1 **Effects of buffer additives and thermal processing methods on the solubility of shrimp**
2 **(*Penaeus monodon*) proteins and the immunoreactivity of its major allergen**

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9 **Abstract**

10 This study examines the potential of two buffer additives (Tween 20 and DTT) to
11 improve the solubility of proteins from shrimp subjected to different heat treatments and the
12 allergenicity of tropomyosin in the extracts. The concentration of soluble proteins extracted
13 by all the buffers from processed shrimp was significantly reduced compared with untreated
14 samples. The concentration of total soluble proteins from heat treated shrimp increased
15 significantly when phosphate buffer containing both surfactant and reducing agent was used
16 as the extraction buffer. However, the concentrations of heat-stable proteins in the buffers
17 were mostly similar. The electrophoretic profile of extracted proteins showed that
18 tropomyosin is very stable under the different heat treatment methods used in this study
19 except for high pressure steaming where the intensity of tropomyosin band was reduced.
20 Competitive inhibition ELISA showed that high pressure steaming reduced the allergenicity
21 of tropomyosin compared with other heat treatments methods.

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23 **Keywords: food allergen, crustacean allergen, shrimp allergen extraction, tropomyosin**
24 **immunoreactivity, food processing effects.**

1. Introduction

Food allergy has been described as an abnormal response by the body's immune system to certain food proteins leading to the occurrence of severe dermatological and physiological reactions in certain individuals (Kamath, Abdel Rahman, Komoda & Lopata, 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, O'Hehir & Lehrer, 2010). Nearly 6.5 million people in the United States have been reported to have an allergic reaction to shellfish (Lopata, O'Hehir & Lehrer, 2010). Symptoms of this reaction include urticaria, angioedema, asthma and life threatening anaphylactic shock (Lopata, O'Hehir & Lehrer, 2010). The major allergen of shellfish is a 34-38 kDa myofibrillar protein known as tropomyosin (Liu, Cheng, Nesbit, Su, Cao & Maleki, 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, Klein & Anklam, 2004). Most ELISA methods require effective solubilization of the allergenic proteins in an extraction buffer prior to detection (Albillos, Al-

Taher & Maks, 2011, Poms, Capelletti & Anklam, 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 methods (Downs & Taylor, 2010, Fu & Maks, 2013, Schmitt, Nesbit, Hurlburt, Cheng & Maleki, 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 effectively extract allergenic proteins from raw or unprocessed samples (Schmitt, Nesbit, Hurlburt, Cheng & Maleki, 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 have strong implication in cases 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, Jackson, Fu & Williams, 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 matrix.

Food processing has also been found to either aggravate or mitigate allergic reactions via alteration of allergen binding with immunoglobulin E (IgE) (Dhakal, Liu, Zhang, Roux, Sathe & Balasubramaniam, 2014, Jiménez-Saiz, Benedé, Molina & López-Expósito, 2014, Noorbakhsh et al., 2010). For instance, Liu et al. (2010) reported that the IgE binding of tropomyosin in an 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 molluscs for 20 minutes was found to increase the reactivity of tropomyosin in these sample extracts towards a monoclonal antibody (Kamath, Abdel Rahman, Komoda & Lopata, 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 the allergic reaction experienced by allergic patients. Understanding the relationship between food processing and allergenicity 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 allergenicity of shrimp tropomyosin have mainly focused on boiling, high intensity ultrasound, gamma irradiation and pulsed ultraviolet light (Li, Lin, Cao & Jameel, 2005, Liu, Cheng, Nesbit, Su, Cao & Maleki, 2010, Shriver, Yang, Chung & Percival, 2011, Zhenxing, Hong, Limin & Jamil, 2007). However, a comparative study on the effect of a variety of processing methods employed during home or retail processing of shrimp on tropomyosin allergenicity has not been conducted to the best of 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 allergenicity 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, Al-Taher & Maks, 2011, Hildebrandt, Steinhart & Paschke, 2008, Seiki et al., 2007, Steinhoff, Fischer & Paschke-Kratzin, 2011, Zhenxing, Hong, Limin & Jamil, 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.

2. Materials and Methods

2.1. Samples and Chemicals

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 purchased from Sigma (St. Louis, MO, USA) and Promega (Madison, WI, USA) respectively. Ammonium sulfate was purchased 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 purchased 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 purchased from Life Technologies (Grand Island, NY., USA). Costar™ 96-well microtitre plates used for immunoassay were purchased from Thermo Fisher (Waltham, MA., USA).

2.2. Extraction Buffers

Buffers used for protein extraction were as follows: Buffer 1 is 20 mM sodium phosphate + 1M NaCl, Buffer 2 is 20 mM sodium phosphate + 1M NaCl + 1% Tween 20, and Buffer 3 is 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.

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.

