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Identification of Common and Novel Major Crab Allergens in *Scylla tranquebarica* and the Allergen Stability in Untreated and Vinegar-treated Crab

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ABSTRACT

Crab allergy is reported as a serious form of food allergy in many countries. This study was aimed to identify the major allergens of the local mud crab, *Scylla tranquebarica (S. tranquebarica),* and subsequently, determine the effect of vinegar treatments on the crab allergens.

Crab muscles were treated with synthetic and natural vinegar. Crab proteins were then extracted from the untreated and vinegar-treated crabs. All extracts were then fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and analyzed by immunoblotting; using sera from crab-allergic patients. The crab proteins were then further fractionated by two-dimensional electrophoresis (2-DE) and analyzed by mass spectrometry (MS).

The untreated crab had 38 protein bands, while that was only a few bands between 18 to 73 kDa for the vinegar-treated crabs. Immunoblotting of untreated crab revealed 20 IgE-binding bands, whereas the vinegar-treated crabs could only retain a few IgE-binding bands. Five major allergens were identified with molecular weightsof38, 42, 49, 63, and 73 kDa in the untreated crab. In contrast, the vinegar-treated crabs had only a few major allergens with molecular weights of 38, 42, and 73 kDa. MS identified the 43 and 49 kDa as arginine kinase, while the 38, 63, and 73 kDa were identified as tropomyosin, actin, and hemocyanin, respectively.

In conclusion, we found three common major allergens for *S. tranquebarica* including tropomyosin, arginine kinase, and actin, and one novel allergen known as hemocyanin. All the major allergens could retain minimal allergenic capability in vinegar-treated crabs, suggesting that vinegar treatments might be useful to reduce crab allergenicity. These data would assist the clinicians in the management of crab-allergic patients worldwide.

Keywords: Acetic acid; Allergens; Immunoblotting; Proteomics

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INTRODUCTION

Currently, food allergies are a growing public health concern.[1,2] Annual reports on food allergy increased from 100 in 1971 to 1200 in 2014.[1] Among foods, shellfish including crab was declared as the important culprit.[3,4] *Scylla* spp. are an important mud crab commonly distributed across the Indo-West Pacific Oceans.[5] Among the four species, *S. tranquebarica*, also known as purple mud crab is highly demanded as an essential protein source by various communities in Asian countries including Malaysia and dominates the fishery industries.[5] However, consumption of crab may also cause seafood allergy among the local population,[2-4] that triggering allergic reactions in the skin, gastrointestinal, respiratory, and cardiovascular systems of hypersensitive individuals.[2-4,6,7] Exposure to crab allergens may occur either through ingestion of crab, inhalation of crab vapor, or skin contact to crab meats while processing, cooking, or working.[7,8] In Singapore, 15.2% of food allergic patients were reported to have crab allergy,[4] while in Malaysia, the prevalence of crab sensitization among allergic rhinitis and asthma patients were 21%.[3]

To date, several clinically relevant crab allergens have been identified.[2] Tropomyosin, a protein at 34 to 38 kDa was demonstrated to be the major and cross-reactive allergen for various species of crabs.[9-11] Tropomyosin is believed to be a pan-allergen of invertebrates including arthropods and molluscs,[9-12] as this protein is a major regulatory protein in both muscle and non-muscle cells which plays essential roles in muscle contraction in all vertebrates and invertebrates.[9-12] Apart from tropomyosin, arginine kinase, an enzyme that catalyzes the reversible transfer of a phosphate group from ATP to arginine in all invertebrates was also identified as a crab major allergen.[10,13] More recently, several new crab allergens have also been identified including α-actin, a sarcoplasmic calcium-binding protein, troponin, myosin light chains, and smooth endoplasmic reticulum Ca^{2+}ATPase.[9]

Crab is usually subjected to processing before consumption to improve food quality, taste, and digestibility.[14] However, processing methods particularly heat processing has been widely reported could increase, decrease, or stabilize the allergenic capacity of certain allergens.[15] The effect of numerous heat treatments such as frying, boiling, roasting, and steaming on the allergenicity of shellfish including crab has been well-established.[15-18] However, studies on the effect of other food preservation methods; using non-thermal techniques on shellfish allergenicity are very limited.[15] There are two categories of non-thermal food preservation methods, either by using natural preservatives such as salt, natural vinegar, and honey, which are much safer for humans and the environment than the use of the synthetic preservative such as synthetic vinegar, sulfites, and benzoates that can cause certain health problems.[14] Vinegar, either natural or synthetic vinegar contains acetic acid and is widely used for food preservations by inhibits bacterial growths in both domestic uses and the food industry.[14,19] Recently, natural vinegar, produced from cereals, fruits, or any starch-based sources has been widely used as natural preservatives of foods.[19] Several studies reported that foods treated with vinegar have lower allergic capacity including prawn, egg, chicken, and lentils, thus this method was suggested as an effective method to reduce food allergenicity.[20-22] However, the potential capacity of vinegar to modify the allergenicity of crab has not been reported.

