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CHARACTERIZATION THE ALLERGENICITY AND STABILITY OF ALLERGENS OF FRESHWATER SNAIL, *Pomacea canaliculata*

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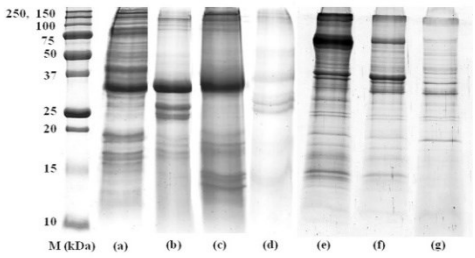
Graphical abstract



Abstract

Snail allergy is considered a serious form of food allergy with prevalence of 1.4 to 22% worldwide. However, allergy to Pomacea canaliculata, a commonly consumed local snail has not been well-described. Hence, this study aimed to characterize the allergenicity and stability of the allergenic proteins of P. canaliculata by proteomics approach. Snail flesh was treated with several thermal and non-thermal treatments prior to overnight protein extraction. The snail proteins were then subjected to SDS-PAGE, followed by immunoblotting, two-dimensional electrophoresis (2-DE), 2-DE immunoblotting and mass-spectrometry analysis. Raw snail demonstrated 31 protein bands between 10 to 250 kDa, with fewer protein bands in treated snails. Boiled snails had the most protein bands among thermally treated snails, while salted and dried snails showed more bands than pickled snails among non-thermal treatments. Immunoblotting of raw extract demonstrated 16 IgE-binding bands, with the 33 and 42 kDa protein bands were identified as the major. The 33 kDa allergen was highly stable to all treatments applied, while the 42 kDa was sensitive to thermal and pickling treatments. Fewer allergenic bands were present in treated snails, with allergenicity ranked as raw > boiled > roasted > fried for thermal treatments, and raw > salted > dried > pickled for non-thermal treatments. Mass spectrometry identified the 33 kDa and 42 kDa allergens as tropomyosin and actin, respectively. In conclusion, P. canaliculata has numerous allergenic proteins with varying stability. This result is essential in facilitating the enhancement of global strategy for diagnosis and management of snail allergic patients worldwide.

Keywords: *Pomacea canaliculata*, freshwater snail, tropomyosin, actin, proteomics

Abstrak

Alergi siput dianggap sebagai bentuk alahan makanan yang serius dengan prevalen antara 1.4 hingga 22% di seluruh dunia. Namun, alahan terhadap Pomacea canaliculata, siput tempatan yang biasa dimakan, belum banyak diterangkan. Oleh itu, kajian ini bertujuan untuk mencirikan alergenisiti dan kestabilan protein alergenik P. canaliculata menggunakan pendekatan proteomik. Daging siput dirawat dengan beberapa kaedah terma dan bukan terma sebelum pengekstrakan protein semalaman. Protein siput kemudian dianalisis menggunakan SDS-PAGE, diikuti oleh imunoblot, elektroforesis dua dimensi (2-DE), imunoblot 2-DE, dan analisis spektrometri jisim. Siput mentah menunjukkan 31 jalur protein antara 10 hingga 250 kDa, dengan sedikit jalur protein pada siput yang dirawat. Siput yang direbus mempunyai jalur protein terbanyak di antara siput yang dirawat secara terma, manakala siput yang diasinkan dan dikeringkan menunjukkan lebih banyak jalur daripada siput jeruk dalam rawatan bukan terma. Imunoblot dari ekstrak mentah menunjukkan 16 jalur pengikatan IgE, dengan jalur protein 33 dan 42 kDa dikenal pasti sebagai alergen utama. Alergen 33 kDa sangat stabil terhadap semua rawatan, manakala alergen 42 kDa sensitif terhadap rawatan terma dan penjerukan. Terdapat sedikit jalur alergenik pada siput yang dirawat, dengan tahap alergenisiti disenaraikan sebagai mentah > rebus > panggang > goreng untuk rawatan terma, dan mentah > masin > kering > jeruk untuk rawatan bukan terma. Spektrometri jisim mengenal pasti alergen 33 kDa dan 42 kDa sebagai tropomiosin dan aktin. Kesimpulannya, P. canaliculata mempunyai banyak protein alergenik dengan kestabilan yang berbeza-beza. Hasil ini penting untuk meningkatkan strategi global bagi diagnosis dan pengurusan pesakit alahan siput di seluruh dunia.