2.4. Thermal processing of shrimp

Shrimp were allowed to thaw, and were deshelled and deveined. They were subsequently subjected to different thermal treatments 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 a control. Samples were treated in triplicate for each processing method. The samples were analyzed immediately after processing or stored at -20 °C prior to analysis.

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 the minimum recommended by United States Food and Drug Administration (FDA) for the safe cooking of seafood.

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 (phosphate buffer and Tris buffer, pH 7-8), a high salt buffer comprising 20 mM 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 $7000 \times g$ for 15 minutes and the resulting supernatant was aliquoted. The aliquots were further centrifuged at $21000 \times g$ for 10 minutes and the clear supernatant was designated total protein extract.

The target protein (tropomyosin) is heat-stable. Thus, in order 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.

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 20 mM Tris-HCl containing 50 mM KCl. The homogenate was centrifuged at 7000 $\times$ 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.

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 7000 $\times$ 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 successively fractionated by adding solid ammonium sulfate to 40-60% saturation and centrifuged at 7000 $\times$ g for 20 minutes. The precipitate was re-dissolved in 20 mM Tris-HCl (pH 7.5) and was passed through 0.45 μ m 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. The solution containing mainly tropomyosin obtained after centrifugation was aliquoted and stored at -20 °C.

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 a range of concentrations that fit a standard curve. Bovine serum albumin (BSA) supplied with the assay kit was used as 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.

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). Each 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 in 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).

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 (0.2 µg/well) was coated onto a high 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 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 average of duplicate analysis and was calculated as follows

$$\% \text{ Inhibition} = \frac{[OD \text{ no inhibitor} - OD \text{ inhibitor}]}{OD \text{ no inhibitor}} \times 100\%$$

2.10. Statistical Analysis

One-way analysis of variance (ANOVA) of treatments was carried out using the JMP software, version 11 (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. Results

3.1. Influence of buffer additives and thermal processing on soluble protein concentration

In general, 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 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 is however obtained from boiled shrimp by this buffer which is only comparable to those obtained from steamed and microwaved 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 shrimp 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. Buffer solutions 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 concentration of soluble proteins from most of the heated samples compared with buffer without these additives or with surfactant alone.

This 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 is higher than those obtained from other heat treatment methods. More so, a combination of surfactant and reducing agent leads to better recovery of soluble protein from shrimp processed by either wet or dry heating method.

3.2. Influence of buffer additives and thermal processing on heat-stable protein concentration

Tropomyosin is a heat-stable protein. Hence, protein extracts are frequently heated to precipitate heat labile proteins in order to obtain a tropomyosin rich extraction method (Seiki et al., 2007, Yu, Cao, Cai, Weng, Su & Liu, 2011). In this study, the concentration of heat-stable proteins in the extracts obtained from raw and heated shrimp were quantified (Table 2). Similar to the total protein extract, soluble protein concentrations from untreated and high pressure steamed shrimp were higher than that obtained from other samples. However, presence of buffer additives has 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. 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, microwaved 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 can be found in steamed samples where buffer 3 has a significantly higher ($p < 0.05$) heat-stable protein concentration compared with other buffers.

3.3. SDS-PAGE Analysis

The buffer containing reducing agent and surfactant had a higher concentration of total proteins in most of the processed samples while the concentration 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 1). Extracts from untreated shrimp shows thick protein bands between (75-250 kDa), as well as prominent 42 kDa band (arginine kinase), a 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 the 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, Huang, Cai, Weng, Su & Cao, 2011, Toomer, Do, Fu & Williams, 2015). In comparison with other bands however, the band intensities of tropomyosin and myosin light chain were enhanced (Fig 1). The protein profile of extracts obtained from high pressure steamed shrimp 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 causes aggregation of myofibrillar proteins. Furthermore, a careful observation of the wells of the electrophoretic gel indicates 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 shrimp was less complex than that of total protein (Fig 2). 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 the absence of protein bands in wells loaded with extracts from untreated and heated samples. However, an exception can be seen in the well loaded with extracts obtained from the 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 has more high molecular weight aggregated proteins compared with other extracts.

Three distinct bands, including a band approximately 100 kDa, tropomyosin (36 kDa) and myosin light chain (20 kDa) can be observed from all extracts (Fig 2). In addition, the intensities of these bands were enhanced in the extract from the 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 the 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 is very stable under the thermal conditions used in this study except for the high pressure steaming process where the tropomyosin band had a lower intensity.

3.4. Inhibition ELISA

Since tropomyosin was retained in the heat-stable fraction of all samples, these extracts were used for the inhibition ELISA assay. The IgE in the pooled sera from three shrimp allergic patients was highly inhibited by the heat-stable extracts from raw and processed shrimp. 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 shows a reduction in IgE binding, especially at protein concentrations between 0.1 – 0.001 µg/ml. Although the 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 has appreciable amounts of epitopes available to bind with anti-tropomyosin antibodies in the pooled sera. Nevertheless, since the extent of this binding is low especially at lower protein concentrations, it can be concluded that the heat-stable extract has less allergenic components compared with extracts from other processed samples.