Allergenic study on *S. tranquebarica* or purple mud crab is unavailable in the literature. Since there is a scarcity of information on the characterization of allergens of *S. tranquebarica,* this study was conducted to identify the major allergens of *S. Tranquebarica* and subsequently determine the effect of several types of vinegar on the allergenicity of these species of crab.

MATERIALS AND METHODS

S. tranquebarica Extracts

The samples of live *S. tranquebarica* were purchased from a local supplier in Tawau, Sabah, Malaysia. The extracts of these crab proteins were prepared from the edible crab meat as explained previously.[23-25] In brief, to prepare the untreated extract, 5 g of untreated crab meat was homogenized in 100 ml phosphate buffer saline (PBS) pH 7.2 (1:10 w/v), extracted overnight at 4ºC, centrifuged, filtered by papers and syringe filters, freeze-dried and stored at -20ºC. Meanwhile, for preparation of the vinegar-treated extracts, 12 g of the crab meats were marinated with six different types of vinegar as listed in Table 1 in the ratio of 1:2 w/v at 4ºC for 16 hours. After treatments, the liquid was removed and the crab samples were rinsed with distilled water and patted dry on a clean paper towel. The treated crab meats were then subjected to protein extraction as mentioned above.

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Table 1.Type of vinegar and its pH used in this study

Type of vinegar	pH
White vinegar (synthetic)	2.4
Pomegranate	2.55
Grape	2.67
Fig	2.94
Apple	3.0
Dates	3.35

Serum Samples

This study used 60 sera from crab-allergic patients from a previous study.[23-25] The crab allergic status of the patients was confirmed by an *in-vivo* test to the crab allergen extract; using skin prick test (wheal size at least 3 mm) and/or in an *in-vitro* test; using Phadia System (Phadia, Sweden) (values above 0.35 kU/L). A set of negative control sera from five healthy, non-allergenic individuals was used in this study. Medical Research and Ethics Committee (MREC), Ministry of Health Malaysia endorsed ethical approval before conducting this study (Approval No. NMRR-11-856-10216).

Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE)

The crab protein profile in the prepared extracts was revealed by SDS-PAGE. Briefly, the extracts were treated with Laemmli buffer (Bio-Rad, USA) and loaded into wells of 12.5% resolving gel with 5% stacking gel. Using a Mini-Protean 3 Apparatus (Bio-Rad, USA), the crab proteins and pre-stained molecular weight markers (GoldBio, USA) were then separated at 120 mA for 50 minutes in the Tris-HCl buffer. After completion, the gels were stained by Coomassie Brilliant Blue R-250 (Bio-Rad, USA). The molecular weights of the proteins were then estimated; using an imaging densitometer (Bio-Rad, USA).

Immunoblotting

Immunoblotting was done as described previously.[23-25] Briefly, the crab proteins in SDS-PAGE gel were electro-transferred to a nitrocellulose membrane; using the Mini Transblot System (Bio-Rad, USA) for 70 minutes at 250 mA. After completion, the membrane was stained with Ponceau S solution (Bio-Rad, USA), cut into small strips, and washed with Tween-20 tris-buffered saline (TTBS). After blocking with 5% low-fat milk in tris-buffered saline (TBS), the strips were probed with the individual sera at 4°C overnight. IgE binding proteins on the strips were then identified by detection systems containing biotinylated goat anti-human IgE (KPL, UK), conjugated streptavidin-alkaline phosphatase (Bio-Rad, USA), and alkaline phosphatase conjugate substrate (Bio-Rad, USA). In this study, 60 sera were used in immunoblotting of untreated extract, while only 10 selected sera were further used in immunoblotting of vinegar-treated crab extracts.

Two-dimensional Electrophoresis (2-DE) and Immunoblotting

In rehydration buffer with 8M urea, 50 mM dithiothreitol (DTT), 4% of 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.0002% of bromophenol blue, and 0.2% of carrier ampholyte with a pH value range of 3-10 (Bio-Rad, USA), 5 mg of the lyophilized crab protein extract was suspended. The crab extract was then used to rehydrate the immobilized non-linear gradient strip length of 7 cm with isoelectric point (pI) value from 3-10, overnight for 16 hours. Using the Protean IEF Cell System (BioRad, USA), the isoelectric focusing was run at 20ºC with the subsequent voltage over time gradient of 100 V for 1 minute, 250 V for 30 minutes, 4000 V for 2 hours, and 4000 V for 10,000 V-h. Before shifting the IPG strips to the second dimension electrophoresis, the strips were equilibrated consecutively for 10 minutes in equilibration buffers that contains 65 mM of dithiothreitol and 135 mM of iodoacetamide (Bio-Rad, USA) in 125 mM of Tris-HCl with a pH value of 6.8, 2% of SDS, 6 M of urea, 0.01% of bromophenol blue and 30% of glycerol. Then, the strips were positioned onto 12.5% SDS-PAGE gel and sealed; using an agarose gel. Subsequently, the Mini Protean 3 Apparatus was used (BioRad, USA) to allow the proteins to be separated for 45 minutes. Using Coomassie brilliant blue R250 (Bio-Rad, USA), the protein spots were observed and Imaging Densitometer GS800 with PDQuest software (BioRad, USA) was used to scan and analyzed the molecular weights and pI of the protein spots. To identify the major IgE-reactive protein spots, a nitrocellulose membrane blotted with 2-DE-separated crab protein components was subjected to a procedure similar to immunoblotting analysis as above; using 10 selected sera.