Kata kunci: Pomacea canaliculata, siput air tawar, tropomiosin, actin, proteomik

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1.0 INTRODUCTION

The prevalence of allergic reactions after ingestion of snails was reported in 1.4 to 22% of food allergic patients worldwide [1-5]. This allergy is among the most serious forms of food allergy, especially among house dust mite allergic patients with severe episodes of asthma [6-7]. Severe asthma was described as the predominant symptom in some of the snail allergic patients [6-10]. Systemic symptoms such as anaphylactic shock have also been documented [8, 11, 12]. However, mild to moderate symptoms including urticaria, eczema, and pruritis have also been reported after snail consumption [1, 7].

Several reports of snail allergy were documented in many countries such as France, Spain, Italy and Portugal where snails such as the *Helix pomatia*, *Helix aspersa*, *Helix terrestre*, *Bolinus brandaris, Cernuella virgate* and *Theba pisana* were commonly ingested by local population as exotic protein sources [7, 8, 13, 14]. Recently, snail allergy has also been described in Malaysia to local snails, *Cerithidea obtusa* and *Pomacea canaliculata* as those species are commonly consumed by local people [1, 15, 16].

Tropomyosin, a 34 to 38 kDa muscle protein has been described as the major allergen in shellfish including snails [13, 14, 17-22], such as *T. cornutus* [13] and *H. aspersa* [14]. This allergen which is associated with troponin complex in muscle contraction was declared as a cross-reactive allergen (pan-allergen) among invertebrates [13, 23-25]. Tropomyosin was described as a highly thermal and chemically stable protein which resist extreme pH and temperatures [18, 26-32].

Boiling, roasting and frying are among the common thermal treatments in food preparation and processing before consumption [15, 26, 28, 33-38], while drying, salting and pickling are also applied as non-thermal treatment methods mainly to preserve the food quality [39-50]. These treatments are furthermore subjected for food safety purposes, to increase the digestibility of proteins and enhance the food texture and taste. However, those treatments were also described as a potential approach to increase or decrease food allergenicity due to modification of allergenic epitopes as the results of protein denaturation and degradation [21-22, 26-32].

Malaysia is rich in exotic seafood including the golden apple snail, *P. canaliculata,* locally named as 'siput gondang'. This snail is a freshwater snail which belongs to Ampullaridae family [51-54], and is frequently found in the rice field regions in Peninsular Malaysia [51-52]. This snail is a source of dietary proteins and minerals [51-52]. Previously, we have described the prevalence of allergy to *P. canaliculata* in Malaysia was 9% among local patients with allergic diseases [1], however its allergenicity has not been well-characterized [16]. Therefore, we conducted this study to characterize the allergenicity of *P. canaliculata* by proteomics approach. In addition, the stability of the allergenic

proteins was also evaluated after subjected to selected thermal and non-thermal treatments.

2.0 METHODOLOGY

2.1 Snail Allergen Extraction

Protein extracts from raw, thermal and non-thermal treated *P. canaliculata* were prepared from the snail muscles, referring to the method by other studies [11, 41, 44, 48]. The snail muscles were treated in three types of heat treatments; boiling at 100˚C for 20 minutes, roasting at 180˚C for 20 minutes in an oven and frying in vegetable oil at ~240°C for 15 minutes. In addition, the snail muscles were also subjected to three types of non-thermal treatments; salting in a salt solution containing 22.5% NaCl, sun-drying for 17 hours at temperatures ranging from 32.5 to 42.5°C and pickling in white vinegar (pH 2.4) overnight (16 hours). Each raw and heat-treated snail was blended and homogenized in phosphate-buffered saline (PBS), pH 7.2 with a ratio of 1:10 (weight/volume). After an overnight extraction at 4˚C in a shaking incubator, the extracts were centrifuged at 14,000 rpm and sterile-filtered using filter paper and a syringe filter. The extracts were then freeze-dried and stored at -20˚C. The content of total proteins in each extract was assessed using Bradford Protein Kit (Biorad, Hercules, CA, USA).