4. Discussion

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, Al-Taher & Maks, 2011, Hildebrandt, Steinhart &

Paschke, 2008, Seiki et al., 2007, Steinhoff, Fischer & Paschke-Kratzin, 2011, Zhenxing, Hong, Limin & Jamil, 2007, Wu et al., 2014). Tween-20 is a non-ionic and non-denaturing surfactant that can help improve protein interactions with solvent by minimizing protein-protein association caused by denaturation. DDT on the 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 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 the extraction solution (Albillos, Al-Taher & Maks, 2011). Using the BCA assay, about a 50% reduction in soluble protein recovery by phosphate buffered saline (PBS) was reported for boiled and autoclaved peanut flour in solution (Fu & Maks, 2013). A study with egg powder reported about 75% reduction in egg soluble proteins in PBS from boiled and autoclaved samples (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 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, Lin, Wang, Lu, Chen, Lin & Li, 2014).

Extraction of soluble proteins from boiled, steamed, microwaved, grilled and fried shrimp with buffers containing both surfactant and reducing agent resulted in a significantly ($p < 0.05$) higher total soluble protein concentration (Table 1). The 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 to buffer without additives. Thus, the presence of DTT in the extraction buffer might be crucial for the recovery of soluble proteins 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 proteins from thermally processed raw materials and from different food matrices (Fieste, Lovberg, Lindvik & Ecaas, 2007, Fu, Maks & Banaszewski, 2010).

In a study to improve the extraction of soluble protein from almonds roasted at temperatures between 230 and 400 °C, phosphate buffered saline with 0.1% Tween-20 (PBST) was found to improve the solubility of proteins from the processed almond compared with PBS and water extracted samples (Albillos, Al-Taher & Maks, 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 shrimp. 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, Fischer & Paschke-Kratzin, 2011). ELISA kits are formulated to extract and detect specific allergenic proteins or total soluble proteins from food samples (Fu & Maks, 2013b, Westphal, Pereira, Raybourne & Williams, 2004). From this study, recovery of total soluble proteins from processed shrimp samples can be improved by adding 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 shrimp.

The band intensities of tropomyosin from most of the processed samples (except that of high pressure steaming) were enhanced during SDS-PAGE analysis. The enhancement of these bands probably indicates the unfolding of these proteins due to thermal processing which increase their interaction with the dye used during gel electrophoresis (Davis & Williams, 1998). Different studies have confirmed the stability of tropomyosin during boiling. Shriver et al. (2011) reported that shrimp extract boiled 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, Abdel Rahman, Komoda & Lopata, 2013). Also, the band corresponding to tropomyosin was found to have greater intensities in the heated extracts (Kamath, Abdel Rahman, Komoda & Lopata, 2013). This study has also shown that shrimp processing methods, including steaming, baking, frying, microwaving roasting and grilling, enhanced the band intensity of tropomyosin.

The intensity of the tropomyosin band as well as other myofibrillar proteins in the extract of shrimp heated at high pressure in an autoclave was reduced. This is in agreement with other studies where a 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 (Yu, Cao, Cai, Weng, Su & Liu, 2011). This suggests that tropomyosin can be denatured under high pressure steaming. 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 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 1 and 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 other 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 a 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 have considerably reduced intensity (Kim, Lee, Song, Kim & Ahn, 2011).

When the allergenicity of tropomyosin in the heat-stable extracts was examined using inhibition ELISA, the percent inhibition of extracts obtained from the 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 in domestic or retail processing cannot be counted on to reduce the allergenicity of shrimp tropomyosin. Studies on the allergenicity 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 allergenicity of peanut allergens (Cong, Lou, Xue, Li, Wang & Zhang, 2007). The authors reported that these processing methods did not alter the allergenicity of major peanut allergens (Cong, Lou, Xue, Li, Wang & Zhang, 2007). Liu et al. (2010) reported that soluble protein extracts from shrimp (*Litopenaeus vannamei*) boiled for 10 minutes produced lower IgE binding than extracts from raw shrimp. However, purified tropomyosin from the same boiled sample gave a higher IgE binding (as shown by dot-blot assay) compare with tropomyosin from raw shrimp (Liu, Cheng, Nesbit, Su, Cao & Maleki, 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 allergenicity of tropomyosin to a certain degree especially at low protein concentrations, but it does not completely abolish the allergenicity 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 inhibition rate compared with extracts from samples that were boiled or treated with combined ultrasound and boiling (Yu, Cao, Cai,

Weng, Su & Liu, 2011). Also, the effect of autoclaving parameters on the allergenicity 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 extreme autoclave conditions (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 bind 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, Teuber, Roux & Sathe, 2002). In the case of these almond proteins, only a prolonged roasting condition (320 °F for 20 and 30 minutes) as well as microwave heating for 3 minutes were able to alter its antigenicity (Venkatachalam, Teuber, Roux & Sathe, 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, Lee, Song, Kim & Ahn, 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 this structural epitopes may also help significantly reduce 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 attribute. 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 murine model or human subject (skin prick test) is desirable. This information is crucial for the successful utilization high pressure steaming to develop hypoallergenic shrimp product which subsequently can be used in clinical trials for desensitization of crustacean allergic patients.

