

Allergenicity potential of protein extract from freshwater and saltwater fish based on heat stability and antibody-binding frequency

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Abstract

Background: Saltwater fish are associated with more allergic reactions compared to freshwater fish. However, the factors contributing to this difference remain unclear.

Objective: To compare the heat stability of freshwater and saltwater fish proteins, and assess their binding affinity to allergen-specific antibodies.

Methods: Protein extracts were isolated from saltwater fish— *Selar crumenophthalmus*, *Euthynnus affinis*, *Ambassis urotaenia*, and freshwater fish i.e., *Rasbora argyrotaenia*, *Monopterus albus*, and *Poecilia reticulata*. Protein extract from *Penaeus monodon* served as a standard allergen source. Both raw and heat-treated protein extracts were subjected to SDS-PAGE analysis. The number of protein bands, their molecular sizes, and intensities were evaluated. Protein binding frequencies to anti-tropomyosin antibodies and IgE-containing serum from allergic patients were measured using ELISA.

Results: The *P. monodon* protein extract < 100 kDa demonstrated heat stability, while *A. urotaenia* proteins < 40 kDa were also heat-stable. Raw protein extracts from *R. argyrotaenia* and *M. albus* exhibited binding frequencies to anti-tropomyosin IgG of $28.18 \pm 1.05\%$ and $14.79 \pm 0.91\%$, respectively. In saltwater fish, raw protein extracts from *A. urotaenia* and *S. crumenophthalmus* showed binding frequencies of $61.74 \pm 1.87\%$ and $34.68 \pm 1.39\%$, respectively. Freshwater and saltwater fish heat-treated protein extracts displayed binding frequencies below 10%. All heat-treated protein samples exhibited higher binding frequencies to polyclonal IgE in patient sera compared to their raw counterparts.

Conclusion: Proteins smaller than 20 kDa exhibit significant heat stability. Raw protein extracts show higher binding frequencies to monoclonal IgG against crustacean tropomyosin, while heat-treated samples have increased binding frequency to IgE-containing human serum.

Key words: Allergen, freshwater fish, heat-stable protein, IgE, cross-reaction

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Introduction

Allergenic proteins are typically small in size (less than 70 kDa) and are resistant to heating and digestion processes.¹ Allergens are widely distributed across various sources, including pollens, dust mites, insect venoms, foods, and drugs.² Food allergens are diverse in origin, encompassing peanuts, tree nuts, milk, eggs, fish, shellfish, soy, and wheat.² Seafood allergy is among the most common food allergies, with the prevalence of fish and shellfish allergies estimated at 0.2%–0.3% and 0.6%, respectively.³ Allergens also exhibit high frequencies of cross-reactivity; for instance, tropomyosin from dust mites can cross-react with IgE antibodies that recognize shrimp tropomyosin.⁴ Individuals with crustacean allergies often experience cross-reactions to various types of shellfish and mollusks, such as gastropods (abalones, snails), bivalves (clams, oysters), and cephalopods (squid, octopus).³

Fish and shrimp are among the most widely consumed food allergens.⁵ Commonly consumed freshwater fish include tilapia and eel, while saltwater fish such as tuna, grouper, and anchovies are also frequently consumed.⁶ The distribution of allergens between marine and freshwater fish is not equal. Despite their higher consumption rates, saltwater fish are associated with a greater number of allergic cases compared to freshwater fish.^{7–9} Food challenges positive reactions to raw *P. monodon* and *M. rosenbergii* were found in 14.63% and 4.35% of cases, respectively. The most common symptoms observed during these challenges occurred in the skin-mucosal system (95.65%).¹⁰

Dorney et al., demonstrated variations in the abundance of allergen types across Australia, China, India, and Indonesia.¹¹ For example, tropomyosin expression was found to be lowest in *P. monodon* grown in Australia.¹¹ Sarcoplasmic calcium-binding protein, myosin light chain, and tropomyosin were identified as the most abundant allergens across all locations.¹¹ An earlier study by Ahlholm (1998) showed that mountain birch pollen consistently expressed similar levels of allergenic proteins. However, specific IgE binding in birch-allergic patients varied, with higher IgE binding observed in pollen from trees grown in gardens with higher mean daily temperatures.¹² These studies suggest that environmental conditions can influence the expression levels of allergenic proteins.

This study aims to investigate food allergenicity, specifically focusing on the number of proteins in the protein extract (as counted by appearance of protein band in SDS-PAGE), their heat stability, and their cross-reactivity with IgE from shrimp-allergic patients. This study compares these characteristics in total protein isolates from freshwater and saltwater fish samples.

Materials and Methods

Protein samples were isolated from saltwater fish species *Selar crumenophthalmus* (locally known as selar), *Euthynnus affinis* (mackerel tuna), and *Ambassis urotaenia* (anchovy)—as well as freshwater species—*Rasbora argyrotaenia*

(locally known as wader), *Monopterus albus* (eel), and *Poecilia reticulata* (locally known as cetol). Protein isolates from *Penaeus monodon* (giant tiger prawn) were used as a standard of potentially allergenic protein. These fish are commonly consumed in Indonesia. Fish were obtained from Yogyakarta (Indonesia), and species were identified at the Animal Systematics Laboratory, Faculty of Biology, Universitas Gadjah Mada (UGM), with statement letter No. 60/BI/SH/1/2023.