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Mass Spectrometry Analysis of Crab Major Allergens

The major IgE-binding spots of all major allergens were analyzed by Apical Laboratories Sdn. Bhd., Malaysia, using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). The spectra were analyzed; using Ludwig NR Database in Matrix Science software to identify the protein spots.

RESULTS

Protein Profiles of Untreated and Vinegar-treated Crab Extracts

Figure 1 shows the comparison of protein profiles between untreated and vinegar-treated extracts of *S. tranquebarica*. Visible protein bands, approximately 38 bands, with a complex protein pattern, mostly within the molecular weight of 10 to 245 kDa were displayed by the untreated extract of crab. Prominent protein bands between 35 to 75 kDa were observed. Compared to the untreated extract, the intensity of crab protein bands in all vinegar-treated extracts became less visible. The prominent band at 38 kDa showed relatively lower intensity in all vinegar treated crabs compared to the untreated extract. The most significant reduction was seen in the synthetic vinegar treated crab, followed by the pomegranate, grape, fig, apple, and date vinegar treated crabs. While, only three extracts; the fig, apple, and date vinegar-treated crabs demonstrated a faint 42 kDa band. All higher molecular weight bands above 42 kDa disappeared in all treated extracts except for the 73 kDa which can still be seen in the grape, fig, apple, and date vinegar-treated crabs as a faint band. While almost all lower molecular weight bands below 38 kDa disappeared except for the 23 and 24 kDa bands that were remained detected in the date vinegar-treated crab.

Immunoblotting of Untreated and Vinegar-treated Crab Extracts

The IgE-binding protein components of untreated crab extract and six different kinds of vinegar-treated crab extracts were detected by immunoblotting as shown in Figure 2 and Figure 3, respectively. Numerous allergenic protein bands between 14 to 135 kDa were noticed in the untreated extract with five proteins at 38, 42, 49, 63, and 73 kDa that could bind to IgE

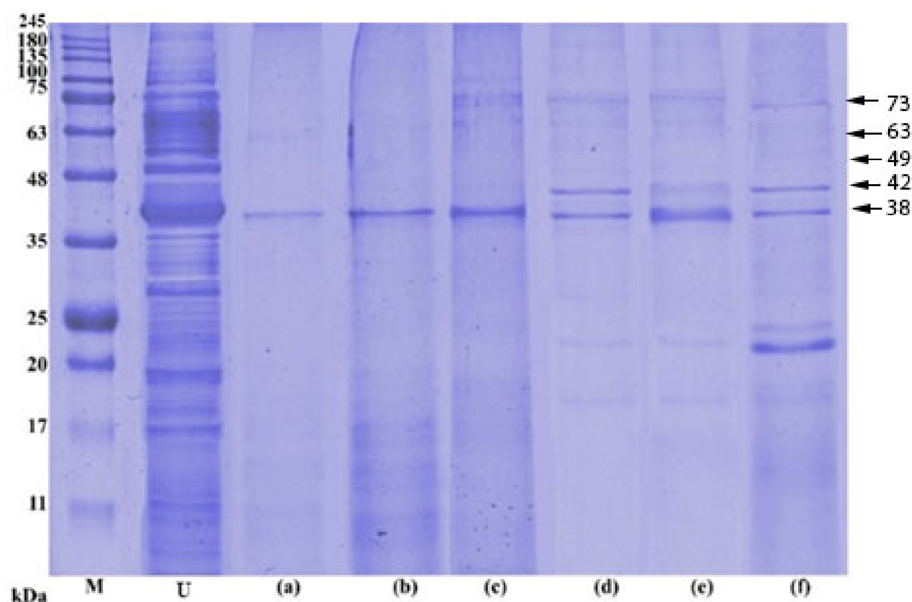

Figure 1.Protein profiles of raw and vinegar treated *Scylla tranquebarica* extracts in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Lane U is untreated crab extract, while lanes a, b, c, d, e, and f are synthetic, pomegranate, grape, fig, apple, and date vinegar treated-crab extracts. Lane M is molecular weight markers in kilodalton (kDa).

antibodies of at least half of the tested sera, hence have been declared as the major allergens for *S. tranquebarica*. Meanwhile, most of the patients' sera showed only single to three IgE-binding bands in the immunoblotting test of all vinegar-treated extracts.

More than 50% of the tested sera retained the IgE-binding to the 38 kDa major allergen band in all vinegar treated-crab extracts. Based on the band intensities, the crab treated with pomegranate, fig, and apple vinegar revealed higher IgE binding intensities to the 38 kDa major allergen band than the other types of vinegar. Meanwhile, the other major allergens at 42, 49, 63 and 73 kDa were sensitive to low pH environments, except in immunoblotting of some sera which can still detect the 42 kDa band in apple and date vinegar-treated crab extracts (sera No 8 and 10) and pomegranate treated-crab (Patient 6) and the 63 kDa in fig vinegar-treated crab extract (sera No 3 and 10) as faint bands.