2.2 Sera Collection

For immunoblotting tests, this study used 20 stored sera from our previous study [1]. The sera were collected from patients with history of shellfish allergy and demonstrated positive skin prick test (SPT) to *P. canaliculata* extract (freshwater snail) [1] and positive ImmunoCAP test (Phadia AB, Uppsala, Sweden) with at least 0.35 IU/ml against snail allergen. For negative control, serum from an individual with healthy and non-allergic status was used. This research was approved by the Medical Research and Ethics Committee (MREC), Ministry of Health Malaysia.

2.3 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The protein profiles of all extracts were analyzed by SDS-PAGE by using the method previously described [15]. The protein extracts were reconstituted with distilled water and heated in a Laemmli buffer for 3 minutes at 97°C. A total of 10 µg per well of the treated protein samples were then separated at 120 mA for 45 min in SDS-PAGE gel containing 12.5% separating gels and 5% stacking gels using a Mini Tetra Cell System (Biorad, Hercules, CA, USA). Pre-stained molecular weight markers (Biorad, Hercules, CA, USA) were run along with the snail samples. After completion, the bands of snail proteins were stained in Coomassie brilliant blue R-250 solution, and the molecular weight of the protein bands were analyzed using Imaging Densitometer GS800 and Quantity One Software (Biorad, Hercules, CA, USA).

2.4 Immunoblotting

Immunoblotting tests were performed to identify the IgE-binding proteins using a method described previously by Rosmilah *et al*. [15]. After electrophoresis, the protein bands of *P. canaliculata* were electro-transferred using the Mini Transblot System (Biorad, Hercules, CA, USA) from the SDS-PAGE gel onto a 0.45 µm nitrocellulose membrane. The blotted protein bands were cut, washed in TTBS solution (tris-buffered saline (TBS) with 0.05% Tween 20), blocked in TBS containing 10% non-fat milk and probed with diluted individual patient's sera (each serum was diluted in TBS with 1:5 dilution ratio) as the primary antibody, overnight at 4°C. A total of 20 sera were used in immunoblotting of raw snail, whereas the immunoblotting of heat-treated snails was conducted using only five selected sera with specific IgE to at least one major allergen of *P. canaliculata* in immunoblotting of raw snail. The secondary antibody of 0.001% biotinylated goat-antihuman IgE (KPL, Gaithersburg, Maryland, USA) and streptavidin-conjugated alkaline phosphatase (Biorad, Hercules, CA, USA) were added to the membrane strips to complete the allergen-antibody interaction. Finally, the membrane strips were incubated in a solution of alkaline phosphatase substrate (BioRad, Hercules, CA, USA) to detect the IgE-binding proteins on the strips. Both blank and negative control strips were included in each immunoblotting test.

2.5 2-Dimensional Electrophoresis (2-DE) and Immunoblotting

The raw snail protein bands were further separated by 2-DE to resolve protein spots. Briefly, an immobilized pH 3-10 non-linear gradient strip (BioRad, Hercules, CA, USA) were rehydrated in 50 µg of the snail extract in rehydration buffer and focused using Protean IEF Cell (BioRad, Hercules, CA, USA) as the first dimension electrophoresis with 4 steps: 100 V for 1 minute, 250 V for 30 minutes, 4000 V for 2 hours and 4000 V for 10 000 v-hr. The separated proteins on the strip were then further separated by SDS-PAGE using the same method as mentioned above, stained with Coomassie brilliant blue R250, and then scanned by an imaging densitometer and PDQuest software. Finally, the IgE-binding spots were then identified by immunoblotting test as above using four selected sera with strong specific IgE to at least one major allergen of *P. canaliculata* in immunoblotting of raw snail. The number of sera was chosen based on their results in immunoblotting of raw snail and sera availability.