Protein isolation

Total protein was isolated using the PRO-PREP™ protein isolation kit (Intron Biotechnology, South Korea) following the manufacturer's protocol. The protein was extracted from the whole body of *P. reticulata*, or the dorsal muscle of the other samples. A 250 mg sample in 2 mL of protein isolation buffer was homogenized at 10,000 rpm, followed by incubation at -20°C for 30 minutes. The homogenized samples were then centrifuged at 13,000 rpm, 4°C for 5 minutes. Supernatants are then subjected to total protein content determination using the Bradford method. Briefly, 5 μL of supernatant was mixed with 250 μL of Bradford reagent (Merck, Germany), shaken for 5 minutes, and incubated at 25°C for 10 minutes. The color intensity was read at 595 nm.

Protein electrophoresis

Protein extracts from each sample were divided into: 1) non-heated (raw) and 2) heated groups (samples heated for 20 minutes at 100°C in a water bath). Before loading into the SDS-PAGE gel, all samples (both raw and heated) were mixed with a sample buffer containing 0.5 M Tris-HCl pH 6.8 (Vivantis, Malaysia), 70% glycerol, 10% SDS, 8% 2-mercaptoethanol, and 1% bromophenol blue (Merck, Germany). The sample-to-buffer ratio was 2:1 (v/v), and the mixture was denatured at 100°C for 5 minutes. Six to seven microliters of protein samples (~ 2 mg/mL) and 6 μL of a protein marker (10–245 kDa, PM5100, ExcelBand™ SMOBIO) were used for electrophoresis. Acrylamide/bisacrylamide and Tris-HCl, components of the polyacrylamide gel, were obtained from Bio-Rad (USA) and Vivantis (Malaysia), respectively. The stacking gel contained 5% acrylamide/bisacrylamide and 0.5 M Tris-HCl (pH 6.8), while the separating gel contained 12% acrylamide/bisacrylamide and 1.5 M Tris-HCl (pH 8.6). Other components of the stacking and separating gels include 0.1% sodium dodecyl sulfate (SDS) (Merck, Japan), 0.1% ammonium persulfate (Sigma-Aldrich, USA), and 0.04% N,N,N',N'-tetramethylethylenediamine (Merck, Germany). Electrophoresis was run for 90 minutes at 200 volts in a buffer containing 25 mM Tris-base (Vivantis, Malaysia), 250 mM glycine (Merck, USA), and 1% SDS (Merck, Japan). Then, the gel was imaged and the intensities of the protein bands were analyzed using Gel Analyzer 19.1 software.

Protein extract binding frequency to anti-shrimp-tropomyosin antibody

Protein binding to anti-shrimp-tropomyosin monoclonal antibody were analyzed using the Sandwich ELISA (Shrimp Tropomyosin ELISA 2.0 system, InBio, USA). The kit includes a rabbit polyclonal antibody (IgG) and a mouse monoclonal IgG (as a capture antibody), both specific to shellfish tropomyosin. Protein samples (2 mg/mL) were mixed with assay buffer at a sample-to-assay buffer ratio of 10:90. Ten ng/mL of tropomyosin was used as a standard. The binding frequency was calculated as follows:

$$\% \text{ binding frequency} = \frac{\text{sample concentration}}{\text{shrimp raw protein concentration}} \times 100\%$$

Protein extract binding frequency to IgE-containing allergic patient serum

Blood serum of shrimp-allergic patients was also used to assess the binding frequency of protein extracts. This experiment was approved by the Medical and Health Research Ethics Committee (MHREC), Faculty of Medicine, Public Health, and Nursing, UGM (FK-KMK UGM), under decision number KE/FK/0136/EC/2023. All the volunteers were given informed consent before they participated in the study. Three allergic respondents and three non-allergic respondents were selected as candidates for serum collection. Ten ml of blood was collected from each respondent, and the samples were stored at 25°C for 60 minutes to allow clot formation. The solution was then separated and centrifuged at 1,250 g for 20 minutes. Total IgE levels were measured using the Human IgE ELISA Kit (Bethyl Laboratories, TX, US, E88-108), following the manufacturer's protocol. The serum with the highest IgE level was used for further experiments. To assess protein binding frequency, 100 µL of raw and heated total protein samples (1 mg/mL) were diluted in dilution buffer at a 53:32 µL ratio. Then, immobilized on a 96-well plate and incubated overnight at 4°C. The plate was then blocked with 5% skim milk in TBST (10 mM Tris-buffered saline, 0.01% Tween 20, pH 8.5) and incubated for 90 minutes at 30°C. After washing the plate with TBST, add 100 µL of patient serum to the immobilized protein and incubate for another 90 minutes at 30°C. After washing, add 85 µL of anti-IgE detection antibody and incubate for 60 minutes at 25°C. Added detection solution (85 µL) containing horseradish peroxidase, followed by incubation for 30 minutes at 25°C. Add TMB solution, incubate for 30 minutes at 25°C, and protect from lights. After adding the stopper solution, the optical density (OD) was recorded at 450 nm. The positive cut-off value for the sample was determined as OD > mean OD of the blank + 3SD (standard deviation). Positive results indicated significant binding between the protein extracts and the serum IgE. The OD values of the samples were converted to protein concentration, which was calculated as tropomyosin protein by referencing the tropomyosin standard.

