteins (total allergenic and non-allergenic proteins) was extracted by the three buffer solutions from the raw shrimp compared with heated shrimp (Table 3.1). Among the heated samples, total soluble protein recovery was higher in the high pressure steamed samples and the addition of surfactant and reducing agent did not improve the recovery of soluble proteins from shrimp subjected to this heat treatment. In the case of buffer solution without additives (Buffer 1), the total soluble protein concentrations recovered by this buffer from most of the differently processed samples were not significantly different (p > 0.05). A much lower protein concentration was however obtained from boiled shrimp by this buffer which is only comparable to those obtained from steamed and microwave shrimp. A similar trend was observed using extraction buffer containing Tween-20 alone (Buffer 2), where the lowest protein concentrations were extracted from boiled and steamed shrimps by this buffer although the concentrations were not significantly different (p > 0.05) from those obtained from microwaved, grilled and baked samples. It should also be noted that the presence of Tween-20 alone did not significantly improve the recovery of soluble proteins from these heated samples when compared with buffers without this additive.
Table 3.1. Total soluble protein concentration (mg/mL) extracted from thermally processed shrimp using buffers with different additives.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Buffer 1</th>
<th>Buffer 2</th>
<th>Buffer 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>8.13±0.12&lt;sup&gt;A&lt;/sup&gt;x</td>
<td>9.10±0.15&lt;sup&gt;A&lt;/sup&gt;y</td>
<td>9.32±0.34&lt;sup&gt;A&lt;/sup&gt;y</td>
</tr>
<tr>
<td>High pressure steaming</td>
<td>1.67±0.09&lt;sup&gt;B&lt;/sup&gt;x</td>
<td>1.84±0.21&lt;sup&gt;B&lt;/sup&gt;x</td>
<td>1.90±0.25&lt;sup&gt;B&lt;/sup&gt;Cx</td>
</tr>
<tr>
<td>Baking</td>
<td>1.35±0.18&lt;sup&gt;C&lt;/sup&gt;Cxy</td>
<td>1.30±0.06&lt;sup&gt;C&lt;/sup&gt;Dy</td>
<td>1.91±0.39&lt;sup&gt;B&lt;/sup&gt;Cx</td>
</tr>
<tr>
<td>Frying</td>
<td>1.34±0.23&lt;sup&gt;B&lt;/sup&gt;Cy</td>
<td>1.53±0.09&lt;sup&gt;B&lt;/sup&gt;Cxy</td>
<td>1.79±0.13&lt;sup&gt;B&lt;/sup&gt;Cx</td>
</tr>
<tr>
<td>Grilling</td>
<td>1.33±0.07&lt;sup&gt;B&lt;/sup&gt;Cx</td>
<td>1.26±0.07&lt;sup&gt;C&lt;/sup&gt;Dx</td>
<td>2.18±0.48&lt;sup&gt;B&lt;/sup&gt;By</td>
</tr>
<tr>
<td>Microwave</td>
<td>1.10±0.03&lt;sup&gt;C&lt;/sup&gt;Dx</td>
<td>1.29±0.15&lt;sup&gt;C&lt;/sup&gt;Dx</td>
<td>1.71±0.18&lt;sup&gt;B&lt;/sup&gt;Cy</td>
</tr>
<tr>
<td>Steaming</td>
<td>1.01±0.03&lt;sup&gt;C&lt;/sup&gt;Dx</td>
<td>1.03±0.12&lt;sup&gt;D&lt;/sup&gt;x</td>
<td>1.38±0.10&lt;sup&gt;B&lt;/sup&gt;Cy</td>
</tr>
<tr>
<td>Boiling</td>
<td>0.87±0.08&lt;sup&gt;D&lt;/sup&gt;x</td>
<td>1.03±0.03&lt;sup&gt;D&lt;/sup&gt;x</td>
<td>1.37±0.18&lt;sup&gt;C&lt;/sup&gt;y</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviation (n = 3)

<sup>ABCD</sup> Mean values with the same superscript letter down each column are not significantly different (p > 0.05)

<sup>xy</sup> Mean values with the same superscript letter across rows are not significantly different (p > 0.05)

Buffer 1: 20 mM sodium phosphate + 1M NaCl
Buffer 2: 20 mM sodium phosphate + 1M NaCl + 1% Tween 20
Buffer 3: 20 mM sodium phosphate + 1M NaCl + 1% Tween 20 + 0.5 mM DTT

Protein concentration was quantified using BCA assay

Buffer solution containing both Tween-20 and DTT extracted similar concentrations of soluble protein from all the heated samples. Interestingly, the concentrations of soluble proteins from most of the heated samples were comparable to that of the sample subjected to high pressure steaming except for boiled samples. In addition, the presence of surfactant and reducing agent in this buffer significantly improved the extraction of soluble proteins from most of the heated samples compared with buffer without these additives or with surfactant alone.

This result shows that different processing methods reduced the solubility of shrimp proteins to varying degrees. Wet cooking methods (boiling and steaming) generally result in lower soluble protein recovery compared with dry heating methods.
such as baking, frying and grilling. Protein solubility of shrimp subjected to high pressure steaming was higher than those obtained from other heat treatment methods. More so, the combination of surfactant and reducing agent led to better recovery of soluble protein from shrimp processed by either wet or dry heating method.

Tropomyosin is a heat-stable protein. Hence, protein extracts are frequently heated to precipitate heat labile proteins to obtain tropomyosin rich extract (Seiki et al., 2007, Yu et al., 2011). In this study, the concentration of heat-stable proteins in the extracts obtained from raw and heated shrimp were quantified (Table 3.2).

Similar to the total protein extract, soluble protein concentration from untreated and high pressure steamed shrimp were higher than that obtained from other samples. However, presence of buffer additives had no effect on the concentrations of heat-stable proteins in the extracts of raw and high pressure steamed samples. In the buffer without additives (Buffer 1), the lowest concentration of heat – stable proteins was found in the extract from boiled and steamed shrimp although the latter was not significantly different (p > 0.05) from the baked shrimp extract.
Table 3.2. Heat-stable protein concentration (mg/mL) extracted from thermally processed shrimp using buffers with different additives

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Buffer 1</th>
<th>Buffer 2</th>
<th>Buffer 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>1.51±0.03&lt;sup&gt;Ex&lt;/sup&gt;</td>
<td>1.57±0.02&lt;sup&gt;ABx&lt;/sup&gt;</td>
<td>1.55±0.12&lt;sup&gt;ABCx&lt;/sup&gt;</td>
</tr>
<tr>
<td>High pressure steaming</td>
<td>1.65±0.16&lt;sup&gt;Ex&lt;/sup&gt;</td>
<td>1.72±0.29&lt;sup&gt;Ax&lt;/sup&gt;</td>
<td>1.76±0.20&lt;sup&gt;Ax&lt;/sup&gt;</td>
</tr>
<tr>
<td>Baking</td>
<td>0.97±0.10&lt;sup&gt;BCx&lt;/sup&gt;</td>
<td>0.96±0.05&lt;sup&gt;Cx&lt;/sup&gt;</td>
<td>0.93±0.09&lt;sup&gt;Dx&lt;/sup&gt;</td>
</tr>
<tr>
<td>Frying</td>
<td>1.03±0.02&lt;sup&gt;CDx&lt;/sup&gt;</td>
<td>0.87±0.05&lt;sup&gt;Cx&lt;/sup&gt;</td>
<td>1.21±0.24&lt;sup&gt;BCDx&lt;/sup&gt;</td>
</tr>
<tr>
<td>Grilling</td>
<td>1.16±0.01&lt;sup&gt;Dx&lt;/sup&gt;</td>
<td>1.21±0.07&lt;sup&gt;ABCx&lt;/sup&gt;</td>
<td>1.63±0.32&lt;sup&gt;ABx&lt;/sup&gt;</td>
</tr>
<tr>
<td>Microwave</td>
<td>0.98B±0.02&lt;sup&gt;Cy&lt;/sup&gt;</td>
<td>1.09±0.17B&lt;sup&gt;Cxy&lt;/sup&gt;</td>
<td>1.27±0.06&lt;sup&gt;ABCDx&lt;/sup&gt;</td>
</tr>
<tr>
<td>Steaming</td>
<td>0.87±0.05&lt;sup&gt;Bx&lt;/sup&gt;</td>
<td>0.90±0.08&lt;sup&gt;Cx&lt;/sup&gt;</td>
<td>1.11±0.04&lt;sup&gt;CDy&lt;/sup&gt;</td>
</tr>
<tr>
<td>Boiling</td>
<td>0.76±0.05&lt;sup&gt;Ax&lt;/sup&gt;</td>
<td>1.17±0.33&lt;sup&gt;Cx&lt;/sup&gt;</td>
<td>1.10±0.06&lt;sup&gt;Dx&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviation (n = 3)

<sup>ABCD</sup> Mean values with the same superscript letter down each column are not significantly different (p > 0.05)

<sup>xy</sup> Mean values with the same superscript letter across rows are not significantly different (p > 0.05)

Buffer 1: 20 mM sodium phosphate + 1M NaCl
Buffer 2: 20 mM sodium phosphate + 1M NaCl + 1% Tween 20
Buffer 3: 20 mM sodium phosphate + 1M NaCl + 1% Tween 20 + 0.5 mM DTT

Heat stable protein extract was an aliquot of total soluble protein extract heated at 100 °C for 10 minutes

Protein concentration was quantified using BCA assay

Other than high pressure steaming, similar heat-stable protein concentrations were retained in the buffer containing surfactant alone. The concentration of heat-stable proteins in the buffer containing surfactant and reducing agent were not statistically different among raw, high pressure steamed, fried, grilled, microwave and steamed shrimp. Presence of surfactant and reducing agent did not influence the recovery of heat-stable proteins from raw and heated shrimp samples although an exception was found in steamed samples where buffer 3 had a significantly higher (p < 0.05) heat-stable protein concentration compared with other buffers.
Three different types of high salt phosphate buffers (20 mM Phosphate + 1M NaCl) maintained at pH 7.5 containing no other additive or with 1% Tween-20 and 0.5 mM DTT with 1% Tween-20, respectively were used to extract proteins from raw and processed shrimp. These additives were selected because a previous study indicated that the addition of a surfactant and a reducing agent helped improve the solubility of proteins from processed foods (Watanabe et al., 2005) and these additives have also been used in other studies for allergenic protein extraction (Albillos et al., 2011, Hildebrandt et al., 2008, Seiki et al., 2007, Steinhoff et al., 2011, Zhenxing et al., 2007, Wu et al., 2014). Tween-20 is a non-ionic and non-denaturing surfactant that can help improve protein interaction with solvent by minimizing protein-protein association caused by denaturation. DDT on the other hand is a reducing agent that cleaves disulfide bonds thus exposing more protein regions for solubilization (Liu & Hsieh, 2008).

A significant reduction in the concentration of total soluble proteins was observed after subjecting shrimp samples to different processing methods (Table 3.1). The significant reduction in soluble protein recovery from the heat processed samples can be attributed to the denaturation of shrimp muscle proteins. Protein unfolding with subsequent exposure of hydrophobic residues and formation of insoluble aggregates are some of the processes that take place during thermal processing and these reduce the solubility of proteins in extraction solution (Albillos et al., 2011). Using the BCA assay, about 50% reduction in soluble protein recovery by phosphate buffered saline (PBS) was reported for boiled and autoclaved peanut flour in solution in comparison with defatted raw peanut flour (Fu & Maks, 2013). A study with egg powder reported about 75%
reduction in egg-soluble proteins in PBS from boiled and autoclaved samples in comparison with raw egg (Fu, Maks & Banaszewski, 2010).

With respect to the raw sample, a significantly higher protein concentration was obtained from the high salt phosphate buffer containing Tween 20 alone and that containing both Tween 20 and DTT when compared with the buffer without these additives. However, no synergistic effect was observed between Tween 20 and DTT for the extraction of soluble proteins from raw shrimp. As shown in Table 3.1, there was no significant difference (p > 0.05) in the concentration of total soluble protein in phosphate buffer containing just Tween-20 and that containing both Tween-20 and DTT for raw shrimp. This may be due to the absence of new covalent and non-covalent interaction between proteins in the raw samples. The effect of surfactant and reducing agent added alone or in combination in order to improve the recovery of soluble proteins from raw shrimp has not been reported to the best our knowledge. However, it has been reported that the addition of DTT to 1M KCl significantly increased protein extraction from raw shrimp (*Litopenaeus vannamei*) when compared with six other buffers (Wu et al., 2014).

Extraction of soluble proteins from boiled, steamed, microwaved, grilled and fried shrimps with buffers containing both surfactant and reducing agent resulted in a significantly (p < 0.05) higher total soluble protein concentration (Table 3.1). Presence of Tween-20 alone in the high salt phosphate buffer did not significantly increase (p > 0.05) the recovery of soluble proteins from most of the heated samples when compared with buffer without additives. Thus, the presence of DTT in the extraction buffer might be crucial for the recovery of soluble protein from processed shrimp. A similar result was reported in a study where sodium dodecyl sulfate (SDS), a denaturing surfactant,
significantly increased the recovery of soluble proteins from raw egg without the presence of 2-mercaptoethanol, a reducing agent (Watanabe et al., 2005). However, SDS alone did not increase protein extractability from boiled egg and fried noodle except in the presence of 2-mercaptoethanol (Watanabe et al., 2005). In addition, different researchers have reported that the extraction buffer supplied with the Morinaga ELISA kit containing both surfactant and reducing agent helps improve the recovery of egg soluble protein from thermally processed raw materials and from different food matrices (Fieste, Lovberg, Lindvik & Ecaas, 2007, Fu et al., 2010).

In a study to improve the extraction of soluble protein from almond roasted at temperatures between 230 and 400 °C, phosphate buffered saline with 0.1% Tween-20 (PBST) was found to improve the solubility of protein from the processed almond compared with PBS and water extracted samples (Albillos et al., 2011). Even though the concentration of Tween 20 in our study was greater than that used in the above study, our result showed that Tween 20 alone did not significantly improve the extraction of soluble proteins from processed shrimps. Therefore, the effect of buffer additives like surfactants on soluble protein recovery might be influenced by the source and nature of the food protein, the food matrix and the processing method applied (Steinhoff et al., 2011). ELISA kits are formulated to extract and detect specific allergenic protein or total soluble proteins from food samples (Fu & Maks, 2013, Westphal, Pereira, Raybourne & Williams, 2004). From this study, recovery of total soluble proteins from processed shrimp samples can be improved by adding a surfactant and reducing agent to the extraction buffer. However, this may not be necessary for ELISA kits targeting tropomyosin or other heat – stable proteins from shrimp because the presence of these
buffer additives did not significantly improve the recovery of heat-stable proteins from most the processed shrimps.

3.3.2. SDS-PAGE Analysis

The buffer containing a reducing agent and surfactant had a higher concentration of total proteins in most of the processed samples while the concentrations of heat-stable proteins in the extract of most of the processed samples were not significantly different (p > 0.05). Hence, the banding pattern of total protein extracts and heat-stable protein extracts from buffer containing Tween 20 and DTT were examined using gel electrophoresis. The total protein extracts from the untreated and thermally processed shrimp shows complex bands consisting of different myofibrillar proteins (Fig 3.1).

Figure 3.1. SDS-PAGE analysis of the soluble proteins from the total protein extracts of raw and thermally processed shrimp.

MR: Molecular weight Marker, Lane 1: Raw, Lane 2: Boiled, Lane 3: Steamed, Lane 4: Baked, Lane 5: Fried, Lane 6: HPS, Lane 7: Microwaved, Lane 8: Grilled, 9-10: Purified tropomyosin, Tm: Tropomyosin (36 kDa), MLC: Myosin light chain
Extract from untreated shrimp shows thick protein bands between 75-250 kDa, as well as a prominent 42 kDa band (arginine kinase), 36 kDa band corresponding to tropomyosin and a 20 kDa band representing myosin light chain. Almost the same bands can be seen in all other extracts (except high pressure steamed sample) although the intensities of the bands were reduced. Prominent protein bands in the extracts from processed samples include 200 kDa (myosin heavy chain), 100 kDa (α-actinin), a 75 kDa band, 42 kDa (arginine kinase), 36 kDa (tropomyosin) and 20 kDa (myosin light chain) (Liu et al., 2011, Toomer et al., 2015). In comparison with other bands however, the band intensities of tropomyosin and myosin light chain were enhanced (Fig 3.1). The protein profile of extracts obtained from high pressure steamed shrimps was very different from those obtained from other processed samples. All the prominent bands (including tropomyosin) found in other extracts appeared vague in the high pressure steamed extract. However, a high molecular weight band (approximately 250 kDa) was observed which might indicate that high pressure steaming caused aggregation of myofibrillar proteins. Furthermore, a careful observation of the wells of the electrophoretic gel indicated that some proteins were retained on the wells loaded with soluble protein extracts from untreated and heated shrimp. The intensity of the retained protein band from extract of untreated shrimp is higher than the protein band intensities of extracts from heated shrimp. These bands might correspond to high molecular weight proteins (in the case of untreated samples) or high molecular weight protein aggregates formed during processing which cannot be resolved on the electrophoretic gel.

The profile of the heat-stable extracts from untreated and processed shrimps was less complex than that of total protein (Fig 3.2).
Figure 3.2. SDS-PAGE analysis of the soluble proteins from the heat-stable extracts of raw and thermally processed shrimp.