Crab 2-DE Profiles and IgE-binding Spots

Figure 4 shows the 2-DE map of both protein spot profiles and allergenic spots of *S. tranquebarica*. Over 50 distinct protein spots between molecular weights of 10 to 245 kDa were observed in *S. tranquebarica* extracts, with an isoelectric point (pI) from 3.0 to 10.0. Subsequently, based on 2-DE immunoblotting results, the major allergenic spots were circled in Figure 4. Five major spots in *S. tranquebarica* were labeled as spots 1a (38 kDa), 2a (42 kDa), 3a (73kDa), 4a (49 kDa), and 5a (63 kDa).

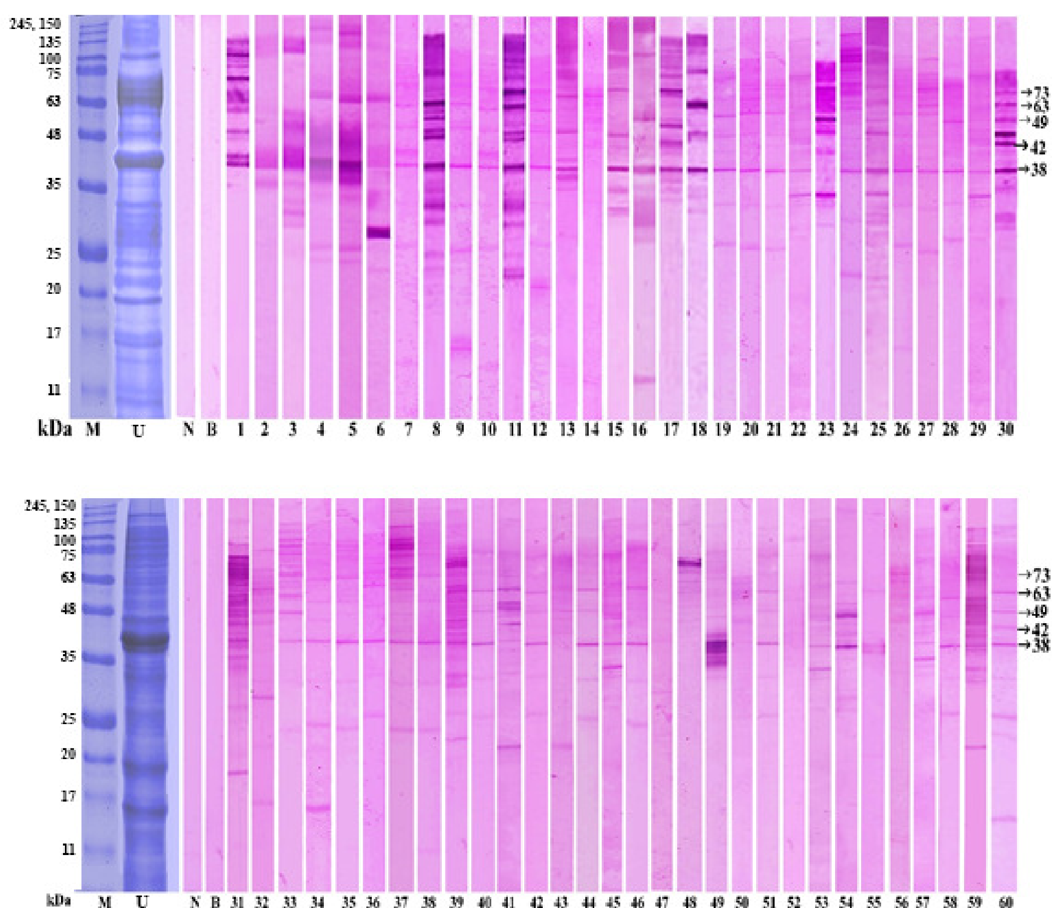

Figure 2. Immunoblotting results of untreated *Scylla tranquebarica* extract; using sera from 60 crab-allergic patients (lane 1 to 60). Lane M is molecular mass markers in kilodalton (kDa); lane U is an untreated extract; lane B is blank and lane N is immunoblot; using a negative control serum. Arrows indicated the molecular weight of major allergens in kDa.