Data analysis

The data obtained were analyzed using both descriptive and quantitative approaches. Protein intensities are presented as the mean ± standard error of the mean (S.E.M.). The total number of protein bands observed after electrophoresis is expressed as a percentage, represented as mean ± S.E.M. The binding frequency of protein extracts to anti-tropomyosin monoclonal antibodies and anti-shrimp protein antibodies from human serum (polyclonal antibodies) is also reported as mean ± S.E.M. from triplicate experiments. Statistical differences between groups were assessed using a *t*-test, performed in Python.

Results

Protein extract size and heat stability profile

Electrophoresis revealed protein bands from both raw and heated extracts, with sizes ranging from 245 kDa to approximately 10 kDa (Figure 1). The raw protein extracts from freshwater fish (Figure 1 A-1, A-7, B-6) exhibited a greater number of protein bands and higher intensities compared to their heated counterparts (Figure 1 A-2, A-8, B-5). In the case of *M. albus*, the raw extract (Figure 1 B-6) displayed 14 protein bands. While the heated sample showed only 6 bands (43%), with protein sizes around 48 and 36 kDa, and four additional proteins below 10 kDa, all in diminished band intensities (Figure 1 B-5). The changes in protein band intensity between raw and heated samples are illustrated in Figure 2. A similar pattern was observed across all freshwater fish samples, where the proportion of heat-stable proteins ranged from 39% to 50%. Protein smaller than 70 kDa constituted 27% to 42% of the heat stable protein. The protein percentage was calculated after the gel intensity readout.

The raw protein extracts from saltwater fish (Figure 1 A-3, A-9, B-4) exhibited a higher number of protein bands and greater intensities compared to the heated protein extracts (Figure 1 A-4, A-10, B-3). For *S. crumenophthalmus*, the raw extract (Figure 1 A-3) contained 17 protein bands, whereas the heated sample retained only 9 bands (53% of that raw extract). A reduction in band intensity was also observed in the heated samples, particularly for proteins sized between 6 and 51 kDa (Figure 1 A-4).

Similar patterns were observed in other saltwater fish samples. Protein bands that appeared exclusively under heated conditions were detected at 69 kDa in *A. urotaenia* and at 165, 146, 72, and 16 kDa in *E. affinis*. The percentage of heat-stable proteins in saltwater fish ranged from 65% to 85%, with heat-stable proteins smaller than 70 kDa constituting 47% to 60% of the total. The protein percentage was made after gel intensity readout.

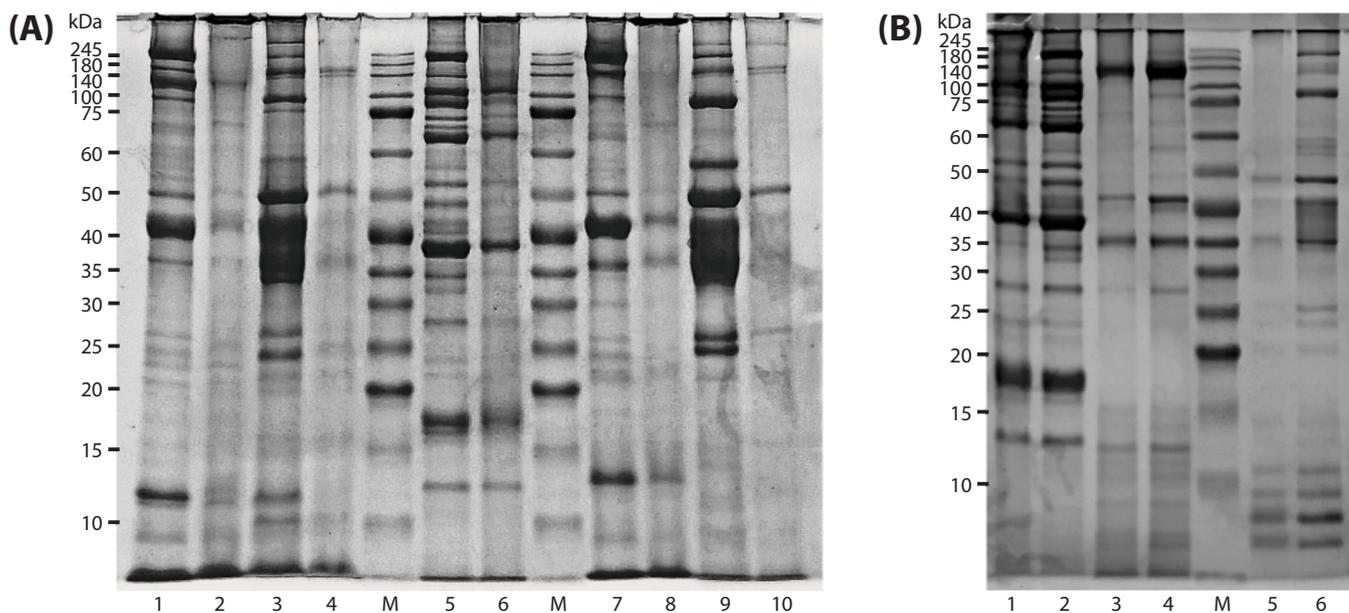


Figure 1. SDS-PAGE profile of total protein extract. (A) Protein extract from *P. reticulata* raw (1) and heated (2); *S. crumenophthalmus* raw (3) and heated (4); *P. monodon* raw (5) and heated (6); *R. argyrotaenia* raw (7) and heated (8); *E. affinis* raw (9) and heated (10). (B) Protein extract from *P. monodon* heated (1) and raw (2); *A. urotaenia* heated (3) and raw (4); *M. albus* heated (5) and raw (6). In panels (A) and (B), (M) is a protein-size marker.