Boiling for 10 minutes resulted in the precipitation of heat labile proteins leaving only heat-stable proteins in the supernatant. More so, unresolved high molecular weight proteins or protein aggregates were removed as indicated by absence of protein bands on the wells loaded with extracts from untreated and heated samples. However, an exception can be seen in the well loaded with extracts obtained from high pressure steamed sample (well 6) where a faint protein band can be observed. This most likely suggests that protein extracts from high pressure steamed shrimp had more high molecular weight aggregated proteins compared with other extracts.

Three distinct bands including a band of approximately 100 kDa, tropomyosin (36 kDa) and myosin light chain (20 kDa) can be observed from all extracts (Fig 3.2). In addition, the intensities of these bands were enhanced in the extract from high pressure steamed sample when compared with that of total protein extract. However, the high molecular weight band observed in the total protein extract from high pressure steamed
sample can also be seen in the heat-stable fraction. This indicates that some of the proteins have been denatured and have rearranged to form aggregates. In general, the intensities of the tropomyosin and myosin light chain bands were the greatest among the observed bands in the heat-stable protein fraction. This confirms that tropomyosin was very stable under the thermal conditions used in this study except for the high pressure steaming process where tropomyosin band had a lower intensity.

Different studies have confirmed the stability of tropomyosin during boiling. Shriver et al. (2011) reported that boiling shrimp extract for 4 minutes did not reduce the band intensity of tropomyosin and was stable under exposure to pulsed ultraviolet light for up to 3 minutes. When the banding pattern of extracts from 11 raw and boiled crustaceans (10 minutes boiling in PBS) was assessed, complex protein bands between 18-20 kDa and 35-40 kDa were reported (Kamath et al., 2013). Also, the band corresponding to tropomyosin was found to have greater intensities in the heated extracts (Kamath et al., 2013). This study has also shown that shrimp processing methods including steaming, baking, frying, microwave roasting and grilling enhanced the band intensity of tropomyosin in extracts.

The intensity of the tropomyosin band as well as of other myofibrillar proteins in the extract of shrimp heated at high pressure in an autoclave was reduced. This agrees with other studies where reduction in band intensity was observed in samples heated at high pressure. In a study on the effect of different processing methods including boiling (100 °C for 20 minutes), combined ultrasound and boiling, and high pressure steaming (0.14 MPa at 121 °C for 20 minutes) on crab (Scylla paramamosain) tropomyosin, the tropomyosin band in the extract from high pressure steamed sample had a lower intensity.
This suggests that high pressure steaming can alter the electrophoretic pattern of tropomyosin. Also, electrophoretic characterization of the protein extract of roasted peanut heated at 121 °C (1.18 atm) and 138 °C (2.56 atm) for 15 and 30 minutes revealed less visible protein bands and a low molecular weight smear (Cabanillas et al., 2012). In general, poor solubility in extraction buffer as well as protein degradation has been suggested to be responsible for the marked difference in electrophoretic patterns of protein extracts from foods treated with high pressure steaming (Tomotake, Yamazaki & Yamato, 2012). However, comparison of the concentration of soluble proteins (Table 3.1 and 3.2) in extracts (buffer 3) from high pressure steaming with extracts obtained from other processing methods shows that similar protein concentrations were extracted. Hence, we submit that another protein modification process might also be responsible for the electrophoretic patterns of soluble proteins from high pressure steamed shrimp rather than just protein insolubilization. For instance, evidence of protein degradation into smaller fragments has been demonstrated in the autoclave treatment of heat-labile porcine serum albumin (PSA) solution (Kim, Lee, Song, Kim & Ahn, 2011). When this solution was heated in an autoclave (121 °C, 1 atm) for 5 and 10 minutes, the PSA band degraded into low molecular weight peptides whereas at 30 minutes, the original PSA band and the low molecular weights peptides had considerably reduced intensity (Kim et al., 2011).

**3.3.3. Inhibition ELISA**

Since tropomyosin was retained in the heat-stable fraction of all samples, these extracts were used for the inhibition ELISA assay (Figure 3.3). The IgE in the pooled sera from three shrimp allergic patients was highly inhibited by the heat-stable extracts from raw and processed shrimp.
Figure 3.3. Inhibition ELISA analysis of heat-stable extracts from raw and processed shrimp

![Graph showing inhibition ELISA analysis](image)

**Legend:**
- □ 0.001
- □ 0.01
- □ 0.1
- □ 1
- □ 10
- □ 100

### Data Table

<table>
<thead>
<tr>
<th>Processing Method</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw</td>
<td>a</td>
</tr>
<tr>
<td>Boiling</td>
<td>b</td>
</tr>
<tr>
<td>Steaming</td>
<td>c</td>
</tr>
<tr>
<td>Baking</td>
<td>d</td>
</tr>
<tr>
<td>Frying</td>
<td>e</td>
</tr>
<tr>
<td>HPS</td>
<td>f</td>
</tr>
<tr>
<td>Microwave</td>
<td>a</td>
</tr>
<tr>
<td>Grilling</td>
<td>b</td>
</tr>
</tbody>
</table>

*abcd* Data are expressed as mean ± standard deviation (n=2). The same letter indicates that the means are not significantly different (p > 0.05).

Serial dilutions (100-0.001 µg/mL) of protein extracts were incubated with pooled sera (1/20 dilution) from three patients with confirmed allergic reaction to shrimp. Peroxidase-conjugated goat anti-human IgE (1/2000 dilution) was used as secondary antibody.

The percent inhibition was similar across the different concentrations of extracts obtained from both raw and processed shrimp. For the highest concentration of heat-stable proteins, the percent inhibition was over 80% thus indicating a strong IgE binding. However, an exception was found in the extract of samples subjected to high pressure steaming which showed a significant reduction (p < 0.05) in IgE binding especially at protein concentration between 0.1-0.001 µg/mL. Extracts from the raw and processed samples also showed significant reduction at the minimum antigen concentration (0.001 µg/mL). At this minimum antigen concentration, the percentage inhibition of tropomyosin (about 60%) of the raw shrimp was comparable to that fried shrimp. The percentage inhibition of other processed samples at 0.001 µg/mL antigen concentration
were significantly ($p < 0.05$) lower than that of raw and fried shrimp. However, the least percentage inhibition (around 20%) was obtained in high pressure steamed sample at the minimum antigen concentration (Figure 3.3).

Although the band intensity of tropomyosin was reduced in the high pressure steamed extract in comparison with other processing methods (likely due to protein denaturation), the iELISA revealed that the tropomyosin in this extract still had an appreciable number of epitopes that bound with anti-tropomyosin antibodies in the pooled sera. Nevertheless, since the extent of this binding was low especially at lower protein concentration, it can be concluded that the heat-stable extract of samples processed by HPS had less allergenic components compared with extracts from other processed samples.

In summary, the allergenic capacity of tropomyosin in the heat-stable extracts as assessed by inhibition ELISA analysis of differently processed samples (except high pressure steaming) was comparable to that of the raw sample. This indicates that the processing methods did not alter the IgE binding epitopes on the tropomyosin structure significantly. Thus, common shrimp processing methods used either at domestic or during retail processing cannot be counted on to reduce the allergenic capacity of shrimp tropomyosin.

Studies on the allergenic capacity of tropomyosin from shrimp prepared using common cooking methods used in this study (except boiling) are scarce or unavailable. However, a similar result was obtained in a study that examined the effects of boiling, frying and roasting on the allergenic capacity of peanut allergens (Cong et al., 2007). The authors reported that these processing methods did not alter the allergenic capacity of
major peanut allergens (Cong et al., 2007). Liu et al. (2010) reported that soluble protein extracts from shrimp (*Litopenaeus vannamei*) boiled for 10 minutes produced lower IgE binding than extract from raw shrimp. However, purified tropomyosin from the same boiled sample gave a higher IgE binding (as shown by dot-blot assay) compared with tropomyosin from raw shrimp (Liu et al., 2010). Hence, it is important for food processors, food service professionals and regulatory agencies to ensure that adequate measures are put in place to prevent exposure of shrimp allergic individuals to these differently processed shrimp products. It is worth noting that high pressure processing helped reduce the allergenic capacity of tropomyosin to a certain degree especially at low protein concentration but it did not completely abolish the allergenic capacity of tropomyosin. Similar results were reported in a previous study where tropomyosin in the protein extract obtained from crab (*Scylla paramamosain*) heated at 121 °C at 0.14 MPa for 20 minutes in an autoclave showed a reduced percent inhibition rate compared with extracts from sample that was boiled or treated with combined ultrasound and boiling (Yu, Cao, Cai, Weng, Su & Liu, 2011). Also, the effect of autoclaving parameters on the allergenic capacity of other food proteins has been reported. Cabanillas et al. (2012) showed that analysis of soluble proteins by indirect ELISA from roasted peanut that was exposed to an extreme autoclave condition (especially at 2.56 atm for 30 minutes) had a reduced IgE reactivity between 9% and 96%. In addition, the Western blot assay from this study showed that 22% of sera from peanut allergic patients bound with some proteins both in the soluble and insoluble fraction obtained from the autoclaved roasted peanut (Cabanillas et al., 2012). This indicates that high pressure steaming does not completely remove the immunoreactivity of certain allergens but the extent of antigen –
antibody binding necessary to cause a reaction is significantly reduced. However, some allergenic proteins have been shown to be able to maintain their stability even after exposure to high pressure steaming. For instance, when the antigenicity of almond (Prunus dulcis L.) soluble proteins was assessed following roasting, blanching, autoclaving and microwave treatment, the autoclaving conditions used in the study did not significantly reduce the antigenicity of the proteins (Venkatachalam et al., 2002). In the case of these almond proteins, only prolonged roasting (320 °F for 20 and 30 minutes) as well as microwave heating for 3 minutes were able to alter their antigenicity (Venkatachalam et al., 2002). Thus, the ability of any processing method to modulate the immunoreactivity of an allergen depends on the type and condition of processing as well as the properties of the allergen.

Food processing can induce conformational changes in allergenic proteins, cause the formation of high molecular weight aggregates and also degrade proteins into smaller fragments (Kim et al., 2011, Davis & Williams, 1998). Since the immunoreactivity of most proteins depends on the retention of their native structure (structural epitopes), food processing methods that can alter or facilitate complete loss of these structural epitopes may also help reduce significantly the ability of these proteins to cause severe allergic reaction. This study has shown that shrimp tropomyosin is stable and still retains its reactivity under conditions that can be used to produce wholesome shrimp through boiling, steaming, frying, microwaving, baking and grilling. However, thermal treatment under high pressure has been shown to modify the structure and immunoreactivity of shrimp tropomyosin. Thus, it is important to understand the nature of the structural changes experienced by tropomyosin during high pressure steaming. More so, the
condition of the high pressure steaming used in this study was harsh since it led to the
loss of structural integrity of the shrimp. Hence, this process needs to be optimized using
pressure, temperature and time levels that will result in processed shrimp with better
sensory attributes. Information on the level of allergic reaction (extent to which IgE is
activated) that can be produced during exposure to high pressure steamed shrimp using a
murine model or human subject (skin prick test) is desirable. This information is crucial
for the successful utilization of high pressure steaming to develop a hypoallergenic
shrimp product which subsequently can be used in clinical trials for desensitization of
crustacean allergic patients.

3.4. Conclusions

In this study, the potential of additives including a mild surfactant and reducing
agent added to an extraction buffer to improve the recovery of soluble proteins from
thermally processed shrimps was investigated. Buffer containing both surfactant and
reducing agent improved the recovery of total soluble proteins from processed shrimp.
However, these additives did not improve the recovery of heat-stable proteins from the
samples. Shrimp’s major allergen, tropomyosin has an enhanced protein band during
SDS-PAGE analysis of extracts from different heat treated shrimps except that of high
pressure steaming where the band intensity was reduced. The IgE binding of tropomyosin
from the extracts of different processed shrimps used in this study was comparable to that
of raw sample except that of high pressure steaming which displayed lower IgE binding.
CHAPTER 4

SHRIMP TROPOMYOSIN RETAINS ANTIBODY REACTIVITY AFTER EXPOSURE TO ACIDIC CONDITION

4.1. Introduction

Crustacean shellfish are good sources of essential nutrients including protein and polyunsaturated fatty acids (Karakoltsidis, Zotos, & Constantinides, 1995). However, they are also responsible for some of the cases of allergic reaction especially among the adult population (Wild & Lehrer, 2005). In the United States, the prevalence of crustacean shellfish allergy among the adult population and young children is about 2% and 0.1%, respectively (Lopata et al., 2010; Sampson, 2004). Ingestion of crustacean shellfish by allergic consumers can cause serious adverse reactions including anaphylaxis (Eischeid, 2016). Consequently, crustacean shellfish is one of the “big 8” food allergens that must be declared on packaged product labels (FDA, 2004).

Different allergenic proteins have been implicated in the allergic reactions to crustacean shellfish. However, a component of the myofibrillar protein known as tropomyosin (34-38 kDa) has been identified as the major crustacean shellfish allergen because at least 80% of individuals with crustacean shellfish allergy reacts to this protein (Liu et al., 2010; Long et al., 2015). Tropomyosin is a coiled-coil dimeric protein having a predominantly α-helix structure and is characterized by stability to heat treatment and digestive proteases, and its isoforms from frog legs showed high stability in acidic condition (Lehrer & Yuan, 1998; Mikita & Padlan, 2007).
Attempts have been made to understand how different methods of processing shrimp affects the immunoreactivity of tropomyosin. These methods include boiling (Liu et al., 2010), gamma irradiation (Zhenxing et al., 2007), pulsed ultraviolet light (Shriver et al., 2011), high intensity ultrasound (Li et al., 2005) as well as combined high pressure and thermal treatments (Long, Yang, Wang, Hu & Chen, 2015). Changes in the allergenic capacity of tropomyosin have been linked to some alteration in its secondary structure via these methods.

Very few reports are available on the effect of food matrix components on the allergenic capacity of shrimp tropomyosin. Some of the commercial products that may contain crustacean shellfish residues include high acid foods (pH < 4.6) such as salad dressing and Worcestershire sauce (Prester, 2016). In these products, organic acids such as vinegar are used to maintain the microbiological quality and sensory attributes of the products (Mejlholm, Devitt & Dalgaard, 2012; Xiong, Xiong, Blanchard, Wang & Tidwell, 2002). However, this low pH condition could also modify the secondary structure of food proteins present in these products and potentially, the immunoreactivity of allergenic proteins. Hence, studies on the immunoreactivity of tropomyosin in a high acid model system are necessary in order to unravel the effects of the acidic environment on its allergenic potential. In addition, the suitability of immunochemical methods such as enzyme-linked immunosorbent assay (ELISA) for the detection of allergens in high acid foods matrix needs to be ascertained.

The design of some studies pertaining to the effect of acidic medium on pH-induced modification (change in secondary structure) of some allergens, raises questions about the actual capacity of this low pH condition to reduce allergenic capacity (Kim et
A low pH condition can indeed alter the allergenic capacity of a protein via alteration of the conformational epitopes. However, a low pH condition might not be effective in reducing the allergenic capacity of a protein where most of the epitopes are linear. More so, pH-induced denaturation of allergenic proteins can reduce their solubility in extraction solution and therefore their detection and accurate quantification via immunochemical methods may be affected (Downs et al., 2016).

Moreover, the antigenic properties of proteins in the insoluble pellet obtained after the recovery of supernatant are often overlooked by investigators.

Thus, the objective of this study was to determine the effects of different acidic pH conditions on the IgE-binding capacity of tropomyosin using a model marinade comprising vinegar at different pH values.

4.2. Materials and Methods

4.2.1. Materials and Chemicals

Frozen shrimp (*Penaeus monodon*), were purchased from a local store and kept at −20 °C until use. Distilled white vinegar (5% acidity) was purchased from a local grocery store and stored at room temperature in the laboratory. The precast gels, nitrocellulose membranes (0.2 μm), sample and running buffers for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were from Bio Rad (Hercules, CA., USA). Sodium dodecyl sulfate, 4-chloro-1-naphthol, diaminobenzidine (CN/DAB) substrate kit and Bicinchoninic assay (BCA) kit were obtained from Thermo Fisher Scientific (Rockford, IL., USA). 3, 3′, 5, 5′-Tetramethylbenzidine (TMB) was obtained from Sigma Aldrich (St Louis, MO., USA) and horseradish peroxidase (HRP) labelled
goat anti-human IgE was from Life Technologies (Grand Island, NY., USA). Sera from
three individuals with confirmed IgE antibodies for shrimp tropomyosin were purchased
from PlasmaLab (Everett, WA., USA).

4.2.2. Marination Process

Prior to the marination process, the frozen shrimp were thawed in a container
under a continuous flow of water at room temperature (approximately 20 °C). The shrimp
shell and intestinal tract were carefully separated from the meat and the shrimp were
patted dry to remove surface moisture. The shrimp had an average mass of 17.6g. The
shrimp were then placed inside a plastic bag (Whirl-Pak Nasco, Fort Atkinson, WI.,
USA) that had been filled with distilled white vinegar of different pH (1.0, 2.5, 3.5 and
4.8) and distilled water (Control). The shrimp to vinegar ratio was approximately 1: 3
(w/v) and each plastic bag had one shrimp. The initial pH of distilled white vinegar was
around 2.7, and the vinegar pH was adjusted with either 6 N HCl or 10 M NaOH
depending on the treatment pH condition. The shrimp were marinated for 1, 3, 6 and 16
hours at 4 °C. These marination conditions (vinegar pH and time) were selected to allow
shrimp muscle to experience varying final core pH values and for different length of
time. After marination, the shrimp were quickly rinsed with distilled water to remove
surface vinegar and then patted dry. All samples were analyzed immediately.