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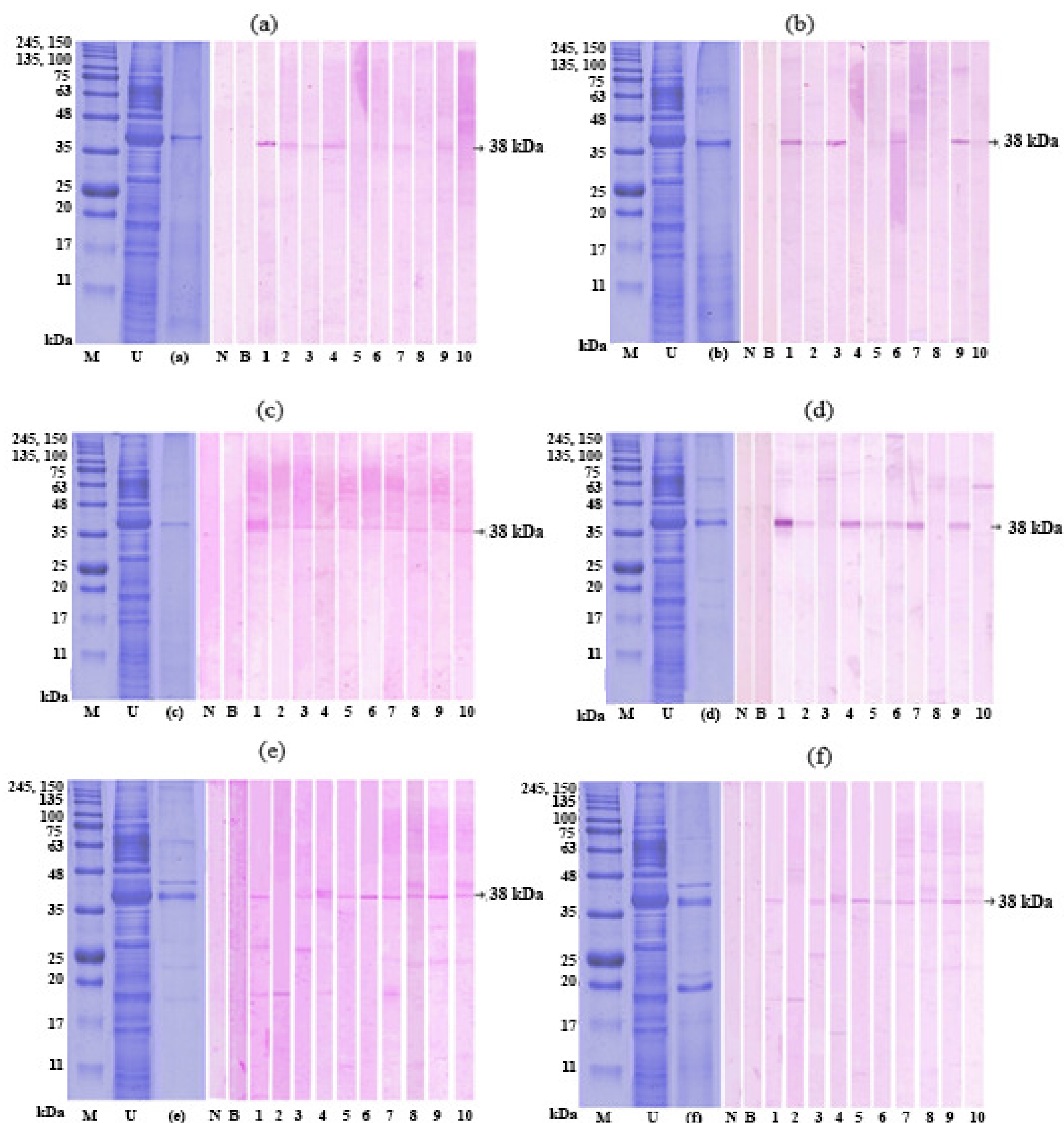

Figure 3. Immunoblotting results of six vinegar treated *Scylla tranquebarica* extracts; which are synthetic (a), pomegranate (b), grape (c), fig (d), apple (e), and date (f) using sera from 10 crab-allergic patients (lane 1 to 10). Lane M is molecular mass markers in kilodalton (kDa); lane U is an untreated extract; lane N is immunoblot; using a negative control serum and lane B is blank. Arrows indicated the molecular weight of major allergens in kDa.

Allergen Identification

Table 2 summarized the MALDI-TOF results of the major allergens of *S. tranquebarica*. The 1a spot at 38 kDa with pI of 4.7 showed a correlation with tropomyosin from *Paralithodes Camtschaticus*, with peptide sequence coverage of 26%. While, the 42 kDa major allergen with a pI of 7.0 (Spot 2a) was identical to arginine kinase from several species of invertebrates including *Ocypode ceratophthalmus (O. ceratophthalmus)*, *S. serrata*, *S. olivacea,* and *Homarus americanus (H. americanus)*, which corresponded to 11 to 26% sequence coverages. Similar to the 42 kDa,