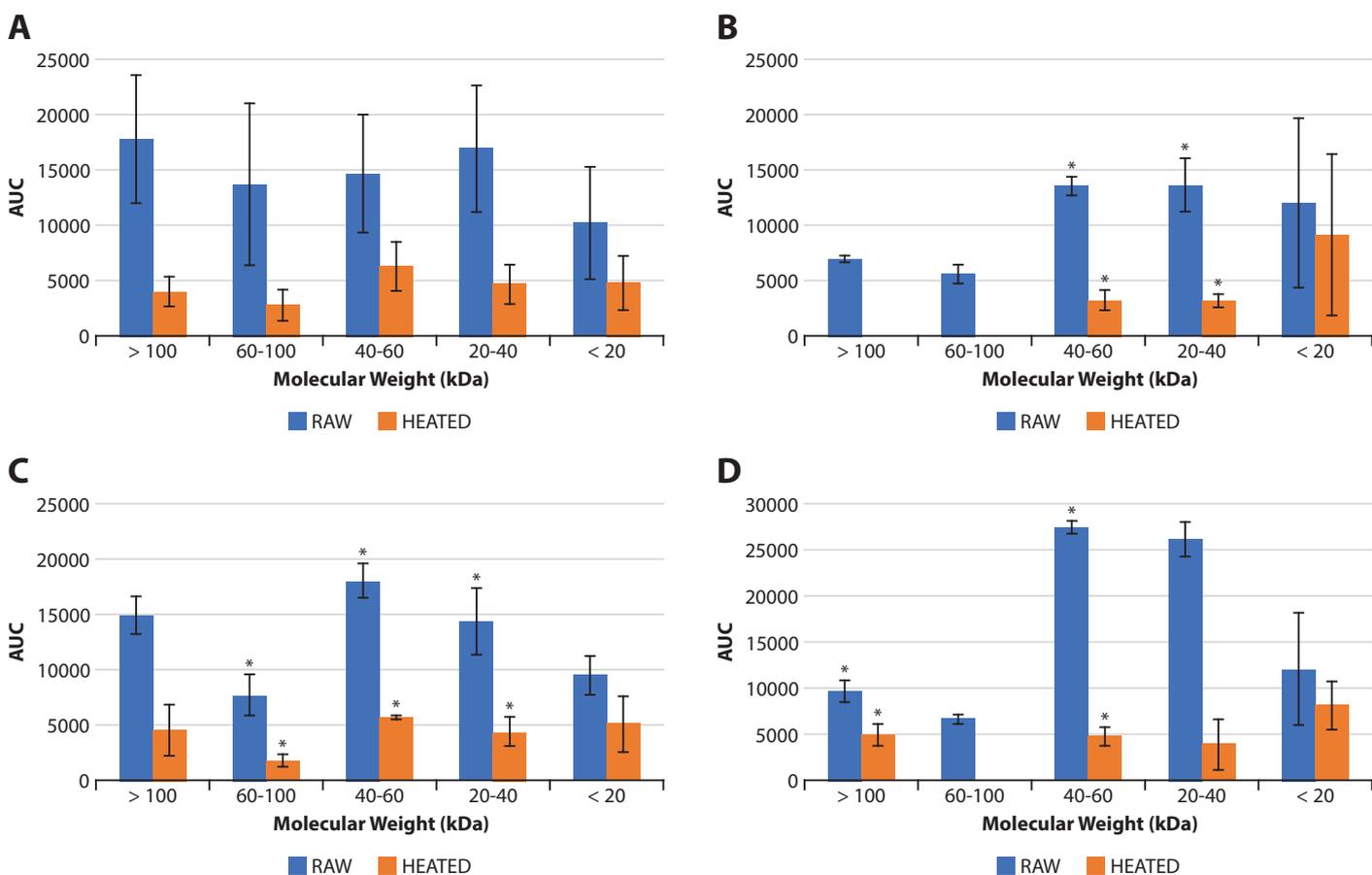


Figure 2. Protein band quantification using Gel Analyzer software. Panels A-G display the protein band quantification for *P. reticulata*, *M. albus*, *R. argyrotaenia*, *S. crumenophthalmus*, *E. affinis*, *A. urotaenia*, and *P. monodon*, respectively. Data are presented as the mean \pm SEM from 3 independent replicates. Significant differences between raw and heated samples are indicated by (*), with $p < 0.05$.