4.2.3. Marinade Uptake

In order to evaluate the influence of marination on the flow of matter into and out
of the shrimp, the mass of shrimp before and after the marinating process was recorded.
The percent marinade uptake was calculated using the formula:
% Marinade uptake = \( \frac{M_2 - M_1}{M_1} \times 100 \)

M1 and M2 represent the mass of shrimp before and after marinating, respectively.

4.2.4. Shrimp Muscle pH

The final pH of marinated shrimp was measured according to the method of Burke & Monahan (2003). The marinated shrimp was minced and 5g were added to 40 ml of deionized water and homogenized with a stomacher for 1 minute. The pH of the homogenate was measured with a pH meter (Orion PerpHecT Model 320) calibrated between pH 4.0 and 7.0.

4.2.5. Extraction of Soluble and Insoluble Myofibrillar Proteins

About 5g of minced shrimp meat was homogenized with a stomacher for 5 minutes in a high salt phosphate buffer (20 mM phosphate buffer containing 1 M NaCl buffered at pH 7.4). The homogenate was centrifuged at 7,000 \( \times \) g for 15 minutes and the clear supernatant (soluble fraction) was stored at -20°C. The concentration of soluble protein in each extract was determined using the bichinchoninic acid (BCA) assay.

Solubilization of the proteins in the pellet obtained after the extraction of soluble myofibrillar protein was carried out according to the method of Schmitt et al., (2010) with a minor modification. About 0.1g of the pellet was vortexed in one milliliter of 125 mM Tris-HCl (pH 6.8) containing 1% SDS and 5 mM DTT for 5 minutes without heating. This homogenate was centrifuged at 10,000 \( \times \) g for 10 minutes in order to separate the clear supernatant (insoluble fraction) from the remaining debris. This supernatant was removed and then stored at -20°C prior to further analyses.
4.2.6. Electrophoretic Profile of Soluble Protein

Proteins extracted from raw and marinated shrimp were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing condition following the method of (Laemmli, 1970). Exactly 7.5 µg of protein was added to each well of a 12% polyacrylamide precast gels and electrophoretic separation was conducted at a constant voltage. GelCode Blue Stain reagent (Pierce Biotechnology Inc, Rockford, IL, USA) was added to the gel to aid visualization of the protein bands.

4.2.7. Indirect ELISA Analysis of Soluble Protein Extract

The IgE-binding capacity of tropomyosin in the extracts of raw and marinated shrimp was evaluated by indirect ELISA using pooled sera from three shrimp allergic patients. Polystyrene 96-well plates (Corning Inc., USA) were coated overnight at 4 °C with shrimp protein extracts (20 ngs/well in duplicate) using phosphate buffer as the diluent. The plates were washed with phosphate buffered saline containing 0.05% Tween-20 (PBST) 3 times and blocked with 1% bovine serum albumin (BSA) in PBST (200 µL per well) at 37 °C for 2h to prevent non-specific binding. Pooled sera from shrimp allergic individuals were diluted in PBS (1: 20) and added in equal amounts to each well (50 µL) and the plate was incubated at 37 °C for 1 h. After washing with PBST, each well was then incubated with secondary antibody, goat anti – human IgE conjugated to horseradish peroxidase HRP (1:2000), for 1 h at 37 °C (50 µL per well). The wells were again washed with PBST, and 3, 3’, 5, 5’-tetramethylbenzidine (TMB) was added (50 µL per well) for color development. The plate was kept in the dark for 15-30 minutes. Fifty microliters of 2 M H₂SO₄ was then added to stop the reaction and the
absorbance was measured with a microplate reader (BioTek Instruments, Inc, Winooski, VT, USA) at 450 nm.

4.2.8. Western Blot Analysis of Insoluble Fraction

Gel electrophoretic separation of the insoluble protein fraction of marinated shrimp was carried out under the same conditions used for the soluble protein fraction as described previously. The same amount of pellet (0.1g) was homogenized in one milliliter of 0.125M Tris buffer containing 1% SDS and 5 mM DTT to solubilize proteins in the pellet. The resulting supernatant was diluted (1:1) with Laemmlie buffer, and the same volumes (15 µl) were loaded into each well. After electrophoretic separation, protein bands on the gel were electrotransfered to a nitrocellulose membrane. The membrane was incubated in a blocking buffer (2% BSA in PBST) for 1 hour at room temperature under slight agitation. After washing with PBST, pooled sera from three shrimp allergic patients (1/20 dilution with 1% BSA in PBST) were added to the membrane and incubated overnight at 4 °C. After five washes with PBST, the membrane was treated with goat anti-human IgE conjugated to horseradish peroxidase (1:2000 dilution) for 1 hour at room temperature. Detection of the antigen-antibody complex on the membrane was achieved with the aid of CN/DAB reagent according to the manufacturer’s instruction.

4.2.9. Statistical Analysis

Each combination of the levels of the independent variables (pH and time) was replicated three times and all quantitative data are reported as means ± standard deviation. Analysis of variance (ANOVA) was used to detect significant effects of the
independent variables on the response variables. A significant effect was reported when p ≤ 0.05. Tukey’s HSD post hoc test was used to determine significant differences among the means of each independent variable. Data were analyzed using JMP statistical software (version 12).

4.3. Results and Discussion

4.3.1. Mass Change in Marinated Shrimp

The effects of vinegar at different pH and time of marination showed that both vinegar pH and marinating time had a significant effect (p < 0.05) on the mass of marinated shrimp (Table 4.1). The shrimp samples experienced an increase in mass when they were marinated in vinegar at pH 1.0 and 2.5 while the samples marinated in vinegar at pH 3.5 and 4.8 had a negative mass change indicating loss of moisture from the samples. However, samples marinated at pH 1.0 had the highest mass increase especially after 3 hours. This agrees with a previous study on the effect of lactic acid and citric acid on beef tenderization which showed that marinade with the highest acid concentration (lowest pH) induced a higher moisture uptake by the samples (Aktaş, Aksu & Kaya, 2003). The mass change of shrimp treated with vinegar at pH 2.5 was significantly lower (p < 0.05) at all marinating times except at 1 hour compared with samples marinated at pH 1.0.
### Table 4.1. Effect of vinegar pH and marinating time on percentage mass change of shrimp

<table>
<thead>
<tr>
<th>Marinating pH</th>
<th>Marinating Times</th>
<th>1 hour</th>
<th>3 hours</th>
<th>6 hours</th>
<th>16 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td></td>
<td>4.63 ± 1.50&lt;sup&gt;axy&lt;/sup&gt;</td>
<td>11.51 ± 2.45&lt;sup&gt;bw&lt;/sup&gt;</td>
<td>16.22 ± 3.73&lt;sup&gt;bx&lt;/sup&gt;</td>
<td>15.66 ± 1.36&lt;sup&gt;bx&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.5</td>
<td></td>
<td>2.00 ± 0.36&lt;sup&gt;ay&lt;/sup&gt;</td>
<td>4.46 ± 1.23&lt;sup&gt;abx&lt;/sup&gt;</td>
<td>4.76 ± 2.56&lt;sup&gt;aby&lt;/sup&gt;</td>
<td>5.12 ± 1.08&lt;sup&gt;by&lt;/sup&gt;</td>
</tr>
<tr>
<td>3.5</td>
<td></td>
<td>-8.13 ± 1.47&lt;sup&gt;az&lt;/sup&gt;</td>
<td>-6.02 ± 1.76&lt;sup&gt;ay&lt;/sup&gt;</td>
<td>-6.94 ± 1.71&lt;sup&gt;az&lt;/sup&gt;</td>
<td>-8.20 ± 1.02&lt;sup&gt;az&lt;/sup&gt;</td>
</tr>
<tr>
<td>4.8</td>
<td></td>
<td>-9.56 ± 0.65&lt;sup&gt;az&lt;/sup&gt;</td>
<td>-10.35 ± 0.75&lt;sup&gt;abz&lt;/sup&gt;</td>
<td>-12.20 ± 0.49&lt;sup&gt;bz&lt;/sup&gt;</td>
<td>-10.34 ± 1.06&lt;sup&gt;abz&lt;/sup&gt;</td>
</tr>
<tr>
<td>Untreated</td>
<td></td>
<td>5.85 ± 0.75&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>6.88 ± 1.11&lt;sup&gt;ax&lt;/sup&gt;</td>
<td>8.99 ± 2.06&lt;sup&gt;ay&lt;/sup&gt;</td>
<td>9.27 ± 2.87&lt;sup&gt;ay&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The percent mass change was calculated using the mass of shrimp before and after marination. All values are reported as the Mean ± Standard deviation, n = 3

<sup>a, b</sup> The same letter across rows indicates that the mean values of percent mass change are not significantly different (p > 0.05)

<sup>w, x, y, z</sup> The same letter down each column indicates that the mean values of percent mass change are not significantly different (p > 0.05)

The extent of moisture loss in shrimp marinated in vinegar at pH 3.5 and 4.8 was mostly similar as indicated by the mean values of the percent mass change which were not significantly different (p > 0.05) throughout the duration of marination. Furthermore, a steady increase in mass was observed among untreated samples (shrimp marinated in deionized water) as the marinating period increased although the values were not statistically different. This hydration of samples marinated in deionized water has been reported in other studies and can be explained by the migration of water into the sample induced by the osmotic pressure difference between the internal components of shrimp (dissolved salts and other biomolecules) and the deionized water (Xiong, 2005).

The swelling or shrinkage of meat products in an acid environment is governed by the net charge of the polypeptides caused by the acid solution. At pH near the isoelectric point of myofibrillar proteins (pH 4.5), the net attractive force between positively and
negatively charged groups of proteins causes the protein filaments to aggregate with the resultant expulsion of moisture (reduced water holding capacity) from the muscle tissue and shrinkage of the sample (Goli, Ricci, Bohuon, Marchesseau & Collignan, 2014). However, below the isoelectric point of these proteins, more of the charged protein residues become positively charged and the polypeptide chains experience intra and intermolecular repulsive force characterized by protein unfolding and an increase in intracellular and extracellular spaces (Burke & Monahan, 2003; Goli, Bohuon, Ricci, Trystram & Collignan, 2011; Ke, Huang, Decker & Hultin, 2009). This condition increases the diffusion of the marinade into the tissues and a resultant increase in mass (Aktaş et al., 2003).

Thus, this study indicated that the higher concentration of hydrogen ions in vinegar at pH 1.0 and 2.5 created a net positive charge on protein moieties which facilitated repulsion of polypeptides and swelling of the shrimp samples. However, the net attractive force among polypeptides in shrimp marinated at pH 3.5 and 4.8 may be greater hence, the loss of mass among these samples. Consequently, crustacean shellfish proteins in high acid foods can either experience intermolecular attraction or repulsion depending on the pH of the food matrix and diffusion of hydrogen ions into the muscle tissue. A high enough intermolecular repulsion and attraction may be able to cause the unfolding and aggregation of the proteins, respectively. It has been hypothesized that these protein modifications may influence the capacity of epitopes on tropomyosin structure to bind with anti-tropomyosin IgE (Lin et al., 2015).
4.3.2. Muscle pH of Marinated Shrimp

Another major consequence of marinating in the presence of organic acid is the reduction in pH of the marinated product (Goli, Bohuon, Ricci & Collignan, 2012). In comparison with the untreated shrimp, there was a significant reduction (p < 0.05) in the pH of marinated shrimp at all marinating conditions (Table 4.2).

Table 4.2. Effect of vinegar pH and marinating time on shrimp muscle pH

<table>
<thead>
<tr>
<th>Marinating pH</th>
<th>1 hour</th>
<th>3 hours</th>
<th>6 hours</th>
<th>16 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>4.22 ± 0.08aw</td>
<td>3.79 ± 0.20bw</td>
<td>3.45 ± 0.13bcw</td>
<td>3.38 ± 0.08cv</td>
</tr>
<tr>
<td>2.5</td>
<td>4.55 ± 0.11awx</td>
<td>4.28 ± 0.05bwx</td>
<td>4.19 ± 0.07bx</td>
<td>3.87 ± 0.09cw</td>
</tr>
<tr>
<td>3.5</td>
<td>4.82 ± 0.08ax</td>
<td>4.50 ± 0.02bx</td>
<td>4.34 ± 0.03cx</td>
<td>4.07 ± 0.06dx</td>
</tr>
<tr>
<td>4.8</td>
<td>5.79 ± 0.08ay</td>
<td>5.41 ± 0.03by</td>
<td>5.22 ± 0.03cy</td>
<td>5.05 ± 0.02dy</td>
</tr>
<tr>
<td>Untreated</td>
<td>7.47 ± 0.21az</td>
<td>7.53 ± 0.36az</td>
<td>7.54 ± 0.18az</td>
<td>7.65 ± 0.05az</td>
</tr>
</tbody>
</table>

The shrimp muscle pH was determined by homogenizing a weighed amount of marinated shrimp in deionized water and measuring the pH of the solution with a pH meter. All values are reported as the Mean ± Standard deviation, n = 3

a, b The same letter across rows indicates that the mean values of the muscle pH are not significantly different (p > 0.05)

w, x, y, z The same letter down each column indicates that the mean values of the muscle pH are not significantly different (p > 0.05)

Rapid reduction in the pH of marinated shrimp can be observed in samples marinated in vinegar at pH 1.0 where the pH of shrimp reduced to 4.2 from the neutral pH of 7.5 within one hour. As the marinating time increased, this pH reduced further even though there was no significant difference (p > 0.05) in the pH at 6 and 16 hours. A similar trend was seen in samples marinated at other pH values where a steady reduction of the core pH was observed throughout the duration of marination. A significant difference in the pH values between samples marinated at pH 1.0 and 2.5 was observed only after
prolonged marination time (6 – 16 hours). Across all the marinating time levels, the final muscle pH of samples marinated in vinegar set at pH 3.5 and 4.8 were statistically different with the latter having a pH that was about one unit greater than the former. Untreated shrimp maintained neutral pH throughout the period of marination.

The reduction in the pH of marinated shrimp can be attributed to the fast migration of hydrogen ion dense vinegar solution into the shrimp tissue. In a study on the effect of marination conditions on the properties of turkey breast, rapid impregnation of turkey breast meat cubes with salt and acetic acid was observed within 20 minutes of marination in a solution containing acetic acid alone and in combination with salt (Goli et al., 2014). The concentrations of salt and acetic acid in the meat cubes were 0.08M and 0.06M, respectively, at 20 minutes which represent one third and one quarter of the initial concentration of salt and acid (Goli et al., 2014). The authors also reported a drop in pH from a value of 6.1 for the control to 5.1 and 4.0 within the first 5 and 360 minutes, respectively of marination with 0.25 M acetic acid (Goli et al., 2014). A “variable grade period” was also observed during the marination of anchovy (Engraulis anchoita) in a marinating solution containing 3% acetic acid and 10% sodium chloride that was characterized by rapid diffusion of acetic acid into the sample and a fall in pH to about 4.2 (Cabrer, Casales & Yeannes, 2002).

The vinegar was adjusted to different pH in this study so that the shrimp muscle proteins could experience final core pH conditions similar to the crustacean shellfish products in high acid foods (pH < 4.6) such as salad dressing and sauces. It can be seen that the marinated shrimp in this study had a final pH values that were less and above 4.6. Thus, the pH regimes used in this study provide a good model to investigate the
allergenic behavior of tropomyosin in crustacean products that are present in some high acid foods.

4.3.3. Myofibrillar Protein Solubility of Marinated Shrimp

The concentration of soluble proteins extracted from marinated shrimp using a high salt phosphate buffer was determined by BCA assay (Table 4.3). Acid marination reduced the concentration of soluble proteins that extracted from shrimp and the lowest protein concentrations (< 1mg/mL) were obtained from shrimp marinated in vinegar with pH 1.0. In addition, no significant difference (p > 0.05) was found in the soluble protein concentration from samples marinated in pH 1.0 vinegar at all levels of marinating time. For samples marinated at pH 2.5 and 3.5, a steady decrease in the soluble protein concentration occurred throughout the duration of marination. In comparison with other marinated shrimp, samples treated with pH 4.8 vinegar had a significantly higher (p < 0.05) soluble protein concentration

The high soluble protein concentration of the samples marinated in pH 4.8 vinegar may indicate that the proteins were denatured to a lesser extent compared with those of pH 1.0-3.5 vinegar.
Table 4.3. Effect of vinegar pH and marinating time on shrimp soluble myofibrillar protein concentration

<table>
<thead>
<tr>
<th>Marinating pH</th>
<th>Marinating Times</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hour</td>
</tr>
<tr>
<td>1.0</td>
<td>0.67 ± 0.14&lt;sup&gt;ax&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.5</td>
<td>1.24 ± 0.23&lt;sup&gt;ax&lt;/sup&gt;</td>
</tr>
<tr>
<td>3.5</td>
<td>1.24 ± 0.10&lt;sup&gt;ax&lt;/sup&gt;</td>
</tr>
<tr>
<td>4.8</td>
<td>7.80 ± 0.37&lt;sup&gt;ay&lt;/sup&gt;</td>
</tr>
<tr>
<td>Untreated</td>
<td>5.91 ± 0.38&lt;sup&gt;az&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Soluble proteins were extracted from unmarinated and marinated shrimp using a high salt phosphate buffer (20 mM phosphate buffer containing 1 M NaCl buffered at pH 7.4). The concentration of protein in the extract was determined by the BCA assay method for protein quantification. All values are reported as the Mean ± Standard deviation, n = 3.