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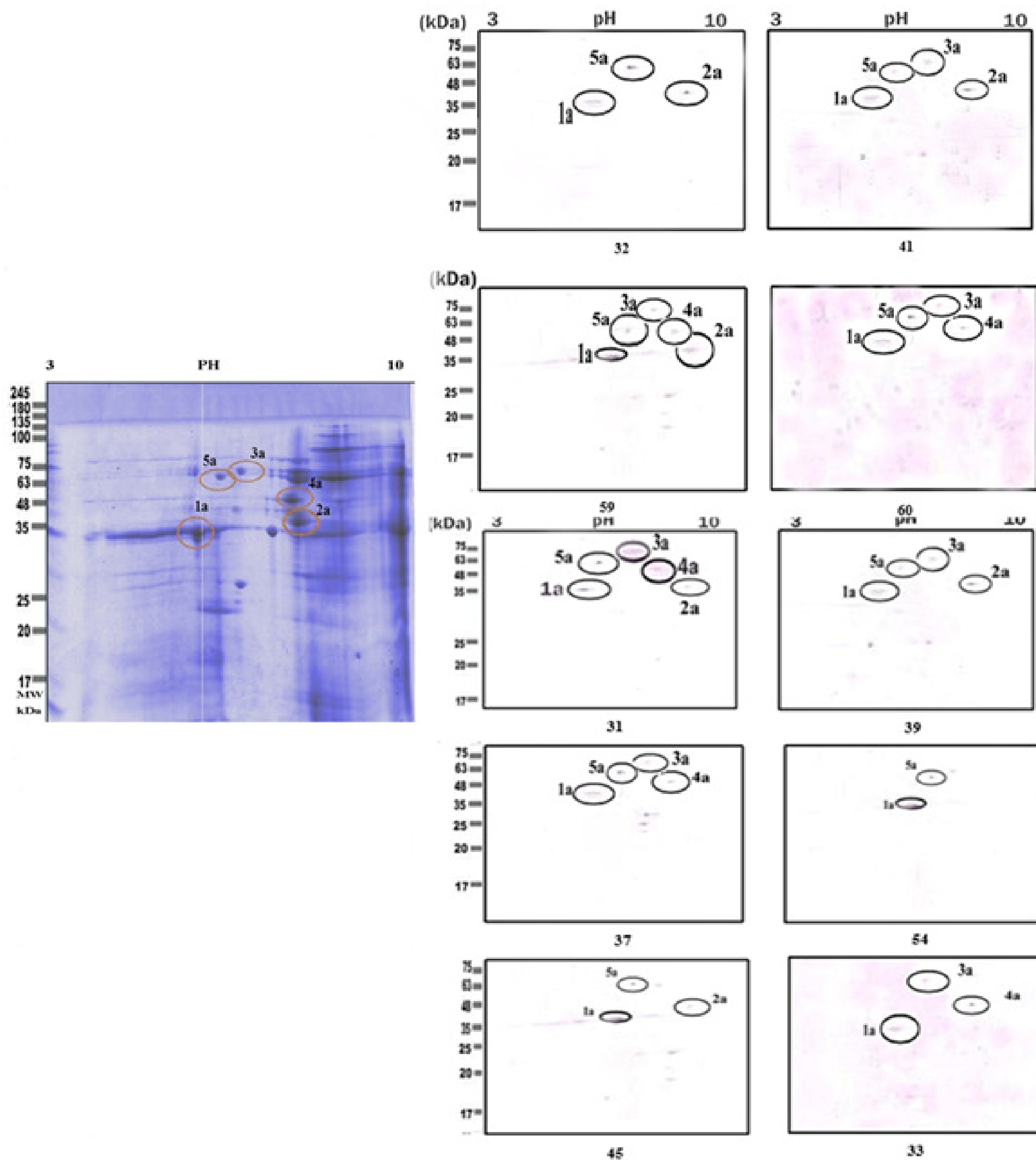

Figure 4. Two-dimensional gel electrophoresis profile (a) and immunoblot results of untreated *Scylla tranquebarica;* using 10 sera (Patients 31 to 60). Lane molecular weight (MW), MW markers in kilodalton (kDa). Circles indicate the major IgE-binding spots of major allergens.

the 4a spot at 49 kDa and pI of 6.5 was identical to arginine kinase from *S. paramamosain*, corresponded to 18% sequence coverage.

Meanwhile, the spot 5a at 63 kDa major allergen with a pI 5.0 was found to be identical to actin from several species of invertebrates including *S. paramamosain*, *S. serrata*, *S. olivacea,* and *H. americanus*, which corresponded to 11% sequence coverage. In this study, the mass spectrometry analysis of 73 kDa major allergen with a pI of 5.7 (spot 3a) of *S. tranquebarica* was identical to hemocyanin from *S. paramamosain* and *S. olivacea*, which corresponded to 12% sequence coverage.

Table 2. The result of mass spectrometry analysis of *Scylla tranquebarica*

Spot No.	MW (kDa, pI) Observed	MW (kDa, pI) Predicted	Protein Identification	Organism	Accession No.	Coverage of Protein sequence (%)	No of matched protein
1a	38, 4.7	37, 4.71	Tropomyosin	*Paralithodes camtschaticus*	A2V734	26	9
2a	42, 7.0	43, 7.03	Arginine kinase	*O. ceratophthalmus* *S.serrata* *S.olivacea*	AIT97437.1	26	6
3a	73, 5.7	73, 5.63	Hemocyanin	*S.paramamosain*	A0A0U1ZZP8	12	6
4a	49, 6.5	43, 6.65	Arginine kinase	*S.paramamosain*	CDO39252.1	18	4
5a	63, 5.0	63, 5.24	Actin	*S.paramamosain* *H.americanus*	XP_008839771.1 B6EAU2	11	3

DISCUSSION

This study found that the untreated *S. tranquebarica* extract has numerous visible protein bands with a complex protein pattern between 10 to 245 kDa. Multiple prominent protein bands were observed between 35 to 75 kDa. These findings are in line with our previous reports on local crab species.[23-26] Immunoblotting showed that 20 protein bands can bind to IgE antibodies from crab allergic patients, but only 5 protein bands at 38, 42, 49, 63, and 73 kDa were identified as the major allergens for *S. tranquebarica,* while the other IgE-binding proteins were revealed as only minor allergens as their IgE-binding frequencies were below 50%.