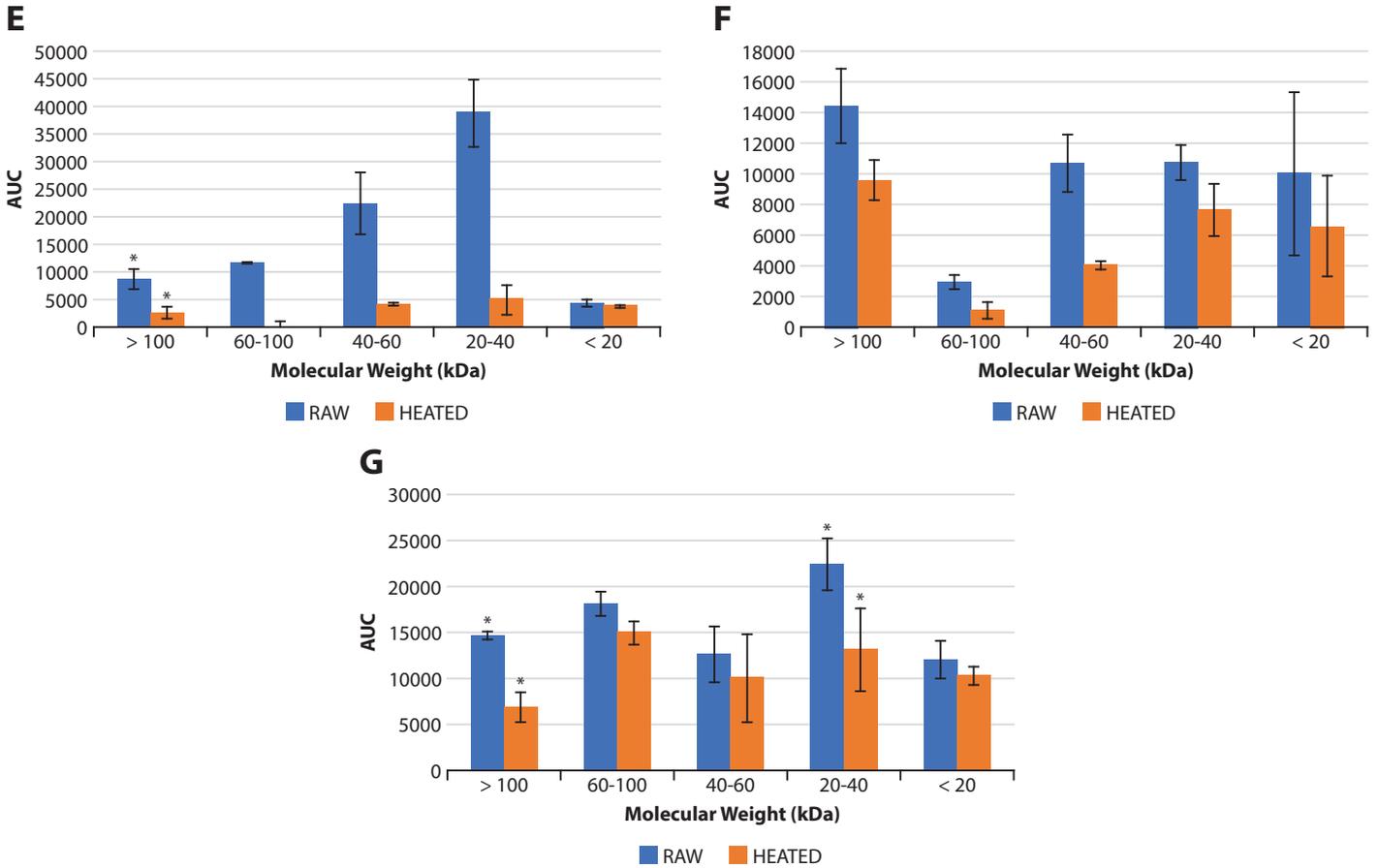


Figure 2. (Continued)

Binding frequency of protein extract against monoclonal anti-tropomyosin IgG

Each sample binds variously to anti-tropomyosin IgG (Figure 3). Since the IgG used in the assay specifically targets crustacean tropomyosin, the binding frequency of the raw shrimp total protein extract was set as the maximum binding frequency (100%). It is reported that crustacean and fish tropomyosin have 54-60% sequence similarities.¹⁸ Thus, it can be expected that the maximum binding frequency of anti-tropomyosin IgG to fish protein extract samples is lower than that of the binding frequency of anti-tropomyosin IgG to shrimp extract (binding frequency < 100%). The heating process results in a statistically insignificant increase in the binding frequency of the shrimp protein extract compared to the binding frequency of the raw shrimp protein extract.

Among saltwater fish, the raw protein extract of *A. urotaenia* showed the highest binding frequency at $61.74 \pm 1.87\%$, while the lowest binding frequency was observed in the raw extract of *E. affinis* at $1.49 \pm 0.05\%$ (Figure 3). Heating significantly reduced the binding frequency in *S. crumenophthalmus* (from $34.68 \pm 1.39\%$ to $3.09 \pm 0.23\%$) and *A. urotaenia* (from $61.74 \pm 1.87\%$ to $11.59 \pm 0.83\%$). In contrast, heating caused a slight, but statistically insignificant, increase in the binding frequency of *E. affinis* protein extract (Figure 3).