<sup>a</sup>, <sup>b</sup> The same letter across rows indicates that the mean values of soluble protein concentration are not significantly different (p > 0.05)

<sup>w</sup>, <sup>x</sup>, <sup>y</sup>, <sup>z</sup> The same letter down each column indicates that the mean values of soluble protein concentration are not significantly different (p > 0.05)

Reduction in the concentration of soluble protein especially from samples marinated in vinegar at pH 1.0 – 3.5 on the other hand may be due to the denaturation of myofibrillar proteins which results in their poor solubility in the extraction buffer (Albillos et al., 2011). In a study involving the recovery of mustard protein from salad dressing (an acidic food matrix) spiked with 0-1000 ppm mustard flour, mustard protein recovery was maximum (94%) in salad dressing adjusted to pH 7 before spiking and at minimum (7.7 – 11%) in salad dressing that was spiked at their natural acidic pH (3.2) (Lee, Niemann, Lambrecht, Nordlee & Taylor, 2009). The authors attributed this low recovery to the poor solubility and extractability of the mustard protein in an acidic food matrix.

The low recovery of soluble proteins from the marinated samples especially those obtained from pH 1.0-3.5 vinegar may also be due to the leaching of vinegar (absorbed into the shrimp meat tissue during marination) into the extraction solution during
homogenization. This has the tendency to reduce the pH of the extraction solution, its buffering capacity and ability to solubilize myofibrillar proteins. Effective solubilization of allergenic protein is essential for the accurate immunochemical detection of allergenic proteins (Downs et al., 2016, Verhoeckx et al., 2015). Therefore, reduced soluble protein recovery may be the main challenge in the immunochemical analysis of acid marinated foods or high acid foods. Monitoring the pH of the extraction buffer after homogenization as well as using a higher concentration of Tris or phosphate buffer (solution with stronger buffering capacity) may help improve extractability of proteins from high acid food matrices.

4.3.4. SDS-PAGE Analysis of Soluble and Insoluble Protein Extracts

Protein components in the soluble and insoluble pellet were separated and visualized via denaturing polyacrylamide gel electrophoresis (Fig 4.1 and 4.2). Figure 4.1A shows the electrophoretic profile of myofibrillar proteins extracted from untreated shrimp (marinated in water). Major salt soluble proteins including myosin heavy chain (MHC), actin, tropomyosin and myosin light chain (MLC) can be seen in the gel. Low recovery of soluble proteins from marinated shrimp by the high salt phosphate buffer was evident from their electrophoretic profiles which showed an absence of most myofibrillar proteins especially from samples marinated with pH 1.0-3.5 vinegar and after prolonged marination time (Fig 4.1B-D). Most high molecular weight myofibrillar protein bands above 20 kDa were not visible on these gels especially in samples obtained from pH 1.0 and 2.5 vinegar (Fig 4.1B-C).
Figure 4.1. SDS-PAGE analysis of the effect of vinegar pH and marinating time on shrimp soluble protein profile.
Figure 4.1E

A: Soluble protein extracts of shrimp dipped in deionised water for 1, 3, 6 and 16 hours. B-E: Soluble protein extracts of shrimp marinated in white vinegar at pH 1.0, 2.5, 3.5 and 4.8, respectively for 1, 3, 6 and 16 hours. Mr: Molecular weight marker. MHC: Myosin heavy chain. MLC: Myosin light chain. Tm: Tropomyosin
Each well was loaded with pooled extracts from three replicate samples

The tropomyosin band was visible in extracts from shrimp marinated in vinegar at pH 1.0-3.5 for 1 hour (Fig 1B-D) after which it became less visible. Extracts obtained from samples treated with pH 4.8 had a profile that is similar to the extracts obtained from shrimp dipped in water (Fig 4.1E). The 36 kDa tropomyosin band as well as other myofibrillar proteins can be seen throughout the marinating period. This correlates well with our results on protein solubility which show that samples marinated at pH 4.8 had higher soluble protein concentrations compared with other samples.

Interestingly, a thick protein band (≈ 20 kDa) can be observed in the soluble protein extract of shrimp marinated in vinegar at all time and pH conditions. Careful observation showed that the band intensity of this protein is higher in extracts obtained from samples marinated at pH 1.0-3.5 than extracts from samples marinated at pH 4.8. This protein persists in the soluble fraction even at marination conditions where other protein bands are not visible (for instance pH 1.0 at 16 hours). Consequently, this protein
may be a good marker to detect the presence crustacean shellfish proteins in high acid food matrices using common extraction solutions like phosphate buffers. Although, the protein has the same molecular weight as myosin light chain, further studies are necessary to ascertain its identity.

The electrophoretic profile of the insoluble fraction indicated that most of the myofibrillar proteins were indeed present in the pellets (Fig 4.2A-D). Both SDS and DTT, used in the extraction buffer were able to dissociate noncovalently – linked protein aggregates in the pellets. High molecular weight protein bands between 100-250 kDa as well as 40 kDa actin and 36 kDa tropomyosin were clearly visible in these gels (Fig 4.2A-D). The intensities of bands were at their lowest levels in the extracts obtained from shrimp marinated in pH 1.0 vinegar and these intensities were more enhanced in extracts from shrimp marinated with higher vinegar pH.

Figure 4.2. SDS-PAGE analysis of the insoluble protein fraction of organic acid marinated shrimp.

A

B
Insoluble protein fraction was obtained by homogenizing the pellet obtained from soluble fraction in 125 mM Tris-HCl (pH 6.8) containing 1% SDS and 5 mM DTT for 5 minutes. Gel electrophoresis was carried under reduced condition with Laemmli buffer containing 5% 2-mercaptoethanol. A-D: Extracts obtained from shrimp marinated in white vinegar at pH 1.0, 2.5, 3.5 and 4.8, respectively.

This agrees with a previous study which showed that purified tropomyosin from short neck clam (*Ruditapes philippinarum*) that was incubated for two hours at both acidic and basic pH conditions, maintained its band intensity at pH 1.0-7.0 (Lin, Li, Lin, Song, Lv & Hao, 2015). However, the researchers observed changes in the secondary structures (alpha helix and beta sheet) of tropomyosin at acidic pH regimes and an increase in its surface hydrophobicity indicating unfolding at these pH conditions (Lin et al., 2015).

Thus, this result suggests that the difference in the profile of tropomyosin from the soluble and insoluble fraction may be due to the difference in the solubility of this protein in the extraction buffers. Although, tropomyosin may have experienced some unfolding or aggregation with other protein filament due to pH alteration, fragmentation or hydrolysis of this protein did not seem to occur. Hence, linear IgE-binding epitopes on the tropomyosin structure may still be preserved.
4.3.5. IgE-Binding Capacity of Tropomyosin in the Soluble and Insoluble Protein Extract

The ability of tropomyosin present in the high salt phosphate buffer and in the insoluble protein fraction to bind with anti-tropomyosin antibodies from the pooled sera of three shrimp allergic individuals was assessed using indirect ELISA and western blot analysis, respectively. The IgE-binding of each soluble extract was measured by ELISA and the results were reported as absorbance at 450 nm (Table 4.4).

In general, the IgE-binding capacity of soluble protein extracts from shrimp marinated in vinegar at pH 1.0-3.5 were significantly lower than those of samples marinated at pH 4.8 and control especially after marinating for three hours. The absorbance values obtained for samples marinated in pH 1.0 vinegar were not significantly different (p > 0.05) throughout the marinating period. This agrees with the result obtained for the soluble protein concentration for these samples which were similar at all marinating times. For samples marinated in vinegar at pH 2.5 and 3.5, a significantly higher (p < 0.05) IgE-binding was seen in samples marinated for 1 hour compared with samples marinated for longer periods.
Table 4.4. Effect of vinegar pH and marinating time on IgE-binding capacity of tropomyosin in soluble protein extract

<table>
<thead>
<tr>
<th>Marinating pH</th>
<th>1 hour</th>
<th>3 hours</th>
<th>6 hours</th>
<th>16 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0.41 ± 0.10&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;x&lt;/sup&gt;</td>
<td>0.48 ± 0.15&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;xy&lt;/sup&gt;</td>
<td>0.51 ± 0.17&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;x&lt;/sup&gt;</td>
<td>0.61 ± 0.22&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;x&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.5</td>
<td>1.39 ± 0.13&lt;sup&gt;y&lt;/sup&gt;&lt;sup&gt;z&lt;/sup&gt;</td>
<td>0.35 ± 0.06&lt;sup&gt;y&lt;/sup&gt;&lt;sup&gt;z&lt;/sup&gt;</td>
<td>0.43 ± 0.22&lt;sup&gt;b&lt;/sup&gt;&lt;sup&gt;x&lt;/sup&gt;</td>
<td>0.21 ± 0.02&lt;sup&gt;b&lt;/sup&gt;&lt;sup&gt;x&lt;/sup&gt;</td>
</tr>
<tr>
<td>3.5</td>
<td>2.05 ± 0.21&lt;sup&gt;z&lt;/sup&gt;</td>
<td>0.82 ± 0.15&lt;sup&gt;b&lt;/sup&gt;&lt;sup&gt;x&lt;/sup&gt;</td>
<td>0.50 ± 0.20&lt;sup&gt;b&lt;/sup&gt;&lt;sup&gt;x&lt;/sup&gt;</td>
<td>0.51 ± 0.21&lt;sup&gt;b&lt;/sup&gt;&lt;sup&gt;x&lt;/sup&gt;</td>
</tr>
<tr>
<td>4.8</td>
<td>2.20 ± 0.20&lt;sup&gt;z&lt;/sup&gt;</td>
<td>1.65 ± 0.11&lt;sup&gt;b&lt;/sup&gt;&lt;sup&gt;x&lt;/sup&gt;</td>
<td>1.96 ± 0.31&lt;sup&gt;y&lt;/sup&gt;&lt;sup&gt;z&lt;/sup&gt;</td>
<td>1.85 ± 0.44&lt;sup&gt;y&lt;/sup&gt;&lt;sup&gt;z&lt;/sup&gt;</td>
</tr>
<tr>
<td>Untreated</td>
<td>1.82 ± 0.08&lt;sup&gt;z&lt;/sup&gt;</td>
<td>1.75 ± 0.15&lt;sup&gt;z&lt;/sup&gt;</td>
<td>1.80 ± 0.04&lt;sup&gt;y&lt;/sup&gt;&lt;sup&gt;z&lt;/sup&gt;</td>
<td>1.93 ± 0.17&lt;sup&gt;y&lt;/sup&gt;&lt;sup&gt;z&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

IgE binding capacity of tropomyosin in the soluble extract was measured by indirect ELISA using pooled sera from three shrimp allergic individuals. Sera (1/20 dilution) were added to bound tropomyosin from extract on microtiter plate. Extent of antigen-antibody binding was determined by probing with HRP-conjugated secondary antibody (1/2000) followed by addition of substrate solution and stop solution. Absorbance was measured at 450 nm with the aid of a plate reader. All values are reported as the Mean ± Standard deviation, n = 3.

The same letter across rows indicates that the mean values of the muscle pH are not significantly different (p > 0.05)

The same letter down each column indicates that the mean values of the muscle pH are not significantly different (p > 0.05)

Relationships between protein concentration in the soluble extract and the extent of IgE-binding or allergen quantification have been observed in different studies (Albillos et al., 2011, Fu & Maks, 2013). In a study to determine the effect of thermal processing on the immunochemical detection of egg, milk and peanut allergens in a baked product matrix, it was found that poor recovery of the soluble allergenic proteins by extraction buffers led to underestimation of the allergen concentration by most ELISA kits (Parker et al., 2015). Similarly, when the single effect of vinegar pH (3, 3.5 and 4.0) on the ELISA detection of mustard protein was investigated, the lowest concentration of mustard protein was detected in samples exposed to the lowest vinegar pH (Lee et al., 2009). This result was due to the reduced solubility of the protein in the extraction buffer caused by acid denaturation (Lee et al., 2009).
The IgE-binding capacity of tropomyosin from shrimp marinated at pH 4.8 and in deionized water (control) was not significantly different across all the marinating times. In addition, there was no significant difference in the IgE-binding capacity of tropomyosin in extracts from samples soaked in vinegar at pH 4.8 and the untreated samples. Due to the higher concentration of protein (caused by minimal denaturation) in these extracts, the IgE-binding capacity was significantly higher than extracts from samples marinated at lower pH. A study on purified tropomyosin from short neck clam showed that the IgE-binding capacity of the allergen was stable after exposure to a pH range from 2.0 to 6.0 according to an indirect ELISA data although the authors observed conformational changes of tropomyosin at the low pH conditions (Lin et al., 2015). This may suggest that tropomyosin epitopes are mostly linear rather than conformational.

The values obtained for the IgE-binding capacity of tropomyosin in the soluble extract were apparently lower in extracts from samples marinated with vinegar at pH 1.0 – 3.5 compared with those obtained from pH 4.8 vinegar which were not significantly different from the control samples. However, these values do not fully represent the differences in the IgE-binding capacity of tropomyosin due to vinegar treatment since they were strongly affected by the concentration of tropomyosin in the soluble fraction. Thus, the IgE-binding capacity of tropomyosin in the insoluble pellet was determined by western blot analysis (Fig 4.3A-D). The tropomyosin bands showed strong binding with IgE from pooled sera at all vinegar pH and duration of marination. Hence, exposure of tropomyosin to low pH condition did not seem to alter its IgE-binding indicating that all or much of the epitopes are linear.
Due to denaturing properties of the SDS-PAGE reagents used prior to western blot, we could not confirm the effect of potential secondary structure modification of tropomyosin in high acid environment on its immunoreactivity. To the best of our knowledge, this is the first study to demonstrate the retention of the IgE-binding capacity of tropomyosin when the actual shrimp sample is exposed to low pH condition found in high acid foods containing crustacean shellfish.

Figure 4.3. Western blot analysis of the insoluble protein fraction of organic acid marinated shrimp.

A-D: Extracts obtained from shrimp marinated in white vinegar at pH 1.0, 2.5, 3.5 and 4.8, respectively for 1, 3, 6 and 16 hours. Mr: Molecular weight marker, Tm: Tropomyosin

Some studies have demonstrated the need for the analysis of the insoluble pellet for accurate determination of the effect of thermal processing on IgE-binding of allergens. For instance, the IgE-binding of peanut allergens (Ara h1, Ara h2 and Ara h3) from boiled, fried and roasted peanut was found to be low in the soluble protein portion but high in the insoluble portion (Schmitt et al., 2010). In general, the authors identified differences in the solubility of the allergens in the two fractions as one of the factors responsible for the difference in antigenicity (Schmitt et al., 2010). Similarly, the IgE-
binding of walnuts allergens heated at 120 or 180 °C for up to 20 minutes was more
enhanced in the insoluble pellet compared with the soluble fraction (Downs et al., 2016). From this study, it was also reported that the allergenic proteins in the insoluble pellet still retained their conformational epitopes as shown by the decrease in IgE-binding of this pellet fraction under reducing condition (Downs et al., 2016). Allergenic proteins may become denatured during food processing which subsequently reduces their solubility in most extraction buffers used for immunochemical detection of allergens (Verhoeckx et al., 2015). As a result, differences in the allergenic capacity observed in the soluble fraction are often due to the variation in the concentration of the allergenic protein in the buffer.

4.4. Conclusions

Exposure of shrimp to vinegar at different acidic pH and time periods caused changes in the mass of shrimp (both increase and decrease), reduction in pH and extractable soluble protein concentration. Reduced IgE-binding of tropomyosin in the soluble protein fraction especially from samples marinated in vinegar at pH 1.0-3.5, was in part due to poor solubility of this protein in the extraction buffer. Probing tropomyosin in the insoluble pellet with human anti-tropomyosin IgE showed that acid marination did not alter the linear IgE epitopes of tropomyosin. This study documents that high to medium acid foods containing crustacean shellfish protein residues pose a risk to crustacean shellfish allergic individuals and should be labeled in order to protect sensitive consumers. It also demonstrates the need to take into account the insoluble protein fraction when attempting to understand the influence of a processing method or matrix
composition on the allergenic capacity of a food allergen. Focusing on the soluble extract alone may give rise to a misleading result on effects of processing on IgE reactivity.
CHAPTER 5

ATTENUATING THE ANTIBODY REACTIVITY OF SHRIMP TROPOMYOSIN
WITH MICROBIAL PROTEASES

5.1. Introduction

Shrimp allergy is an immunoglobulin E (IgE)-mediated hypersensitivity to certain shrimp proteins which can result in adverse health reactions including anaphylaxis which can lead to death (Gámez et al., 2011). Different allergenic proteins that have been identified in shrimp include sarcoplasmic calcium-binding protein, tropomyosin, arginine kinase, myosin light chain, and triosephosphate isomerase (Ayuso et al., 2010; Bauermeister et al., 2011). However, tropomyosin, a 36 kDa myofibrillar protein is considered the major allergenic protein in shrimp because over 80% of individuals with shrimp allergy are reactive to this protein (Lehrer, Ayuso & Reese, 2003). Structurally, tropomyosin is a dimeric coiled-coil protein made up of mostly α-helix secondary structure and has been shown to possess both conformational and linear IgE-binding sites or epitopes (Kamath et al., 2014; Lehrer et al., 2003).