However, immunoblotting of SDS-PAGE protein bands only permits the recognition and not the identification of the IgE-binding components as the IgE binding bands may contain more than one protein.[27,28] Therefore, this study used 2-DE to further resolving the major allergenic bands for mass-spectrometry analysis. Over 50 distinct protein spots between molecular weights of 10 to 245 kDa were observed in *S. tranquebarica*, with pI from 3.0 to 10.0. These results showed that all of the major allergens were further fractionated to more than one spot by 2-DE, suggesting that the 2-DE separation method had resolved the protein bands to higher-resolution protein spots. Therefore, the relative amount of each protein can be determined and individual protein can be identified easily by immunoblotting technique.[28]

Mass spectrometry has successfully identified all the major allergens of *S. tranquebarica*. As expected, the 38 kDa major allergenic spot with pI of 4.7 was identified as tropomyosin, which showed a correlation with tropomyosin from *Paralithodes camtschaticus*. Tropomyosin had been widely claimed as the most significant major allergen that accountable for the allergenic and cross-reaction between various types of shellfish,[12,22-26] including in the local mud crab, *Scylla* spp.[29]Based on SDS-PAGE and immunoblotting results, the tropomyosin band was detected as the most prominent band, indicating that tropomyosin presents in crab tissues in high amount than other allergens.

Meanwhile, both the 42 and 49 kDa major allergens were identified as arginine kinase. The 42 kDa band was identical with arginine kinase from several species of invertebrates including *O. ceratophthalmus*, *S. serrata*, *S. olivacea,* and *H. americanus*, while the 49 kDa band was identical to arginine kinase from *S. paramamosain*. Arginine kinase, an enzyme is known as phosphokinase enzyme which regulates the level of ATP in the muscles of invertebrates[10,13] was commonly recognized as the notable allergenic protein in invertebrates including shrimp and crab.[6,10,12,13,23,24]

Meanwhile, the 63 kDa major allergen with a pI 6.5 of *S. tranquebarica* was found to be identical to actin from several species of invertebrates including *S. paramamosain*, *S. serrata*, *S. olivacea,* and *H. americanus*, which corresponded to 11% sequence coverage. Actin is a part of muscle cells in all eukaryotes, which is essential in muscle contraction[9] and has been increasingly identified as a significant allergen in both crustaceans and molluscan shellfish.[12,23,24]

While, the 73 kDa major allergen with a pI of 5.7 of *S. tranquebarica* was found to be identical to hemocyanin from *S. paramamosain* and *S. olivacea*,

which corresponded to 12% sequence coverage. Hemocyanin with a molecular weight range of 60 to 80 kDa was detected as a new major non-cross-reactive allergen in several species of prawns including giant freshwater prawn; *Macrobrachium nipponense* at 75 kDa and pI 5.42[30] and *Macrobrachium rosenbergii* at 65 kDa,[31] banana shrimp (*Fenneropenaeus merguiensis*) at 75 kDa and pI 5.42[32] and Lanchester's freshwater prawn (*Macrobrachium lanchesteri*) at 67 kDa[30]. To the best of our knowledge, this is the first report on the identification of allergenic hemocyanin in mud crab species. Hemocyanin, a hexamer of six identical subunits is a colorless copper-containing respiratory pigment in the blood plasma of various arthropods and mollusks including lobsters, octopuses, horseshoe, and crabs,[30-33] and is conserved among all arthropods.[33]

The stability of the protein profiles, major and minor allergens of *S. tranquebarica*in in low pH environments via synthetic and natural vinegar treatments was identified in this study. Compared to the untreated extract, the intensity of crab protein bands in all vinegar treated-extracts in SDS-PAGE gel became less visible. The intensity of the 38 kDa band (tropomyosin) in all vinegar-treated crabs was generally reduced. This agrees with other studies that also observed a reduction in band intensity of tropomyosin band in vinegar-treated shrimp extracts.[20,22] In this study, the most significant reduction of 38 kDa band was seen in the synthetic vinegar-treated crab, while the least affected was apple vinegar-treated crab. Based on the band intensity, the order of denaturation effect on the 38 kDa band was synthetic>date=fig> pomegranate>grape>apple vinegar treated crabs. This study showed that tropomyosin is highly stable in acidic environments, similar to other reports[20], most possibly due to its dimer structure of α-helical coiled-coil.[17-18,20]