The raw protein extract of *R. argyrotaenia* exhibited the highest IgG binding frequency among the freshwater fish species, with a binding frequency of $28.18 \pm 1.05\%$ (Figure 3). In contrast, the lowest binding frequency was observed in the raw protein extract of *P. reticulata*, at $0.88 \pm 0.05\%$. Significant decreases in binding frequency were noted after heating in both *R. argyrotaenia* (from $28.18 \pm 1.05\%$ to $5.85 \pm 0.39\%$) and *M. albus* (from $14.79 \pm 0.91\%$ to $3.09 \pm 0.10\%$). Additionally, the binding frequencies of both freshwater and saltwater fish samples were significantly lower than those of the shrimp samples (Figure 3).

Binding frequency of protein extract against IgE-containing allergic patient serum (polyclonal IgE)

The shrimp allergic patient with the highest IgE level (1092.92 ± 69.01 ng/ml) was selected as the respondent for IgE isolation, while healthy donor serum served as the control. Unlike the binding frequency expressed as a percentage in Figure 3, the binding frequency of protein extracts against IgE-containing serum is presented as the concentration of proteins binding to the antibody. A higher protein concentration indicates a greater binding frequency. The binding frequency against the allergic patient serum is not expressed as a percentage, as the serum contains polyclonal antibodies, which have the possibility of different epitopes from the same allergen, and also a high potential

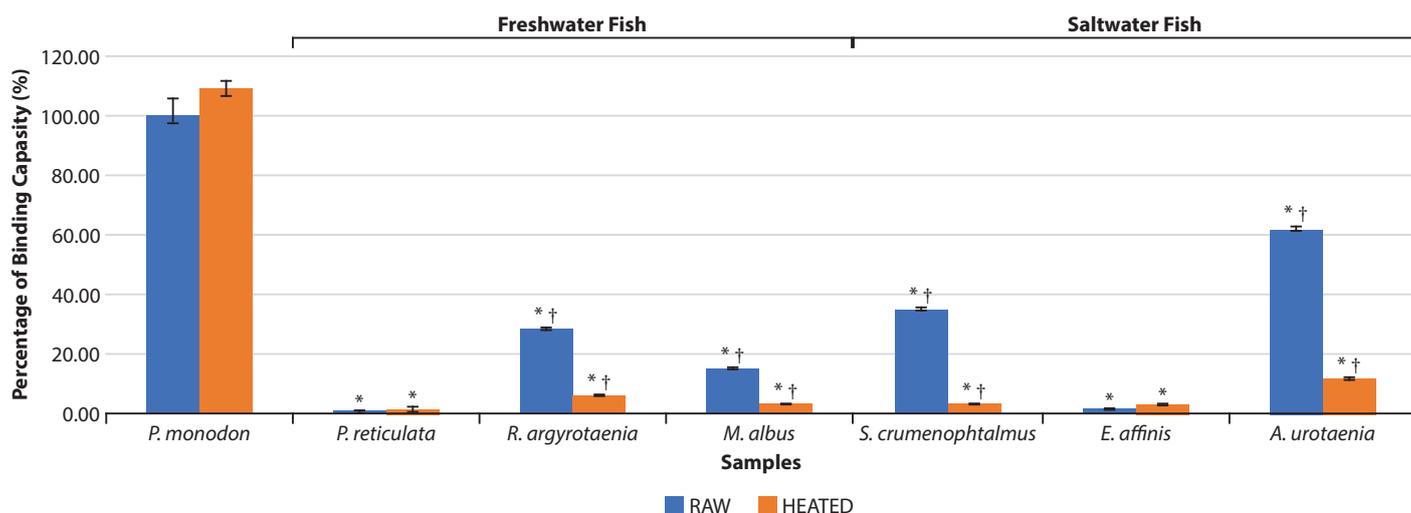


Figure 3. Binding frequency of freshwater and saltwater fish total protein extracts against monoclonal anti-tropomyosin IgG specific to shrimp. Significant differences are indicated between samples and raw shrimp ($p < 0.05$, *), as well as between raw and heated extracts of the same protein ($p < 0.05$, †). Data are presented as mean \pm SEM from 3 independent replications.