Like other allergens, there is currently no known cure for shrimp allergy. Consequently, individuals with shrimp allergy need to totally avoid this food or products containing crustacean shellfish ingredients (Kamath et al., 2014). In addition to avoidance, attempts have also been made to utilize different processing techniques to weaken the ability of tropomyosin to bind IgE which is responsible for the activation of a series of reactions that culminate in the elicitation of allergic response. This strategy capitalizes on the capacity of these processing techniques to disrupt or mask the linear and conformational IgE-binding epitopes on the tropomyosin structure. Some of the
processing techniques that have been used to modulate the immunoreactivity of shrimp tropomyosin include different cooking methods such as boiling, frying, steaming, baking and microwave roasting, organic acid treatment, gamma irradiation, pulsed ultraviolet light treatment, high intensity ultrasound and pressure assisted heat treatment (Lasekan & Nayak, 2016; Li et al., 2005; Long et al., 2015; Shriver et al., 2011; Zhenxing et al., 2007). Some of these methods did not influence the IgE-binding capacity of tropomyosin while others were only able to modify its conformational epitopes leaving the linear epitopes intact. Hence, tropomyosin retains significant IgE reactivity after exposure to certain food processing conditions due to the conservation of its linear epitopes.

Enzymatic hydrolysis is a commonly used method in the food industry for the development of products with functional and bioactive properties as well as with improved digestibility (Shi et al., 2013). In addition, different reports have shown that enzymatic hydrolyses have the capacity to significantly disrupt both conformational and linear epitopes of food allergens thereby significantly reducing their IgE-binding capacity (Rahaman et al., 2016; Bahna, 2008; Cabanillas et al., 2012; Li, Yu, Goktepe & Ahmedna, 2016). However, the efficacy of this enzymatic hydrolysis method to modulate the immunoreactivity of food allergens depends on the enzyme type, degree of hydrolysis, hydrolysis condition, the allergenic protein and sensitivity of the allergic individual (Rahaman et al., 2016; Li et al., 2016). For instance, in an extensive study by Panda, Tetteh, Pramod & Goodman (2015), it was shown that hydrolysis of soybean proteins with Alcalase, papain, trypsin, chymotrypsin and bromelain did not abolish IgE reactivity of many soy allergic patients to the hydrolysate. In addition, it was demonstrated that hydrolysis of peanut protein extracts with an endoprotease (Alcalase)
significantly reduced the IgE reactivity and levels of peanut allergens while hydrolysis with an exoprotease (Flavourzyme) caused an increase (Cabanillas et al., 2012).

However, with respect to crustacean shellfish tropomyosin, the focus has been on the susceptibility of this allergen to digestive proteases. For instance, Toomer et al., (2015) identified immunogenic as well as pepsin and pancreatin-stable shrimp protein using an in vitro digestibility model. Also, Liu et al. (2011) compared the in vitro digestibility of tropomyosin from grass prawn and Pacific white shrimp and concluded that tropomyosin from the latter is more stable to digestive protease and thus, more allergenic. Consequently, reports on the utilization of non-digestive protease to modulate the IgE reactivity of tropomyosin is scarce or unavailable to the best of our knowledge. More so, very few studies utilized the whole food sample rather than the protein extracts to test the capacity of an enzyme treatment method to reduce allergenic potential.

Thus, in this study, the ability of three commercial microbial enzymes with GRAS status (alkaline protease, neutral bacterial protease and fungal protease) to hydrolyze tropomyosin in shrimp tissue was evaluated. In vitro assessment of the IgE-binding capacity of tropomyosin in the protein extracts of these enzyme – treated shrimp was examined via indirect ELISA and western blot assay using IgE from human plasma and polyclonal anti-tropomyosin. It was hypothesized that this enzymatic hydrolysis method could be used to develop a hypoallergenic shrimp product which can be utilized for oral immunotherapy to determine its capacity to desensitize shrimp allergic individuals.
5.2. Materials and Methods

5.2.1. Materials and Chemicals

Black tiger shrimp (*Penaeus monodon*) was from a local seafood store (Bangor, ME) and samples were kept frozen until use. The enzymes that were used in this study include alkaline protease L-660 from *Bacillus licheniformis* (640 KDAPU/g Minimum), neutral bacterial protease 160 B from *Bacillus subtilis* (152,000-184,000 PC/gram) and fungal protease 400 from *Aspergillus oryzae* (375,000-440,000 HUT/g). These enzymes were provided by Enzyme Development Corporation (New York, NY, USA). Modified Lowry Protein Assay kit and Pierce BCA Protein Assay Kit were from Thermo Scientific (Rockford, IL, USA). Trichloroacetic acid (TCA), 3, 3′, 5, 5′-Tetramethylbenzidine (TMB), Folin & Ciocalteu’s phenol reagent and goat anti-rabbit IgG conjugated with horseradish peroxidase were purchased from Sigma-Aldrich (St. Louis, MO, USA). Reagents and precast gels used for gel electrophoresis were obtained from Bio Rad (Hercules, CA, USA). Human plasma from three shrimp allergic individuals were from PlasmaLab (Everett, MA, USA) while the Horseradish Peroxidase-labelled goat anti-human IgE is a product of Life Technologies (Grand Island, NY, USA). An anti-mud crab tropomyosin raised in rabbit used as primary antibody was provided Dr. Maleki.

5.2.2. Treatment of Whole Shrimp with Microbial Enzymes

Solution of alkaline protease, fungal protease and neutral bacterial protease were made by either dissolving enzyme powder or by adding enzyme solution to deionized water. The total volume of these enzyme solutions was approximately 40 mL. The pH of these enzyme solutions was adjusted to 9.5 for alkaline protease and 7.5 for fungal
protease and neutral bacterial protease based on information from the enzyme supplier and in-house trials which showed that these enzymes exhibit optimal activities at these pH conditions. These solutions were prepared such that the final enzyme concentration ranged between 3-5% for alkaline protease and neutral bacterial protease while the concentration range for fungal protease was set at 3-6% based on the shrimp weight. Thawed, deshelled and weighed shrimp were added to these enzyme solutions and the enzyme treatment was carried out for 3, 6 and 16 hours under agitation with the aid of rotary shaker (Fisher Scientific, Pittsburgh, PA, USA) and at 4 °C. Marination of shrimp in the enzyme solution was carried out in triplicate for each combination of enzyme concentration and marination time. Control samples were shrimp soaked in deionized water. Immediately after enzyme treatment, the samples were rinsed with deionized water and subjected to further analysis.

5.2.3. Extraction of Soluble Myofibrillar Proteins

Raw and enzyme-treated shrimp were minced manually and 5g of the minced tissue was added to an extraction buffer comprising 20 mM phosphate buffer containing 1 M NaCl adjusted to pH 7.5. The mixture was homogenized with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY, USA) at 60 rpm for 1 minute. The homogenate was then shaken for 1 hour at 4 °C with the aid of a rotary shaker to increase the solubilization of proteins. The homogenate was then centrifuged at 7,000 × g for 20 minutes and the supernatant was stored in aliquots at – 20 °C. Soluble protein concentration of the extracts was determined using the BCA protein assay method.
5.2.4. Determination of TCA-Soluble Peptide Concentration

The Modified Lowry Method was used to determine the concentration of TCA-soluble peptides in the protein extracts of raw and enzyme–treated shrimp (McDonald & Chen, 1965). Deproteinization of the extracts was first carried out by mixing equal volume of the protein solution with 20% TCA. The mixture was vortexed and allowed to stand at room temperature for 15 minutes to ensure protein precipitation. The mixture was centrifuged and the supernatant was carefully transferred into separate tube. To quantify the concentration of peptides in the supernatant, dilution series of bovine serum albumin (BSA) standard were made with deionized water while series of dilution of the TCA-peptide solution were made with 10% TCA. Exactly 40 µl of the diluted standards and TCA-peptide solution was added to 200 ul of the Modified Lowry reagent (Thermo Scientific, Rockford, IL, USA) in a 96 well plate, mixed for 30 seconds and allowed to stand at room temperature for exactly 10 minutes. Folin reagent (20 µl) was added immediately, mixed for 30 seconds and incubated at room temperature for 30 minutes. The absorbance was read with a plate reader (BioTek Instruments, Inc, Winooski, VT, USA) at 750 nm and the peptide concentrations in mg/mL were determined from the standard curve. All analyses were carried out in duplicate.

5.2.5. Gel Electrophoresis Separation of Soluble Proteins

Separation and visualization of proteins in the soluble extracts of raw and enzyme-treated shrimp was performed with the aid of reducing SDS-PAGE based on the method of Laemmli (1970). The shrimp protein extracts were first diluted with the extraction buffer to achieve a final concentration of 1 mg/mL. These diluted extracts were
then mixed with equal volumes of loading buffer comprising Tris-HCl (pH 6.8), glycerol, 2% SDS and 0.01% bromophenol blue and the mixtures were immediately heated at 100 °C for 5 minutes. Exactly 15 µl of the protein-loading buffer mixture was loaded onto each well of a 4-20% precast gel (Bio Rad, Hercules, CA, USA) and the electrophoretic separation was carried out at 170V for 40 minutes. The gels were subsequently rinsed and stained with GelCode Blue Stain reagent (Pierce Biotechnology Inc, Rockford, IL, USA) to aid visualization of the protein bands.

5.2.6. Determination of IgE Reactivity of Tropomyosin by Indirect ELISA

The IgE reactivity of tropomyosin in the protein extracts of raw and enzyme – treated shrimp were evaluated by indirect ELISA. The protein extracts were serially diluted with 50 mM sodium carbonate/bicarbonate buffer (pH 9.6) to a final concentration of 1µg/mL and 100 µl (100 ng/well) were added to each well of a high binding microplate and incubated at 37 °C for 2 hours. After washing 5 times with phosphate buffered saline containing 0.05% Tween-20 (PBST), 200 µl of 1% bovine serum albumin (BSA) in PBST was added to the wells and incubated at 37 °C for 2 hours. The wells were washed and 50 µl of diluted pooled plasma (1/20) from shrimp allergic individuals were added to each well and the plate was incubated for 1 hour at 37 °C. After another round of washing with PBST, 50 µL of goat anti – human IgE with attached horseradish peroxidase (1:2000) was added to each well and incubated for 1 hour at 37 °C. Following 5 washing cycle with PBST, 50 µl of the substrate solution (TMB) for the peroxidase conjugate was added to the wells and incubated for 15 minutes to allow for complete color development followed by termination of the reaction by adding equal volume of 2 M H₂SO₄. The absorbance was read at 450 nm with the aid of a
microplate reader (BioTek Instruments Inc., Winooski, VT, USA). Each extract was analyzed in duplicate.

5.2.7. Western Blot Analysis of Shrimp Protein Extract with Human IgE and Rabbit Anti-Mud Crab Tropomyosin

Western blot analysis was carried out to visualize the effect of enzyme treatments on the IgE-binding capacity of shrimp tropomyosin. Electrophoretic separation of the soluble proteins was first carried out as described in the previous section. The protein bands in the gel were fixed onto a nitrocellulose membrane via wet electrotransfer method at 100V for 1 hour. Following blocking with PBST containing 2% BSA for 1 hour at room temperature, the membrane was washed with PBST and pooled plasma (1/20 dilution) from three shrimp allergic individuals was added to the membrane and incubated overnight at 4 °C. After repeated washes with PBST, secondary antibody conjugated with peroxidase enzyme (1/2000) was added to the membrane and incubated for 1 hour. The secondary antibody solution was discarded and the membrane was washed five times with PBST. Tropomyosin IgE-binding was detected by adding CN/DAB reagent according to the manufacturer’s instruction (Thermo Fisher Scientific, Rockford, IL., USA).

A polyclonal anti-mud crab tropomyosin raised in rabbit was also used as the primary antibody. After blocking and washing of the membrane, the polyclonal antibody (1/3,000 dilution) was added to the membrane and incubated for 1 hours at room temperature. A goat anti-rabbit IgG conjugated with horseradish peroxidase (1/100,000) was subsequently added to the membrane and incubated for 1 hour room temperature.
The membrane was washed extensively and tropomyosin-IgE binding was detected by CN/DAB reagent.

5.2.8. Statistical Analysis

Analyses of Variance (ANOVA) were performed on quantitative data using JMP Statistical Software, Version 12 (SAS Institute, Cary, NC, USA) to determine the significant effect \( (P \leq 0.05) \) of the independent variables, enzyme concentration and marination time, on the response variables such as TCA-peptide concentration and IgE reactivity via indirect ELISA. Tukey's HSD post hoc test was also used to compare the means across the levels of each independent variable to identify the groups that are statistically different.

5.3. Results and Discussions

5.3.1. Capacity of Different Microbial Proteases to Hydrolyze Myofibrillar Proteins in Shrimp Tissue

Shrimp were treated with three microbial enzymes to establish their capacity to hydrolyze the myofibrillar proteins (including the allergenic protein, tropomyosin) in the shrimp tissue and ultimately determine the resulting IgE-binding capacity of tropomyosin in the protein extracts of the enzyme-treated shrimp. This hydrolytic capacity was measured by quantifying the concentration of peptides in the protein extracts that is soluble in 10% TCA (Figures 5.1A-B).

At the minimum concentration of the enzymes (3%) used in this study, both alkaline protease and fungal protease displayed better hydrolytic capacity compared with
neutral bacterial protease as shown by the significantly higher (p < 0.05) concentration of TCA-soluble peptides present in the extracts of shrimp marinated with these enzymes (Figure 5.1A). In addition, there was no significant difference (p > 0.05) in the TCA peptide concentration of samples treated with alkaline protease and fungal protease after 6 and 16 hours. It should also be noted that the maximum TCA-soluble peptide concentrations for alkaline and fungal protease were around 1.5 mg/mL while that of neutral bacterial protease was around 1 mg/mL. When the shrimp samples were soaked in solutions containing alkaline protease and neutral bacterial protease at 5%, and fungal protease at 6%, only the TCA-soluble peptide concentration of the neutral bacterial protease increased slightly in comparison to samples treated at a lower enzyme concentration (Figure 5.1B). However, the TCA-soluble peptide concentrations of samples treated with alkaline and fungal proteases were significantly higher (p < 0.05) than that neutral bacterial protease except at 3 and 16 hours.

Figure 5.1. TCA-soluble peptide concentration (mg/mL) of shrimp protein extract A

![Graph showing TCA-soluble peptide concentration over time for different proteases.](image)
The same letters within each level of treatment time indicates no significant difference in the mean values of the TCA-soluble peptide concentration of samples treated with the three proteases ($p > 0.05$). All values are reported as means ± standard deviation of triplicate analysis of sample ($n=3$)

A = treatment at minimum enzyme concentration (3%), B = treatment at maximum enzyme concentration (5% or 6%)

Result of the analysis of variance (ANOVA) also indicated that the levels of the independent variables (enzyme concentration and marination time) did not significantly affect ($p > 0.05$) the TCA-soluble peptides in the extracts of shrimp treated with alkaline protease. This shows that the maximum limit of hydrolytic capacity for alkaline protease was already attained at the concentration and marination periods that were used for this enzyme. Hence, further increase in the levels of the independent variables would not result in any significant increase in TCA-soluble peptides. However, only the enzyme concentration had a significant effect ($p < 0.05$) on the TCA-soluble peptide concentration of shrimp treated with fungal protease and neutral bacterial protease. Consequently, increasing the treatment times beyond 3 hours did not significantly ($p > 0.05$) increase the concentration of TCA-soluble peptides by all the three enzymes.
whereas an increase in enzyme concentration resulted in higher peptide concentration in samples treated with fungal protease and neutral bacterial protease.

Different enzymes are often used to hydrolyze food proteins to improve their functional and bioactive properties (Balti et al., 2011). The extent to which a protease will cleave an intact protein will depend on the type of protease (endoprotease or exoprotease), its specificity for the protein and treatment conditions including pH, time and temperature (Kristinsson & Rasco, 2000). The alkaline protease used in this study is an endoprotease from *Bacillus licheniformis* and this type of protease has been reported to have broad specificity and to preferentially cleave hydrophobic amino acids including leucine, phenylalanine and tyrosine (Kumar & Takagi, 1999). The neutral bacterial protease from *Bacillus subtilis* has been described as a serine protease and a metalloprotease with broad specificity although it cleaves mainly the Leu-, Phe–NH₂, and Tyr–COOH peptide bonds (Hsu, Lu & Jao, 2009). Protease from *Aspergillus oryzae* on the other hand is noted for its affinity to cleave hydrophobic amino acid residues hence its application as a debittering agent (Sumantha, Larroche & Pandey, 2006).