5. Conclusion

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 shrimp was investigated. Buffers 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 shrimp except that of high pressure steaming where the band intensity was reduced. The IgE binding of tropomyosin from the extracts of different

533 processed shrimp used in this study was comparable to that of raw sample except that of high
534 pressure steaming which displayed lower IgE binding.

535

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654

Table 1

Total soluble protein concentration (mg/ml) extracted from thermally processed shrimps using buffers with different additives

Treatments	Buffer 1	Buffer 2	Buffer 3
Untreated	8.13±0.12 ^{ax}	9.10±0.15 ^{ay}	9.32±0.34 ^{ay}
High pressure steaming	1.67±0.09 ^{bx}	1.84±0.21 ^{bx}	1.90±0.25 ^{bcx}
Baking	1.35±0.18 ^{bcxy}	1.30±0.06 ^{cdy}	1.91±0.39 ^{bcx}
Frying	1.34±0.23 ^{bcy}	1.53±0.09 ^{bcxy}	1.79±0.13 ^{bcx}
Grilling	1.33±0.07 ^{bcx}	1.26±0.07 ^{cdx}	2.18±0.48 ^{by}
Microwave	1.10±0.03 ^{cdx}	1.29±0.15 ^{cdx}	1.71±0.18 ^{bcy}
Steaming	1.01±0.03 ^{cdx}	1.03±0.12 ^{dx}	1.38±0.10 ^{bcy}
Boiling	0.87±0.08 ^{dx}	1.03±0.03 ^{dx}	1.37±0.18 ^{cy}

^{abcd} Means within the same column with different uppercase letters are significantly different (p < 0.05)

^{xy} Means within the same row with different lower case letters are 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

Table 2

Heat stable protein concentration (mg/ml) extracted from thermally processed shrimps using buffers with different additives

Treatments	Buffer 1	Buffer 2	Buffer 3
Untreated	1.51±0.03 ^{ex}	1.57±0.02 ^{abx}	1.55±0.12 ^{abcx}
High pressure steaming	1.65±0.16 ^{ex}	1.72±0.29 ^{ax}	1.76±0.20 ^{ax}
Baking	0.97±0.10 ^{bcx}	0.96±0.05 ^{cx}	0.93±0.09 ^{dx}
Frying	1.03±0.02 ^{cdx}	0.87±0.05 ^{cx}	1.21±0.24 ^{bcdx}
Grilling	1.16±0.01 ^{dx}	1.21±0.07 ^{abcx}	1.63±0.32 ^{abx}
Microwave	0.98B±0.02 ^{cy}	1.09±0.17 ^{bcxy}	1.27±0.06 ^{abcdx}
Steaming	0.87±0.05 ^{bx}	0.90±0.08 ^{cx}	1.11±0.04 ^{cdy}
Boiling	0.76±0.05 ^{ax}	1.17±0.33 ^{cx}	1.10±0.06 ^{dx}

^{abcd} Means within the same column with different uppercase letters are significantly different (p < 0.05)

^{xy} Means within the same row with different lower case letters are 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

Fig. 1. SDS-PAGE analysis of the soluble proteins from the total protein extracts of raw and thermally processed shrimps. MR: Molecular weight Marker, Lane 1: Raw, Lane 2: Boiled, Lane 3: Steamed, Lane 4: Baked, Lane 5: Fried, Lane 6: High pressure steaming, Lane 7: Microwaved, Lane 8: Grilled, 9-10: Purified tropomyosin; Tm: Tropomyosin (36 kDa).

Fig. 2. SDS-PAGE analyses of the soluble proteins from the heat stable extracts of raw and thermally processed shrimps. MR: Molecular weight Marker, Lane 1: Raw, Lane 2: Boiled, Lane 3: Steamed, Lane 4: Fried, Lane 5: Microwaved, Lane 6: High pressure steaming, Lane 7: Baked, Lane 8: Grilled. Tm: Tropomyosin (36 kDa)

Fig. 3. Inhibition ELISA analysis of heat stable extracts from raw and processed shrimps.

Serial dilutions (100 – 0.001 $\mu\text{g/ml}$) 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.