Meanwhile, this study showed that the other protein bands were significantly reduced or disappeared after being treated with vinegar. Only three extracts; the fig, apple, and date vinegar-treated crabs demonstrated a weak 42 kDa major allergen band in SDS-PAGE gel. While, all higher molecular weight bands above 42 kDa disappeared in all treated extracts except for the 73 kDa which can still be seen in the grape, fig, apple, and date vinegar treated-crabs as a faint band. All lower molecular weight bands below 38 kDa were also not detected in all vinegar-treated crab except for the 23 and 24 kDa bands that were remained detected in the figure, apple, and date vinegar-treated crab. The reduction in the number and intensity of protein bands in the vinegar-treated extracts might be due to an alteration in the protein components after treatment with vinegar due to a partial loss of protein structures as the result of protein degradation in an acidic environment.[20-22] This study also indicated that different types of vinegar contributed to different effects on protein profiles of this crab as all vinegar have different pH values. Based on the effect on the protein bands, we found that the synthetic vinegar causes the most significant influence on protein profiles of *S. tranquebarica* than the other vinegar, while, among natural vinegar tested, date vinegar gave the weakest effect, while pomegranate vinegar can be said as the strongest natural vinegar.

Several studies reported that vinegar can decreases the allergenic responses in food allergy.[20-22,34,35] Similarly, our study also found that the number of IgE-binding protein components and band intensities of *S. tranquebarica* decreased in immunoblotting of all vinegar treated crabs compared to the untreated crab. Most of the patients showed only up to three IgE-binding bands in all vinegar treated-crab extracts, relatively very low when compared to immunoblotting of untreated extract. The reduction of the immunoreactivity may be caused by proteolysis of the antigenic proteins causing either blocking or eliminating the allergenic epitopes by acidic proteases in crab muscles, which was activated by vinegar. Hence, a dramatic reduction in IgE-binding responses had been shown in the crab treated with vinegar. This result is following other studies, which reported acid ingredients used as everyday food flavorings might have the possibility to decrease the allergenicity of several food allergens including shrimp,[20,22] egg,[22,33] lentil[20], and peanuts.[34-35]

However, more than 50% of the tested sera retained the IgE-binding to the 38 kDa major allergen band. This is because of the nature of this band, the tropomyosin, to retain its antibody reactivity even at the acidic condition, supporting the study by Lasekan et al.[20] which reported that the IgE-binding capacity of tropomyosin was significantly lower in the shrimp marinated at pH 1.0 to 3.5, compared to the control.[20] Another study also reported that the solubility of tropomyosin in the shrimp treated with vinegar decreased, which then triggering the reduction of IgE

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binding capacity of tropomyosin in the soluble extract.[22] However, analysis of the insoluble protein fraction indicated the retention of significant IgE-binding by tropomyosin following exposure to vinegar.[20,22] It was reported that shrimp tropomyosin can retain its allergenic capacity due to the conservation of its linear epitopes after being treated in low pH conditions.[20] Based on the band intensities, the crab treated with pomegranate, fig, and apple vinegar revealed higher IgE-binding intensities to the 38 kDa tropomyosin band than the other types of vinegar, suggesting that the reactions of this vinegar are slightly lower than the other vinegar tested.

This study revealed that the other major allergens at 42, 49, 63, and 73 kDa are sensitive to low pH environments, except in immunoblotting of some sera which can still detect a very weak 42 kDa band in the apple and date vinegar treated-crab extracts (sera No 8 and 10) and the 63 kDa in fig vinegar treated-crab extract (sera No 3 and 10) as faint bands, indicating that all these major allergens are sensitive to low pH treatments. This is not surprising as the pH of fig, apple, and date vinegar are 2.94, 3.0, and 3.35 respectively as shown in the result section higher than the pH of the other vinegar tested. Therefore, some of the major allergens could retain some of their allergenic epitopes in these acidic environments. The 42 kDa major allergen band was identified as arginine kinase, therefore, as an enzyme, this major allergen will be sensitive to low pH, as indicated in this study. Likewise, the other major allergens at 63 kDa and 73 kDa, identified as actin and hemocyanin, respectively were also sensitive to low pH. Actin and hemocyanin had only been previously reported as resistant to heat denaturation by other studies.[31,36] Thus, to the best of our knowledge, this is the first study that reported that actin and hemocyanin are sensitive to acidic environments.

In general, this study indicated that different types of vinegar contributed to a slightly different effect on crab allergenicity. Based on the number of allergenic bands, major allergens, and band intensities, we suggest the synthetic and grape vinegar causing the most significant impact on allergenicity than the other vinegar tested. The allergenicity of vinegar treated-crabs in decreasing order are as follows: fig>pomegranate>apple>grape>date>synthetic vinegar.

In conclusion, five major allergens at 38, 42 and 49, 63, and 73 kDa of *S. tranquebarica* were identified in the untreated *S. tranquebarica*. The 38 kDa was identified as tropomyosin, the 42 and 49 kDa were arginine kinase, and the 63 kDa was actin, while the 73 kDa was identified as hemocyanin, respectively. This study demonstrated that vinegar treatments; using all types of tested vinegar revealed a decrease in recognition of the allergenic protein bands but retained minimal IgE-binding at the major allergen bands. Based on this finding, we suggested that vinegar treatments without regard to the type of vinegar can be used to reduce the crab allergenicity. This study will be useful for clinicians in the management of crab allergic patients worldwide. However, clinical trials should be conducted to confirm these earlier studies' results.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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