The hydrolytic capacity of these enzymes on some food proteins has been demonstrated in different studies. In a study that was aimed at producing soy protein hydrolysate with antioxidant activities using a neutral protease from *Bacillus subtilis*, protease from *Aspergillus oryzae*, and an alkaline protease from *Bacillus licheniformis*, the alkaline protease was reported to have the highest degree of hydrolysis (25.1) while the neutral protease gave the least degree of hydrolysis (13.4%) thus agreeing with our result (Zhang, Li & Zhou, 2010). When these same proteases were used to hydrolyze milk proteins, the fungal protease from *Aspergillus oryzae* gave the highest degree of hydrolysis
(35.5%) followed by the alkaline protease (30.7%) (Hogan, Zhang, Li, Wang & Zhou, 2009) thus indicating the influence of the substrate type on hydrolytic capacity of the enzymes. With respect to seafood, a study showed that a crude enzyme preparation from *Bacillus licheniformis* NH1 gave the highest degree of hydrolysis in comparison to a commercial enzyme (Alcalase) and a fungal protease when these enzymes were used to hydrolyze protein in the by-product of Sardinelle (*Sardinella aurita*) (Bougatef et al., 2010). Although both enzymes are alkaline proteases, the crude protease preparation from *Bacillus licheniformis* NH1 has multiple proteases which may have contributed to its hydrolytic capacity.

Treatment of whole shrimp with microbial proteases has the potential to cleave the myofibrillar proteins including the allergenic protein, tropomyosin in the shrimp tissues. However, the effectiveness of this enzyme treatment to reduce the IgE-binding capacity of tropomyosin will depend on the extent to which this allergenic protein is hydrolyzed by the individual enzyme used in this study. Consequently, it is hypothesized that the difference in the hydrolytic capacity of these enzymes as shown by the concentration of peptides generated will influence the IgE reactivity of tropomyosin in shrimp.

### 5.3.2. SDS-PAGE Analysis of Soluble Proteins from Enzyme-Treated Shrimp

Following the treatment of shrimp with microbial proteases, the myofibrillar proteins in these samples were extracted using high salt phosphate buffer and were subsequently separated under reducing gel electrophoresis. The SDS-PAGE patterns of the soluble proteins of shrimp treated with alkaline protease, fungal protease and neutral bacterial protease are presented in Figures 5.2A-C, respectively.
Figure 5.2. SDS-PAGE analysis of soluble protein extracts of raw and enzyme-treated shrimp

A

SDS-PAGE profile of samples treated with alkaline protease
Lane a: control, Lane b – d: extracts from samples treated at 3% enzyme concentration for 3, 6 and 16 hours, Lane e – g: extracts from samples treated at 5% or 6% (in case of fungal protease) enzyme concentration for 3, 6 and 16 hours. Tm: Tropomyosin.

B

SDS-PAGE profile of samples treated with fungal protease
Lane a: control, Lane b – d: extracts from samples treated at 3% enzyme concentration for 3, 6 and 16 hours, Lane e – g: extracts from samples treated at 5% or 6% (in case of fungal protease) enzyme concentration for 3, 6 and 16 hours. Tm: Tropomyosin
Among the three enzymes, alkaline protease displayed the strongest capacity to hydrolyze shrimp myofibrillar proteins including tropomyosin (Fig 5.2A). In comparison with the untreated samples, treatment of shrimp with alkaline protease resulted in the loss of protein bands between 37 – 250 kDa, including the 36 kDa tropomyosin. It is worth noting that the SDS-PAGE profiles for samples treated with alkaline protease were similar regardless of the time and concentration of the enzyme which agrees with the TCA-soluble peptide result indicating rapid hydrolysis of these proteins. The oligopeptides generated by the hydrolysis of the high molecular weight proteins is probably the smear band that accumulated in the 10 – 15 kDa region of the gel. The gel electrophoresis profile of soluble proteins from shrimp treated with fungal protease showed faint bands of almost all the major myofibrillar proteins including tropomyosin (Fig 5.2B) indicating lower hydrolytic capacity in comparison with the alkaline protease. However, it should be noted that the band intensity of tropomyosin in the extract of shrimp marinated with the fungal protease is weaker in comparison to other protein bands.
especially those migrating below the 37 kDa region. The extracts of sample treated with neutral bacterial protease on the other hand showed significant reduction in the band intensities of proteins that typically migrate between the 250 – 100 KDa region while clear protein bands can be seen between 75 – 15 kDa including the dimeric bands of 36 KDa tropomyosin (Fig 5.2C).

The alkaline protease used in this study demonstrated the greatest capacity to hydrolyze shrimp myofibrillar proteins including tropomyosin among the three enzymes. The strong hydrolytic capacity of alkaline proteases from Bacillus licheniformis towards shellfish proteins has been reported in different research studies (Baek & Cadwallader, 1995, Dey & Dora, 2014). Also, it has been shown that the specificity of enzymes as well as their activities under a fixed hydrolysis condition could influence their capacity to cleave protein substrates. For instance, the hydrolysis of yellow stripe trevally with an alkaline protease (Alcalase) resulted in the disappearance of high molecular weight protein bands including myosin heavy chain even at a low (5%) degree of hydrolysis (Klompong, Benjakul, Kantachote, Hayes & Shahidi, 2008). In addition, Pacheco-Aguilar, Mazorra-Manzano & Ramírez-Suárez (2008) reported the appearance of a low molecular weight smear on SDS – PAGE gel following the hydrolysis of Pacific whiting (Merluccius productus) muscle proteins with Alcalase. A study on the enzymatic tenderization of beef muscle indicated that both neutral bacterial protease from Bacillus subtilis and the fungal protease from Aspergillus oryzae had greater hydrolytic capacity towards myofibrillar proteins than collagenous proteins although the extent of this hydrolytic potential could not be ascertained from the study (Sullivan & Calkins, 2010). Investigation of the proteolytic activities of one bacterial protease and two fungal
proteases (fungal 31K protease and 60K protease) revealed that the fungal proteases are specific in their activities toward myofibrillar proteins compared with bacterial protease and papain which cleaves all myofibrillar proteins indiscriminately (Ha, Bekhit, Carne & Hopkins, 2013). The authors also observed a time dependent rate of hydrolysis of myofibrillar proteins by one of the fungal proteases in comparison with the bacterial protease which cleaved almost all the myofibrillar proteins in the first five minutes of enzymatic treatment (Ha et al., 2013). Similarly, an alkaline protease, Pescalase 560, was found to more rapidly hydrolyse fish proteins than two neutral proteases (HT200 and Protease N) from Bacillus subtilis due to the broad specificity of the former (Rebeca, Pena-Vera & Diaz-Castaneda, 1991). The result of the hydrolytic capacity of seven commercial protease preparations including Protamex, a neutral proteinase from Bacillus subtilis, towards Atlantic cod (Gadus morhua L.) viscera proteins also corroborated the minimal hydrolysis of muscle proteins by neutral protease when compared with alkaline protease (Aspmo, Horn & Eijsink, 2005). The SDS-PAGE analysis of the viscera protein hydrolysate indicated the retention of high molecular weight protein bands in samples hydrolysed with Protamex after 24 hours whereas these same proteins were effectively cleaved by Alcalase.

Thus, the broad specificity of the alkaline protease used to treat shrimp ensured the effective cleavage of shrimp meat proteins including tropomyosin to low molecular weight oligopeptides which formed a smear at the bottom of the SDS-PAGE gel. The fungal protease also showed the ability to cleave most of the shrimp meat proteins (especially those between 250 – 37 kDa) although the extent of hydrolysis was lower compared to the alkaline protease. The neutral bacterial protease on the other hand only
indicated specific hydrolytic capacity towards few high molecular weight proteins while leaving the low molecular weight proteins including tropomyosin relatively intact. It is hypothesized that the extent to which each of these enzymes cleaved the shrimp tropomyosin and the exact point of cleavage on the linear structure of this protein will influence its IgE reactivity.

5.3.3. IgE Reactivity of Tropomyosin from Shrimp Treated with Microbial Enzymes

The capacity of tropomyosin in the protein extracts of shrimp treated with three microbial enzymes to bind with IgE was determined via indirect ELISA using pooled human plasma from three shrimp allergic patients. The absorbance value that was read at 450 nm was used as the quantitative measure of the IgE-binding capacity as influenced by the enzyme type, enzyme concentration and treatment time (Figures 5.3A-B).

Treatment of whole shrimp with alkaline protease (at 3% enzyme concentration) resulted in a significantly lower IgE binding (p < 0.05) in comparison with samples treated with other proteases (Figure 5.3A). However, the IgE binding capacity of tropomyosin in extracts treated with alkaline protease was similar to that of the untreated control. The IgE binding capacity of tropomyosin in the extracts of samples treated with fungal and neutral bacterial protease on the other hand were significantly higher (p < 0.05) than that in the extracts of alkaline protease-treated samples and control.
Figure 5.3. Effect of enzyme treatment on the IgE-binding capacity of shrimp tropomyosin

A

Treatment at minimum enzyme concentration (3%)
All values correspond to absorbance values measured at 450 nm and are reported as means ± standard deviation of triplicate analysis of sample (n=3).

The same letters within each level of treatment time indicates no significant difference in the mean values of the IgE binding capacity (measured as absorbance at 450 nm) of samples treated with the three proteases (p > 0.05).

B

Treatment at maximum enzyme concentration (5% or 6%)
All values correspond to absorbance values measured at 450 nm and are reported as means ± standard deviation of triplicate analysis of sample (n=3).

The same letters within each level of treatment time indicates no significant difference in the mean values of the IgE binding capacity (measured as absorbance at 450 nm) of samples treated with the three proteases (p > 0.05).
At the maximum level of enzyme concentration, tropomyosin in the extracts of samples treated with alkaline protease had a significantly lower (p < 0.05) IgE binding capacity in comparison with samples treated with other enzymes and untreated samples (Figure 5.3B). In addition, the minimum IgE binding capacity was attained within three hours of treatment of shrimp with alkaline protease and increasing the marination time beyond three hours did not significantly (p > 0.05) affect the IgE reactivity of tropomyosin. More so, the IgE reactivity of tropomyosin in samples treated with neutral bacterial protease reduced to the level of the tropomyosin in extracts from untreated samples across the treatment times. The IgE reactivity of tropomyosin in the extracts of samples treated with fungal protease on the other hand showed gradual reduction as the time of marination increases.

Enzymatic hydrolysis has been used to attenuate the allergenic capacity of different food allergens (Verhoeckx et al., 2015). The advantage of this method lies in its potential to disrupt both conformational and linear epitopes of allergens (Rahaman et al., 2016). However, reports from previous studies have shown that the influence of enzymatic hydrolysis on the immunoreactivity of food allergens depends on some factors including properties of the allergenic protein, the enzyme that was used, extent of hydrolysis and on the allergic individual (Panda et al., 2015; Rahaman et al., 2016). With respect to shrimp tropomyosin, application of enzymatic hydrolysis to modify its IgE reactivity has mainly focused on the utilization of digestive proteases such as pepsin, trypsin and chymotrypsin which were aimed at unravelling its digestive stability (Gámez et al., 2015; Huang et al., 2010; Liu et al., 2010). Hence, to the best of our knowledge,
this is the first report on the utilization of microbial proteases to hydrolyze and modify the allergenic capacity of tropomyosin using a whole shrimp rather than a protein extract.

The ability of the alkaline protease from *Bacillus licheniformis* used in this study to significantly reduce the IgE binding capacity of shrimp tropomyosin may be due to its broad specificity which probably increased its likelihood of cleaving most of the linear epitopes of tropomyosin. The allergenic properties of protein hydrolysate derived from commercial alkaline proteases have been reported by other investigators. For instance, when the IgE reactivity of buckwheat protein hydrolyzed with seven enzymes was analyzed using rabbit antiserum to buckwheat protein and pooled sera from 21 patients that are allergic to buckwheat, an alkaline protease from *Bacillus licheniformis* was found to be effective at reducing the immunoreactivity of the buckwheat allergens (Sung et al., 2014). Other allergenic proteins with reduced in vitro IgE reactivity after treatment with alkaline protease include wheat gliadins, kidney bean, black gram, peanut allergens and soybean meal (Kasera, Singh, Lavasa, Prasad & Arora, 2015; Li et al., 2016; Wang et al., 2014). However, it should be noted that studies that utilized assays that could demonstrate the appearance of clinical symptoms of food allergy have shown that while enzymatic hydrolysis could reduce the IgE-binding capacity of food allergens, the hydrolytic fragments (peptides) could still bind to effector cells and cause the release of mediators that are responsible for allergic symptoms (Panda et al., 2015; Shi et al., 2013). Hence, application of biological assay such as basophil activation or mediator release assay is necessary to confirm the hypoallergenic properties of shrimp tropomyosin hydrolyzed with alkaline protease.
5.3.4. Western Blot Profile of the IgE Reactivity of Tropomyosin from Enzyme Treated Shrimp

To visualize the binding of human IgE to the tropomyosin in the protein extracts from enzyme – treated shrimp, a western blot analysis was conducted following the electrophoretic separation of the proteins and transfer to nitrocellulose membrane (Fig 5.4A-C). In agreement with the indirect ELISA result, the western blot profile of the separated proteins from the extracts of shrimp marinated with alkaline protease showed very weak binding of antibodies to the band that corresponded to tropomyosin (Figure 5.4A). Rather than a single band, the western blot profile indicated that the antibodies bound to some protein bands that are directly below the 36 KDa band which may correspond to some of the peptide fragments of tropomyosin. Although the band intensities were low, they were still visible among those extracts derived from shrimp marinated with alkaline protease at 3% enzyme concentration. However, these bands were barely visible for samples marinated at higher enzyme concentration indicating significant loss of IgE-binding capacity (Figure 5.4A).
Figure 5.4. Western blot analysis (using pooled plasma from shrimp allergic individuals) of soluble protein extracts of raw shrimp and enzyme-treated shrimp.

A

A = Western blot profile of the extracts of samples treated with alkaline protease
Lane a: control, Lane b – d: extracts from samples treated at 3% enzyme concentration for 3, 6 and 16 hours, Lane e – g: extracts from samples treated at 5% or 6% (in case of fungal protease) enzyme concentration for 3, 6 and 16 hours. Tm: Tropomyosin.

B

B = Western blot profile of the extracts of samples treated with fungal protease
Lane a: control, Lane b – d: extracts from samples treated at 3% enzyme concentration for 3, 6 and 16 hours, Lane e – g: extracts from samples treated at 5% or 6% (in case of fungal protease) enzyme concentration for 3, 6 and 16 hours. Tm: Tropomyosin.
C = Western blot profile of the extracts of samples treated with neutral bacterial protease
Lane a: control, Lane b – d: extracts from samples treated at 3% enzyme concentration for 3, 6 and 16 hours, Lane e – g: extracts from samples treated at 5% or 6% (in case of fungal protease) enzyme concentration for 3, 6 and 16 hours. Tm: Tropomyosin.

In the case of shrimp treated with fungal protease, a strong binding of the antibody with tropomyosin was observed especially at the lowest enzyme concentration while the band intensity became weaker in the extracts of sample treated with the highest concentration of the enzyme (Figure 5.4B). Interestingly, antibody from the pooled plasma was also observed to bind with certain high molecular weight proteins between 250 – 75 KDa. However, it should be noted that this binding was also observed in the untreated sample, appearing as a thick band (≈ 250 – 200 KDa). Hence, the multiple bands in the treated samples could be the degraded fragments of this single band. Other proteins that have been implicated in crustacean shellfish allergy include myosin light chain (17 – 20 kDa), calcium binding sarcoplasmic protein (20 – 25 kDa), actin (31 – 42 kDa), arginine kinase (40 – 45 kDa), troponin C (20 kDa), triosephosphate isomerase (28 kDa) and myosin heavy chain (227 kDa) (Abdel Rahman et al., 2013, Faber et al., 2016).
Although less commonly reported as a shrimp allergen, our result indicates that the allergic individuals from which the plasma was obtained are reactive to both tropomyosin and myosin heavy chain. Consequently, since this high molecular weight allergen was not completely hydrolyzed by the fungal protease (unlike the alkaline protease which ensured effective hydrolysis of almost all the proteins), antibody binding to the peptide fragments of the myosin heavy chain may have also contributed to the absorbance value obtained in the indirect ELISA analysis of tropomyosin IgE reactivity. This could also explain why some of the enzyme-treated samples had higher absorbance values during the indirect ELISA analysis of their extracts in comparison with the untreated sample. In the case of the untreated sample, the antibodies will bind to the few exposed epitopes in the intact myosin heavy chain protein while in the enzyme treated samples, the antibodies will bind to more epitopes in the peptides liberated from the intact protein thus increasing the intensity of the color obtained during the analysis.

The high IgE binding capacity of tropomyosin in shrimp treated with the neutral bacterial protease is also evident by the clear dimeric tropomyosin bands that can be seen on the membrane at all enzyme concentration and marination times (Figure 5.4C). This agrees with the indirect ELISA result which showed that this enzyme did not reduce the IgE reactivity of shrimp tropomyosin. More so, the binding of the antibody in the pooled plasma to myosin heavy chain is also observed among samples treated with neutral bacterial protease. However, unlike the proteins that were hydrolyzed by the fungal protease, this high molecular weight bands were less fragmented and thus maintained their position at around 250 KDa. However, the binding of the antibodies to this protein and its peptides would have also contributed to the absorbance values obtained via
indirect ELISA analysis. Thus, to ascertain the direct effect of this enzyme treatment on tropomyosin, a polyclonal antibody that is specific for tropomyosin was also utilized for the western blot analysis of these extracts (Figure 5.5A-C).

Unlike the antibody from the human plasma, the binding of this polyclonal antibody to tropomyosin in extracts from samples treated with alkaline protease was not visible on the membrane (Figure 5.5A). This may be due to extensive hydrolysis of this allergenic protein by the enzyme resulting in weakened color development. However, the binding of this antibody with residual tropomyosin and likely tropomyosin fragment was observed among extracts treated with Fungal and neutral bacterial proteases (Figure 5.5B-C).