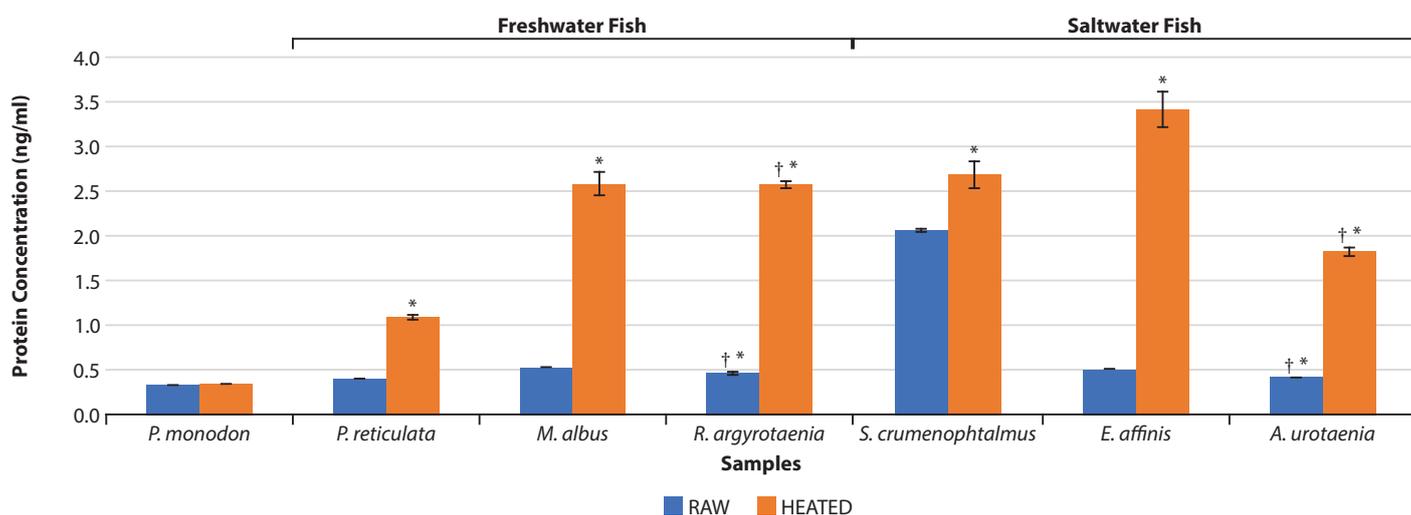


Figure 4. Protein concentration is the binding frequency of freshwater and saltwater fish total protein extracts against IgE-containing serum from allergic patients. Significant differences between samples and raw shrimp extracts are indicated ($p < 0.05$ (*)), and significant differences between raw and heated samples of the same protein extract are also indicated ($p < 0.05$ (†)). Data are presented as mean \pm SEM from three replications.

for cross-reactivity with the other allergens. Therefore, converting to a percentage relative to a particular sample may not be appropriate.

Among the saltwater fish species in this study, *E. affinis* heated protein extract exhibited the highest binding frequency to the IgE-containing serum, with a protein concentration of 3.42 ± 0.35 ng/ml (Figure 4). Conversely, the raw protein extract of *A. urotaenia* demonstrated the lowest binding frequency within the saltwater group, with a protein concentration of 0.39 ± 0.01 ng/ml (Figure 4). While heating generally increased the binding frequency across all samples, the changes were not statistically significant, except for *A. urotaenia*, which showed a notable increase from 0.41 ± 0.02 ng/ml to 1.82 ± 0.09 ng/ml (Figure 4).

In the freshwater fish species, the highest binding frequency was observed in the heated protein extract of *M. albus*, with a protein concentration binding to serum antibodies of 2.58 ± 0.23 ng/ml. Conversely, the lowest binding frequency was exhibited by the raw protein extract of *P. reticulata*, with a protein concentration of 0.39 ± 0.01 ng/ml binding to serum antibodies. A significant increase in binding frequency following the heating process was noted for *R. argyrotaenia*, which rose from 0.45 ± 0.03 ng/ml to 2.57 ± 0.07 ng/ml (Figure 4). Unexpectedly, the binding frequency of both raw and heated shrimp protein extracts was found to be lower compared to that of marine and freshwater fish (Figure 4).

Discussion

Despite generally lower consumption rates compared to marine products, reports of allergic reactions caused by freshwater organisms remain limited. *M. rosenbergii* (freshwater shrimp) has been identified as an allergen source, with only two main allergenic proteins reported: Mac r 1 and Mac r 2.¹³ In contrast, saltwater shrimp are more commonly associated with allergy, with multiple allergens, including Pen m 1, Pen m 2, and Pen m 3 from *Penaeus monodon*; and Cra c 1, Cra c 2, and Cra c 4 from *Crangon crangon*.¹³

Freshwater shrimp, such as *M. rosenbergii* and *M. lanchesteri*, can bind to IgE from individuals with crustacean allergies.¹⁴ The amino acid sequence of tropomyosin in both shrimp species is highly conserved among crustaceans.¹⁴ A study on the salmon species *Oncorhynchus nerka* revealed that freshwater salmon protein pattern and IgE-binding frequency are similar to that of red salmon inhabiting saltwater and river.¹⁵ Comparative studies also highlight the relative abundance of allergenic proteins between different species or among individuals of the same species living in varying conditions.^{11,12,14}

The strong heat stability of *P. monodon* protein extracts sized below 100 kDa may correlate with the high incidence of *P. monodon* allergy. Particularly, sarcoplasmic calcium-binding protein, myosin light chain, and tropomyosin are among the shrimp allergens that are heat stable and sized below 100 kDa.¹⁶ Among the fish samples, *R. argyroteenia* exhibits significant heat stability, with proteins sized below 100 kDa (excluding the 40-60 kDa range, which is heat labile). Heat-stable protein sized below 20 kDa that exist in *S. crumenophthalmus*, *P. reticulata*, *E. affinis*, *A. urotaenia*, *M. albus*, protein extracts could be parvalbumin (size 10-13 kDa), a major allergen in fish.¹⁷ Heat-labile properties of proteins sized between 20-100 kDa across samples suggest their limited contribution to allergenicity, or they are not the major allergenic proteins. Many allergenic proteins in both freshwater and saltwater fish fall within this size range, such as aldolase (~40 kDa), β -enolase (~50 kDa), and triosephosphate isomerase (28 kDa).^{16,18} Despite the different species analyzed for heat stability, the environmental conditions can influence heat resistance, protein structure, and allergenicity characteristics of the proteins from each fish. Previous studies have shown that the diversity of parvalbumin isoforms among various fish species impacts both heat resistance and allergenicity.¹⁹

Due to difficulties in obtaining appropriate samples for antibody binding experiments specific to each fish species, the experiments were conducted using IgG against shrimp tropomyosin and serum from shrimp-allergic patients. *P. reticulata* and *E. affinis* did not bind to anti-shrimp-tropomyosin IgG. Whereas, raw protein extracts from other samples cross-react with this IgG. The conformational epitope is crucial for the allergens binding to anti-tropomyosin IgG, and previous studies have highlighted the significance of IgE binding and cross-reactivity.²⁰ Raw protein extracts in this study may similarly have cross-reactive epitope to anti-shrimp-tropomyosin IgG.