Figure 5.5. Western blot analysis (using polyclonal antibody from mud crab raised in rabbit) of soluble protein extracts of raw and enzyme-treated shrimp

A

A = Western blot profile of the extracts of samples treated with alkaline protease
Lane a: control, Lane b – d: extracts from samples treated at 3% enzyme concentration for 3, 6 and 16 hours, Lane e – g: extracts from samples treated at 5% or 6% (in case of fungal protease) enzyme concentration for 3, 6 and 16 hours. Tm: Tropomyosin.
Unlike the antibody from the human plasma, the binding of this polyclonal antibody to tropomyosin in extracts from samples treated with alkaline protease was not visible on the membrane (Figure 5.5A). This may be due to extensive hydrolysis of this
allergenic protein by the enzyme resulting in weakened color development. This may also be due to the extensive dilution of the secondary antibody (1: 100,000) used in the development of the western blot assay. A lower dilution factor will be utilized in subsequent studies to aid visualization of the antigen-antibody binding on the western blot membrane. However, the binding of this antibody with residual tropomyosin and likely tropomyosin fragment was observed among extracts treated with Fungal and neutral bacterial proteases (Figure 5.5B-C). In the case of extracts from samples treated with fungal protease (Fig 5.5B), protein-antibody binding can be observed at a point that is directly beneath the region that tropomyosin normally migrates. These bands (a likely tropomyosin fragment) can be seen from in the extracts of samples treated at both enzyme concentration although it becomes vague in the extract of sample treated at 6% enzyme concentration for 16 hours. Another band migrating around the 15 KDa region also bind to this polyclonal antibody indicating that it may be another fragment of tropomyosin. This band can be found at all enzyme concentrations and treatment times. A significant binding of the antibody with tropomyosin in the extracts of shrimp treated with neutral bacterial protease is observed as shown by the thick smear around the 36 KDa region. However, the intensity of this smear reduced at higher enzyme concentration and treatment time.

5.4. Conclusions

The capacity of three microbial enzymes including alkaline protease, neutral bacterial protease and fungal protease to hydrolyze the proteins in whole shrimp as well as their ability to modify the IgE-binding capacity of shrimp tropomyosin was evaluated. Protein extracts from shrimp treated with alkaline protease and fungal protease had
higher concentration of peptides that were soluble in 10% TCA compared with extracts obtained from samples treated with neutral bacterial protease. However, the IgE-binding capacity of shrimp tropomyosin was significantly reduced only in samples that were treated with the alkaline protease. This shows that a shrimp product with reduced immunoreactivity could be produced via treatment with alkaline protease. Future studies should focus on separating the peptide fragments generated by this enzyme and establishing their amino acid sequence. Also, the ability of these peptides to bind effector cells and cause the release of mediators need to be ascertained using a cell-based method.
CHAPTER 6

COMPARING THE ANTIBODY REACTIVITY OF TROPOMYOSIN FROM SHRIMP TREATED WITH MICROBIAL AND PLANT PROTEASES

6.1. Introduction

Food allergy is an abnormal response or hypersensitivity of the human immune system to harmless dietary food proteins (allergens) (Sampson, 2004). The first stage of food allergy is characterized by the production of a type of antibody known as immunoglobulin E (IgE) during the initial exposure of the allergen to the immune system which subsequently bind to effector cells including mast cells and basophils (Kamath et al., 2014). Following repeated exposure, the allergen will then crosslink to the IgE on the surface of mast cells and basophils which will trigger the degranulation of these effector cells causing the release of mediators including histamine, leukotriene and prostaglandin which are responsible for the symptoms of food allergy (Kamath et al., 2014).

Epidemiological studies have shown that the prevalence of food allergy is on the rise especially in western countries affecting around 5% of children and 3 – 4% of the adult population (Sicherer & Sampson, 2010).

Crustacean shellfish such as shrimp is one of the major sources of dietary proteins that functions as allergens in certain individuals. About 2% of the world population have been reported to have allergic reaction to crustacean shellfish (Lopata et al., 2010).

Allergic reactions to crustacean shellfish are characterized by cutaneous, oral, digestive, respiratory and systemic symptoms like anaphylaxis which can lead to death (Pedrosa, Boyano-Martínez, García-Ara & Quirce, 2015). Over the years, more than ten crustacean shellfish allergens have been identified although the major allergen is a 33-38 KDa heat-
stable myofibrillar protein known as tropomyosin (Kamath et al., 2014). An immunoinformatic analysis of shrimp (*Penaeus monodon*) tropomyosin showed the presence of ten linear epitopes with greater occurrence of tyrosine, glutamic acid, arginine, phenylalanine and serine residues in those epitopes (Zheng et al., 2011).

Food processing methods that could inhibit or limit the binding of IgE to tropomyosin epitopes could potentially reduce the capacity of this protein to cause an allergic reaction. Hence, processing techniques including different cooking methods, irradiation, ultrasound, pulsed ultraviolet light and combined heat and pressure treatment have been applied to reduce the extent of IgE binding to tropomyosin (Pedrosa et al., 2015). Most of these processing methods affects the secondary structure of tropomyosin without disrupting the IgE-binding capacity of the linear epitopes. Consequently, the ability of digestive enzymes to cleave these linear epitopes have also been investigated. For instance, Yu et al. (2011) demonstrated that high pressure steaming accelerated the peptic, tryptic and chymotryptic digestion of tropomyosin in the protein extract of crab (*Scylla paramamosain*) extracts. In another study, resistance of tropomyosin to hydrolysis by enzymes in simulated gastric fluid and simulated intestinal fluid system was observed and this only led to some reduction in the IgE-binding capacity (Liu et al., 2011). However, the narrow specificity of these digestive enzymes could be a limiting factor in their ability to significantly cleave tropomyosin epitopes. Hence, enzymes with broader specificities such as plant and microbial enzymes may exert greater hydrolytic capacity on shrimp tropomyosin and its epitopes resulting in significant reduction in IgE binding. More so, treatment of whole food samples (rather than protein extracts) with both digestive and non-digestive proteases including trypsin, chymotrypsin, papain and
alcalase have been shown to be effective at reducing the allergenic capacity of foods (Li et al., 2016; Yu, Ahmedna et al., 2011).

Thus, the aim of this study was to treat whole shrimp with a microbial enzyme and two plant enzymes and subsequently compare the IgE reactivity of tropomyosin in the protein extracts from these samples using IgE from shrimp allergic individuals and a polyclonal antibody with specificity for tropomyosin.

6.2. Materials and Methods

6.2.1. Samples and Chemicals

Frozen shrimp (*Penaeus monodon*) were obtained from a local seafood store (Bangor, ME). Alkaline protease L – 660 from *Bacillus licheniformis* (640 KDAPU/g Minimum) was obtained from Enzyme Development Corporation (New York, NY, USA) while papain from papaya latex (1.5-10 units/mg solid) and bromelain from pineapple stem (> 3 units/mg protein) were from Sigma-Aldrich (St. Louis, MO, USA). Trichloroacetic acid (TCA), 3, 3’, 5, 5’-Tetramethylbenzidine (TMB), Folin &Ciocalteu’s phenol reagent and goat anti-rabbit IgG conjugated with horseradish peroxidase were also purchased from Sigma-Aldrich (St. Louis, MO, USA). Reagents and precast gels used for gel electrophoresis were obtained from Bio Rad (Hercules, CA., USA). Human plasma from three shrimp allergic individuals were from PlasmaLab (Everett, MA., USA) while the Horseradish Peroxidase-labelled goat anti-human IgE is a product of Life Technologies (Grand Island, NY., USA). An anti-mud crab tropomyosin raised in rabbit used as primary antibody was provided Dr Maleki (United States Department of Agriculture, New Orleans, LA).
6.2.2. Treatment of Whole Shrimp with Plant and Microbial Proteases

Solutions of alkaline protease, papain and bromelain were made using deionized water up to a final volume of 40 mL. The pH of these enzyme solutions was adjusted to 9.5 for alkaline protease and 9.0 for both papain and bromelain. Thawed and deshelled shrimp were then added to these enzyme solutions such that a final enzyme concentration of 5% (based on the weight of shrimp) was achieved. The shrimp were treated with these enzyme solution for 3 hours under controlled agitation with the aid of a rotary shaker. The values of the enzyme concentration and the treatment time were based on results of in-house experimental trials. Following the enzyme treatment, the shrimp were quickly rinsed with deionized water and subjected to the protein extraction protocol. The control samples were shrimp dipped in just deionized water.

6.2.3. Determination of Salt-Soluble Protein Concentration

Untreated and enzyme-treated shrimp were minced manually and 5g of the minced tissue was added to 50 mL of 20 mM phosphate buffer containing 1 M NaCl adjusted to pH 7.5. The mixture was homogenized with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY, USA) at 60 rpm for 1 minute. The homogenate was then shaken for 1 hour at 4 °C with the aid of a rotary shaker to increase the solubilization of proteins. The homogenate was then centrifuged at 7000 × g for 20 minutes and the supernatant was stored in aliquots at – 20 °C. Soluble protein concentration of the extracts was determined using the BCA protein assay method.
6.2.4. Determination of TCA-Soluble Peptide Concentration

The Modified Lowry Method was used to determine the concentration of TCA-soluble peptides in the protein extracts of raw and enzyme-treated shrimp (McDonald & Chen, 1965). Deproteinization of the extracts was first carried out by mixing an equal volume of the protein solution with 20% TCA. The mixture was vortexed and allowed to stand at room temperature for 15 minutes to ensure protein precipitation. The mixture was centrifuged and the supernatant was carefully transferred into a separate tube. To quantify the concentration of peptides in the TCA solution, dilution series of bovine serum albumin (BSA) standard were made with deionized water while series of dilution of the TCA-peptide solution were made with 10% TCA. Exactly 40 µl of the diluted standards and TCA-peptide solution was added to 200 ul of the Modified Lowry reagent (Thermo Scientific, Rockford, IL, USA) in a 96 well plate, mixed for 30 seconds and allowed to stand at room temperature for exactly 10 minutes. 1x Folin reagent (20 µl) was added immediately, mixed for 30 seconds and incubated at room temperature for 30 minutes. The absorbance was read with a plate reader (BioTek Instruments, Inc, Winooski, VT, USA) at 750 nm and the peptide concentrations in mg/mL were determined from the standard curve. All analyses were carried out in duplicate.

6.2.5. Gel Electrophoresis Separation of Soluble Proteins

Separation and visualization of proteins in the soluble extracts of raw and enzyme-treated shrimp was performed with the aid of reducing SDS-PAGE based on the method of Laemmli (1970). The shrimp protein extracts were first diluted with the extraction buffer to achieve a final concentration of 1 mg/mL. These diluted extracts were
then mixed with equal volumes of loading buffer comprising Tris-HCl (pH 6.8), glycerol, 2% SDS and 0.01% bromophenol blue and the mixtures were immediately heated at 100 °C for 5 minutes. Exactly 15 µl of the protein – loading buffer mixture was loaded onto each well of a 4 – 20% precast gel (Bio Rad, Hercules, CA, USA) and the electrophoretic separation was carried out at 170V for 40 minutes. The gels were subsequently rinsed and stained with GelCode Blue Stain reagent (Pierce Biotechnology Inc, Rockford, IL, USA) to aid visualization of the protein bands.

6.2.6. Determination of IgE Reactivity of Tropomyosin by Indirect ELISA

The IgE reactivity of tropomyosin in the protein extracts of untreated and enzyme-treated shrimp were evaluated by indirect ELISA. The protein extracts were serially diluted with 50 mM carbonate/bicarbonate buffer (pH 9.6) to a final concentration of 5µg/mL and 100 µl were added to each well of a high binding microplate and incubated at 37 °C for 2 hours. After washing 5 times with phosphate buffered saline containing 0.05% Tween 20 (PBST), 200 µl of 1% bovine serum albumin (BSA) in PBST was added to the wells and incubated at 37 °C for 2 hours. The wells were washed and 50 µl of diluted pooled plasma (1/20) from shrimp allergic individuals were added to each well and the plate was incubated for 1 hour at 37 °C. After another round of washing with PBST, 50 µL of goat anti-human IgE with attached horseradish peroxidase (1:2000) was added to each well and incubated for 1 hour at 37 °C. Following 5 washing cycle with PBST, 50 µl of the substrate solution (TMB) for the peroxidase conjugate was added to the wells and incubated for 15 minutes to allow for complete color development followed by termination of the reaction by adding equal volume of 2
M H₂SO₄. The absorbance was read at 450 nm with the aid of a microplate reader (BioTek Instruments Inc., Winooski, VT, USA). Each extract was analyzed in duplicate.

6.2.7. Western Blot Analysis of Shrimp Protein Extract with Human IgE and Rabbit Anti-Mud Crab Tropomyosin

Western blot analysis was carried out to visualize the effect of enzyme treatments on the IgE-binding capacity of shrimp tropomyosin. Electrophoretic separation of the soluble proteins was first carried out as described in the previous section. The protein bands in the gel were fixed onto a nitrocellulose membrane via wet electrotransfer method at 100V for 1 hour. Following blocking with PBST containing 2% BSA for 1 hour at room temperature, the membrane was washed with PBST and pooled plasma (1/20 dilution) from three shrimp allergic individuals was added to the membrane and incubated for 1 hour at room temperature. After repeated washes with PBST, secondary antibody conjugated with peroxidase enzyme (1/2000) was added to the membrane and incubated for 1 hour at room temperature. The membrane was washed five times with PBST and tropomyosin – IgE binding was detected by CN/DAB reagent according to the manufacturer’s instruction (Thermo Fisher Scientific, Rockford, IL, USA).

A polyclonal anti-mud crab tropomyosin raised in rabbit was also used as the primary antibody. After blocking and washing of the membrane, the polyclonal antibody (1/3000 dilution) was added to the membrane and incubated for 1 hours at room temperature. A goat anti-rabbit IgG conjugated with horseradish peroxidase (1/10000) was subsequently added to the membrane and incubated for 1 hour room temperature. The membrane was washed extensively and tropomyosin – IgE binding was detected by CN/DAB reagent.
6.2.8. Statistical Analysis

Analyses of Variance (ANOVA) were performed on quantitative data using JMP Statistical Software, Version 12 (SAS Institute, Cary, NC, USA) to determine the significant effect \( P \leq 0.05 \) of the independent variable, enzyme type, on the response variables such as TCA-peptide concentration and IgE reactivity via indirect ELISA. Tukey's HSD post hoc test was also used to compare the means of response values across the independent variables to identify the groups that are statistically different.

6.3. Results and Discussions

6.3.1. Effects of Enzyme Treatment on Total Soluble Protein Concentration of Shrimp

Following enzymatic treatment of shrimp with microbial and plant proteases, the salt soluble proteins were extracted from the samples and quantified using BCA assay (Figure 6.1) in which bovine serum albumin (BSA) was used to develop the standard curve by diluting the stock solution to create concentrations that ranged from 2 mg/mL to 0.125 mg/mL.

The untreated samples had a significantly higher \( (p < 0.05) \) soluble protein concentration than the enzyme-treated samples. However, among the enzyme-treated samples, those treated with alkaline protease had a significantly lower \( (p < 0.05) \) soluble proteins concentration compared with raw sample and papain-treated samples. Reduction in soluble protein concentration in sample treated with alkaline protease was about 50% less than that of the control whereas the reduction was less than 30 and 20%, respectively for samples treated with bromelain and papain.
Figure 6.1. Total soluble protein concentration from untreated and enzyme-treated shrimp.

![Bar graph showing protein concentration (mg/g shrimp) for different treatments.]

The values represent the average of three replicate samples (n=3) and are reported as mean ± standard deviation. Ap: alkaline protease, Bro: Bromelain, Pap: Papain. Ap^x The same letters indicate no significant difference in the mean values.

This reduction in soluble protein concentration may be due to the leaching of hydrolyzed proteins into the enzyme solution during the treatment period. Some studies have indicated that processes that facilitate the hydrolysis of proteins often lead to loss of oligopeptides or nitrogen fraction into the medium in which the sample was placed. For instance, Szymczak & Kołakowski (2012) reported increased total nitrogen loss from herring meat marinated in solution containing sodium chloride and acetic acid for up to 18 days. In addition, when raw peanut kernels were treated with α-chymotrypsin, a slight decrease in the soluble protein concentration was observed (Yu et al., 2011). However, some investigators have reported increase in protein solubility following enzymatic treatment of food samples. For example, Li et al., (2016) and Gokoglu, Yerlikaya, Ucak and Yatmaz (2017) reported increase in protein solubility during enzyme treatment of wheat flour and squid, respectively. In the first study, the wheat flour was hydrolyzed in a phosphate buffer medium and the whole mixture was analyzed for protein which may
then account for the increased protein concentration because the process did not result in loss of proteins. In the second study on the other hand, the relatively low concentration of enzymes (0.001-0.004%) as well as short treatment time of 20 minutes may have prevented extensive leaching of oligopeptides into the liquid medium.

Thus, it was hypothesized that the amount of soluble proteins extractable from the shrimp following enzyme treatment could be an indicator of the capacity of each enzyme to hydrolyze shrimp proteins to oligopeptides and small peptides which can leach out into the surrounding solution. Since samples treated with alkaline protease had the least soluble protein concentration, it was hypothesized that this enzyme likely led to the generation of small peptide fractions which leached into the liquid medium.

6.3.2. Effects of Enzyme Treatment on the Concentration of TCA-Soluble Peptides

The capacity of the microbial and plant proteases used in this study to cleave shrimp proteins into small peptides was assessed by quantifying the concentration of peptides soluble in 10% tricholoroacetic acid using the modified Lowry’s method (Figure 6.2). As expected, the untreated shrimp samples had a significantly lower (p < 0.05) concentration of peptides soluble in 10% TCA because the protein extract from these samples will have intact proteins that will precipitate out in the presence of the acidic reagent. Among the enzyme-treated samples, those treated with alkaline protease and papain had a significantly higher (p > 0.05) TCA-soluble peptide concentration in comparison with the control and bromelain-treated samples (Figure 6.2).
Figure 6.2. TCA-soluble peptide concentration of untreated and enzyme-treated shrimp protein extract.

The values represent the average of three replicate samples (n=3) and are reported as mean ± standard deviation. Ap: alkaline protease, Bro: Bromelain, Pap: Papain. 

*The same letters indicates no significant difference in the mean values

This suggests that both alkaline protease and papain cleaved more shrimp proteins into sizes that could remain in 10% TCA solution in comparison with bromelain. Some studies have been conducted to ascertain the mechanism of peptide solubility in 10% TCA as well as the molecular weight of peptides soluble in this acidic solution. For instance, Greenberg & Shipe (1979) reported that the molecular weights of peptides soluble in 10% TCA range from 330-380 Da which correspond to about 3 – 4 amino acid residues. However, Yvon, Chabanet and Pélissier (1989) argued that the peptide size was not a reliable indicator of peptide solubility in 10% TCA since peptides containing 7-30 amino acid residues may be soluble, insoluble or partially soluble in up to 12% TCA solution. However, the authors found that all peptides with fewer than 7 amino acid residues were soluble in 2-12% TCA solutions. They also established that the increase in the hydrophobicity of peptides (which caused aggregation) caused by TCA is mainly
responsible for the precipitation of proteins in TCA solution (Yvon, Chabanet & Pélissier, 1989). Thus, the slightly lower concentration of TCA soluble peptides in the bromelain treated samples may be an indication of more high molecular weights oligopeptides in the extracts compared with those of alkaline protease and papain.

In a study that compared the capacity of ten commercial enzymes comprising alkaline and neutral proteases (such as papain and bromelain) to hydrolyze crayfish processing by-products, the alkaline proteases including Alcalase, Prozyme and Optimase showed higher hydrolytic capacity than the neutral proteases (Baek & Cadwallader, 1995). Also, Alcalase was found to hydrolyze herring (*Clupea harengus*) proteins to higher degree of hydrolysis in comparison with papain (Hoyle & Merrltt, 1994). In contrast, however, the enzymatic hydrolysis of Alaska pollock frame using ten commercial enzymes showed that the extent of hydrolysis was low in samples hydrolyzed with alkaline protease, Alcalase, papain and bromelain in comparison with trypsin and mixed enzymes for animal proteolysis (Hou, Li, Zhao, Zhang & Li, 2011). These results may be due to the difference in the activities of enzymes used in different studies as well as the differences in samples. In addition to seafood, treatment of substrates with alkaline proteases often lead to higher degree of hydrolysis owing to its broad specificity.

Although enzymatic treatment of whole shrimp with microbial and plant proteases led to the generation of TCA soluble peptides and insoluble oligopeptides, the sizes of the peptide fragment of tropomyosin generated after this enzymatic treatment will strongly influence the IgE binding capacity of this allergen. Using an immunoinformatic methods, ten peptides have been predicted as the IgE binding epitopes of tropomyosin and the minimum and maximum amino acid residues in these linear epitopes were 12 and 20
residues, respectively (Zheng et al., 2011). This shows that the best enzyme will be one that can cleave tropomyosin into very small peptides with residues significantly lower than those found in the linear epitopes.

6.3.3. SDS-PAGE Analysis of Soluble Proteins from Untreated and Enzyme-Treated Shrimp

To aid the visualization of the effects of plant and microbial enzymes on the hydrolysis of shrimp proteins, the salt-soluble proteins were separated on a SDS-PAGE gel (Fig 6.3).

Figure 6.3. SDS-PAGE analysis of protein extracts

![Figure 6.3](image)

Raw shrimp (Lane a), and enzyme treated with alkaline protease (Lane b), Bromelain (Lane c) and Papain (Lane d), respectively. Mr: Molecular weight marker, Tm: Tropomyosin

The major proteins that can be seen in the extract of the untreated sample include high molecular weight protein bands between 75-250 KDa, a 41 KDa band immediately followed by the dimeric tropomyosin bands between 36-37 KDa (Figure 6.3, Lane a). Other protein bands include the 30, 20 and 15 KDa bands having lower band intensities. Among the three enzymes, alkaline protease and papain showed the greatest capacity to
hydrolyze shrimp myofibrillar proteins to smaller peptides although this capacity seems
to greatest with alkaline protease (Figure 6.3, Lane b and d). However, both enzymes
could not completely hydrolyze the protein migrating around the 30 KDa region and
treatment with papain led to the accumulation of more proteins at the 10 KDa region
compared with extracts obtained from samples treated with alkaline protease which
showed a comparatively weak band around 10 KDa. Bromelain on the other hand could
cleave most of the proteins between 30-250 KDa although faint bands of some of these
proteins could be seen. More so, a thick protein band around 25 KDa and a smear
between 10-15 KDa was also observed which could represent accumulated oligopeptide
fragments of some of the cleaved high molecular weight proteins.

The mechanism of peptide cleavage by alkaline proteases especially the serine –
type has been shown to involve nucleophilic attack of peptide bond via the serine amino
acid residue present in the active site of the enzyme (Jellouli, Ghorbel-Bellaaj, Ayed,
Manni, Agrebi & Nasri, 2011). The extensive hydrolytic capacity of alkaline proteases
especially alcalase enzyme has been demonstrated in different substrates including
Atlantic salmon muscle protein (Kristinsson & Rasco, 2000) where a smear of protein
bands around 10 KDa was observed on the SDS-PAGE gel, In addition, a commercial
alkaline protease (Alcalase) displayed the best capacity to liberate soluble proteins from
shrimp (Penaeus monodon) waste comprising head and shell while also providing the
highest degree of hydrolysis (33%) among the four enzymes that were tested (Dey &
Dora, 2014). In general, studies have shown that enzymes with broader substrate
specificity can hydrolyze proteins to smaller peptides (Kristinsson & Rasco, 2000).
alkaline proteases especially those from Bacillus licheniformis are known for their broad

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specificity for peptide bonds especially those of aromatic or hydrophobic amino acid residues such as tyrosine, phenylalanine and leucine at P1 position (Gupta, Beg & Lorenz, 2002, Klompong, Benjakul, Kantachote, Hayes & Shahidi, 2008). This property probably account for the absence of most of the shrimp proteins from the SDS-PAGE gel due to their hydrolysis to very small peptides by alkaline protease.

Papain has also been described as an endopeptidase with broad specificity and preference for amino acids with large hydrophobic group at the P2 position as well as strong catalytic activity towards salt – soluble myofibrillar proteins (Berger & Schechter, 1970; Kang & Rice, 1970a; Tavano, 2013). Bromelain on the other hand is made up of a group of thiol endoproteases with molecular weights between 20-31 KDa which preferentially cleave peptide bonds containing leucine, alanine and glycine residues (de Lencastre Novaes et al., 2015). Other authors have reported that stem bromelain cleaves the peptide bond in which arginine residues are present at P1 and P2 positions while fruit bromelain cleaves the substrate containing arginine, valine and phenylalanine at P1 – P3 positions (Hale, Greer, Trinh & James, 2005). Based on the electrophoretic profile of the protein extract from shrimp treated with papain and bromelain, the former seems to have smaller peptides which could not be retained on the SDS-PAGE gel in comparison with the latter which has bands of oligopeptides between 30-10 KDa. A similar observation was reported during hydrolysis of cattle plasma with plant and fungal proteases in which hydrolysis of albumin with papain led to the generation of peptides with molecular weights < 20 KDa in one hour while the peptide sizes for the substrate hydrolyzed with bromelain hydrolysate range from 20 – 60 KDa after 24 hours (Bah, Bekhit, McConnell & Carne, 2016). More so, Kang & Rice (1970) demonstrated that papain strongly
hydrolyzed the myofibrillar protein fraction of beef muscle while bromelain had a greater affinity for the insoluble stromal protein fraction. Thus, differences in the ability of these enzymes to hydrolyze different muscle proteins as well as the extent of hydrolysis could influence their effect on the IgE binding capacity of shrimp tropomyosin.

6.3.4. Effects of Enzyme Treatment on the IgE Binding Capacity of Tropomyosin

The IgE reactivity of tropomyosin in the protein extract of untreated and enzyme treated shrimp was first evaluated with indirect ELISA using pooled plasma from shrimp allergic individuals. The absorbance values measured at 450 nm was used as indication of the extent of tropomyosin binding with IgE (Figure 6.4).

Figure 6.4. IgE binding capacity of tropomyosin in the protein extracts of untreated and enzyme-treated shrimp.

The values represent the average of three replicate samples (n=3) and are reported as mean ± standard deviation. Ap: alkaline protease, Bro: Bromelain, Pap: Papain.

The same letters indicates no significant difference in the mean values

Tropomyosin in the protein extracts from shrimp treated with alkaline protease had a significantly lower (p < 0.05) IgE binding capacity, with a reduction of about 87% in comparison with the control. Tropomyosin in the extracts from samples treated with
papain and bromelain also showed slight reduction in IgE binding capacities but were not significantly different (p > 0.05) from that of the untreated shrimp. This shows that the broad specificity of alkaline protease and probably its capacity to cleave shrimp proteins including tropomyosin to small peptide fragments in comparison to papain and bromelain ensured significant reduction of the IgE reactivity of tropomyosin. Although bromelain and papain also cleave tropomyosin as shown by the SDS-PAGE analysis of the extract, significant amount of intact tropomyosin and tropomyosin peptides with IgE binding epitopes may still be present in the samples treated with these enzymes. During the enzyme treatment of shrimp, the pH of the enzyme solutions was not maintained at the initial value. This may have affected the hydrolytic capacity of bromelain and papain while the activity of alkaline protease remained unchanged despite the changes in pH. In addition, the different activities of the enzymes used in this study may also be influencing their capacity to cleave tropomyosin sufficiently to reduce its IgE binding.

To the best of our knowledge, this is the first study comparing the efficacy of plant and microbial protease to attenuate the IgE reactivity of shrimp tropomyosin. However, these proteases have been utilized in studies aimed at reducing the allergenic capacity of certain food allergens. For instance, when the capacity of six proteases (alcalase, papain, flavourzyme, pepsin, trypsin and chymotrypsin) to reduce the concentration wheat gliadin was assessed, papain and alcalase were found to cause the greatest reduction in the content of this allergenic protein (Li et al., 2016). Using a cell based method to ascertain the allergenic capacity of soybean protein isolate following enzymatic treatments with alcalase, trypsin, chymotrypsin, bromelain and papain, it was reported that these enzymatic treatments did not result in hypoallergenic soybean protein
isolate and some of the enzymes (chymotrypsin and bromelain) were found to increase allergenic capacity (Panda et al., 2015). Hence, further studies need to focus on the capacity of tropomyosin from shrimp treated alkaline protease to bind effector cells and activates the release of inflammatory compounds such as histamine.

To visualize the binding of tropomyosin and its peptide fragments with anti-tropomyosin antibody, western blot analysis was carried out using IgE from human plasma and a polyclonal antibody specific for tropomyosin (Figure 6.5A and B, respectively).

Figure 6.5. Western blot analysis of protein extracts using IgE from human plasma and anti-mud crab tropomyosin raised in rabbit

Raw shrimp (Lane a), and shrimp treated with alkaline protease, Bromelain and Papain (Lane b – d), respectively. The human plasma was diluted 1/20 and the secondary antibody was at 1/2000 dilution. The polyclonal antibody was diluted 1/3000 prior to use while its corresponding secondary antibody was diluted 1/10000.

The western blot profile utilizing human IgE indicated the binding of the antibody to the 36 KDa tropomyosin band although the band intensities were different. Strong binding was observed in the case of untreated shrimp while the band intensity was weak in the bands that correspond to samples treated with alkaline protease and papain. The western blot banding pattern from the extracts of shrimp treated with bromelain was distinct in
that the antibody seems bind to certain high molecular weight proteins (75-37 KDa) in addition to the protein band corresponding to tropomyosin (Figure 6.5A). The western blot profile utilizing the anti-mud crab tropomyosin raised in rabbit gave a better picture of the binding of tropomyosin and potentially its peptide fragments with the polyclonal antibody (Figure 6.5B). As expected, the dominant band from the untreated sample is the 36 KDa tropomyosin. In the case of sample treated with alkaline protease, the antibody reacted to residual intact tropomyosin in the 36 KDa region forming a thin band. Some antibody reactivity with certain bands can also be observed right below the tropomyosin band followed by a strong reactivity with certain proteins migrating between 20-15 KDa. It is assumed that these low molecular weight proteins forming a smear in this region are enzymatic hydrolysis fragments of tropomyosin. The western blot profile of samples treated with bromelain showed a complex banding patterns as indicated by the reaction of the polyclonal antibody with a protein band around 70 KDa, a group of proteins between 36-24 KDa followed by another band near the 15 KDa region. The lane corresponding to samples treated with papain showed a similar banding pattern with that of samples treated with alkaline protease which comprise a 36 KDa band (residual intact tropomyosin), 25 KDa band and a band near the 15 KDa region. However, the intensities of these bands were higher than that of alkaline protease.

In general, the western blot profile agrees with the ELISA result which showed that treatment with alkaline protease caused significant reduction in the IgE binding capacity. This may be due to the appreciable reduction in the amount of intact tropomyosin present in the samples treated with alkaline protease in comparison to the other enzymes. Based on the western blot profile utilizing the polyclonal antibody, the
band corresponding to tropomyosin is thicker in both bromelain and papain treated samples which indicated higher residual intact tropomyosin in these samples. More so, further study is required to ascertain the high molecular weight protein in bromelain treated samples that also bind with the antibody.

6.4. Conclusions

Treatment of whole shrimp with alkaline protease, papain and bromelain lead to the hydrolysis of several shrimp proteins including tropomyosin as well as generation of peptides soluble in TCA. However, only the treatment with alkaline protease resulted in significant decrease in IgE reactivity as shown by indirect ELISA and western blot analysis. alkaline protease seems to reduce the amount if residual intact tropomyosin in comparison with other enzymes. Hence, this enzyme is a good candidate for the development of hypoallergenic shrimp product using enzymatic hydrolysis method. However, it is important to utilize these enzymes at the same activity level as this will provide a good basis for comparing their IgE binding reduction capacity. In addition, the ability of the enzyme treated shrimp to cause the activation of effector cells needs to be evaluated before this product can be truly hypoallergenic.
CHAPTER 7

OVERALL CONCLUSIONS AND RECOMMENDATIONS

In this study, the effects of simple thermal processing methods, exposure to high acid conditions and treatment with plant and microbial proteases on the IgE reactivity of shrimp tropomyosin were evaluated. Tropomyosin maintained its immunoreactivity after thermal and acid treatment of whole shrimp. This likely indicates that the linear epitopes of tropomyosin (which remained intact following exposure to heat and organic acid) contribute significantly to the allergenic capacity of this protein. In addition, the importance of evaluating the allergenic capacity of tropomyosin in the soluble and insoluble protein fractions were also evident in this study. Vinegar treatment on tropomyosin IgE reactivity showed that, reliance on the soluble protein fraction alone may give misleading results due to poor solubility of proteins.

Treatment of whole shrimp with plant and microbial proteases is a promising method of reducing the allergenic capacity of shrimp tropomyosin because of the capacity to cleave the linear tropomyosin epitopes. Among the enzymes tested, alkaline protease gave the best result in terms of the extent of reduction of IgE reactivity. Although other enzymes including papain, bromelain, fungal protease and neutral bacterial protease could hydrolyze shrimp proteins just as alkaline protease as shown by the gel electrophoresis profile, only the latter could attenuate the IgE reactivity of tropomyosin. One of the limitations of this study is that, the activities of all the enzymes were not standardized. Hence, it remains unclear whether alkaline protease exerted significant influence on tropomyosin IgE reactivity because it had the highest activity among the enzymes or because it had specificity towards its allergenic epitopes.
Thus, further studies should normalize the activity of these enzymes to confirm their capacity to hydrolyze tropomyosin epitopes. In addition, a chromatographic separation method should be applied to profile the sizes and sequence of peptides generated by the enzyme treatment of whole shrimp. The generated peptides may also be compared to the known allergenic peptides of shrimp tropomyosin to identify the enzymes that can cleave these epitopes. With respect to the treatment of whole shrimp with these proteases, the pH of the enzyme solution could be maintained at the optimal or initial pH by adjusting with a base. Changes in the pH of the enzyme solution during the marination process may have affected the hydrolytic capacity of the enzymes we used in this current study.

Reduction in the IgE binding of an allergen following processing does not guarantee its safety. It is important to investigate the capacity of the residual tropomyosin and enzymatic hydrolytic fragments to bind effector cells and cause the release of mediators. Hence, enzyme treated shrimp could only be confirmed as hypoallergenic after in-vivo assays such as cell mediator release assay, basophil activation test and skin prick test have been conducted.
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