Heating that represents cooking processes is vital for disrupting the epitopes that interact with IgG. Cooking decreases the allergenicity of food proteins.²¹

The heating process can modify epitope conformation by damaging or changing the protein structure. This process can expose hidden epitopes in a linear structure, thereby altering allergenicity. Newly exposed epitopes contribute to increased IgE binding frequency in allergens like *M. tuna*.²² For other allergens, disruption of conformational epitopes can lead to diminished IgE binding frequency. Levels of tropomyosin were higher under various food processing conditions (boiled, grilled, and steamed) compared to raw conditions in *L. vannamei*.²³ Similarly, the heating processes of steaming, frying, and grilling decreased the immunoreactivity of tropomyosin in tilapia (*Oreochromis niloticus*), while not affect the tropomyosin of grouper (*Epinephelus coioides*).²⁴ Amino acid sequence variations result in different thermal stabilities (melting points), indicating that the heating process can significantly influence IgE binding frequency.^{25,26}

Antibodies present in allergic patient serum are naturally polyclonal. Polyclonal antibodies recognize more epitopes than monoclonal antibodies, which potentially leads to cross-reactivity not observed with a monoclonal antibody. This condition is observed when investigating the protein extract binding to anti-tropomyosin antibody and IgE containing patient serum. When using a monoclonal antibody (IgG against tropomyosin), the binding capacity of the antibody with protein extract is higher in the shrimp protein extract group. However, when IgE-containing allergic patient serum was used, the shrimp protein extract group did not bind to the largest amount of IgE. In addition to the polyclonal nature of serum antibodies, a higher number of allergenic proteins in fish compared to shrimp could also contribute to the more binding of IgE-containing serum to fish protein extract. Fish contained more allergenic protein compared to shrimp.^{3,7}

The unexpected cross-reactivity of raw protein extract samples to monoclonal antibodies against tropomyosin, as well as the binding of heated protein extract samples to allergic patient serum, indicates the ability of allergic patients IgE binds to extracted protein. However, it remains unclear whether this binding is primarily due to the abundance of the proteins or protein structure that enhances the avidity and affinity of antibodies to the allergenic proteins.

Cross-reactivity occurs due to shared epitopes of the allergens.²⁶ The sequence- or structural homology and physicochemical stability contribute to this phenomena.²⁶ Several fish species used in this study belong to the superclass Actinopterygii, which have the potential for cross-reactivity with shrimp.²⁷⁻²⁸ The structural similarity of B-cell epitopes predicts potential cross-reaction between shrimp tropomyosin and fish or shellfish proteins.²⁹ Shrimp tropomyosin has less than 20% structural similarity to fish tropomyosin.²⁹ While other studies report a 54-60% similarity between crustacean and vertebrate (fish) tropomyosin.¹⁸

P. monodon protein extract is the only sample that demonstrates high heat stability. These results correlated with the high incidence of allergic reactions associated with *P. monodon*. The low binding frequency of *P. monodon* protein extract to IgE-containing serum from allergic patients is still elusive. Furthermore, the heated protein extracts exhibit greater binding to the IgE of allergic patients than the raw protein extracts is also intriguing. The heating process notably increased the intensity of IgE binding for protein isolates from *M. rosenbergii*, *Cherax quadricarinatus*, *Procambarus clarkii*, and *M. lanchesteri*.¹⁴ Further studies are necessary to elucidate our findings.

The lack of specific antibodies to identify particular allergenic proteins (as there are no specific antibodies for some of the species used in this study) is the main limitation of this study. This condition precludes a precise determination of the allergenic protein investigated. Another limitation arises from the use of different ELISA systems for investigating protein binding frequency to antibodies. Investigation with allergic patient serum-containing IgE was performed with direct ELISA which is less specific than the sandwich ELISA system that is used for protein-antibody binding of antibodies against tropomyosin. The difference between direct and sandwich ELISA applied in this study could result in different binding specificity between antibody and protein extract.

This study demonstrates that proteins sized below 20 kDa, whether from saltwater or freshwater fish, are relatively heat stable. This protein is highly possible as parvalbumin, the major allergen from fish.¹⁸ Further investigation into protein expression, structural diversity, and allergic sensitization among allergenic proteins from fish inhabiting different environments—such as marine and freshwater—might explain why certain proteins are more allergenic than others.

Conflict of interest

None

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Author contributions

- AHE, ZA, and MNAS conceived the research, conducted the research, analyzed the data, and wrote the manuscript
- BBAS conceived the research, and conducted the research
- IPS conceived the research and analyzed the data.

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