2.6 MALDI-TOF Analysis

Major allergenic spots of the major allergens were identified by MALDI-TOF MS after being digested by trypsin. The MALDI-TOF Spectra were then analysed and compared with Ludwig NR Database to identify the snail proteins using Matrix Science software (Mascot sequence matching).

3.0 RESULTS AND DISCUSSION

3.1 Protein Profiles of *P. canaliculata*

The total protein content in each extract of *P. canaliculata* ranged between 2.01 to 9.65 mg/mL. Overall, it was found that raw extract contained higher protein concentration compared to the treated extracts. This may be due to protein degradation during the treatment processes [31, 32].

Meanwhile, SDS-PAGE as shown in Figure 1 resolved the protein mixtures in raw and six treated extracts of *P. canaliculata* to numerous protein bands between 10 to 250 kDa. Raw extract of *P. canaliculata* expressed 31 protein bands between molecular weights of 13 to 250 kDa, with four noticeable bands at 33, 42, 74 and 250 kDa.

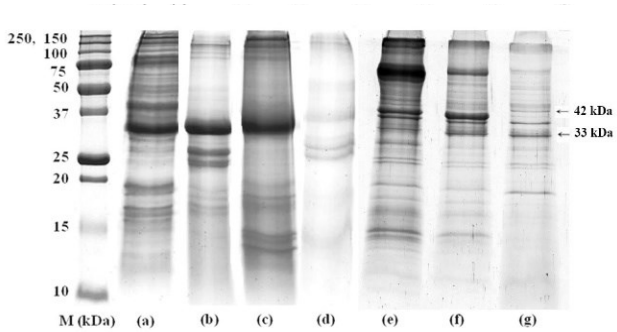

Figure 1 SDS-PAGE results showing the protein profiles of *Pomacea canaliculata*. Lane a, b, c, d, e, f and g are the raw, boiled, roasted, fried, salted, dried and pickled extracts, respectively. Lane M is the protein markers in kiloDalton (kDa). Arrows indicate the major allergens

All treatments induced alterations in the protein profiles of *P. canaliculata* by reducing the number of bands and band intensities. The protein bands in the raw snail were reduced to 8 to12 bands in the thermal-treated snails. Among the thermal-treated snails, the boiled snail retained the most protein bands (12 bands), followed by roasted (11 bands) and fried (8 bands). Meanwhile, the protein bands in the raw snail were reduced to 9 to 20 bands in the non-thermal-treated snails. The salted snail has the highest protein bands (20 bands), followed by dried (15 bands) and pickled (9 bands).

The fried and pickled snails presented the most significant decrease in the quantity and intensity of protein bands with the increase of smearing regions. This result is not surprising as, during the process of frying at approximately 240°C and the pickling process in white vinegar at pH 2.4, protein denaturation may occur and usually it was irreversible [26, 28, 32].

Thermal treatments of foods at extreme temperatures usually generate permanent protein denaturation at their secondary, tertiary and/or quaternary protein structure levels [17, 21]. The disappearance of the thermolabile proteins of snails might also be due to the formation of insoluble protein components or an aggregation process [17, 22].

Meanwhile, the reduction of the number of protein bands in the salted snail was possibly due to the protein denaturation during the salting process. The salting process generated the diffusion of sodium chloride (NaCl) from the brine to snail muscles due to the osmotic pressure differences, thus triggering protein dehydration [39-41]. This may lead to a higher degree of protein denaturation and decrease the stability of both myosin and actin [39-41]. Drying is a common processing method to maintain the quality of food for a long period [44-45]. This study demonstrated a significant influence of sun-drying on the protein profiles of *P. canaliculata*. It is possible due to the removal of moisture and warming of snail muscle activity during the process which generated protein degradation. While the reduction of the number of protein bands in pickled snails can be explained by an alteration in the proteins due to the partial loss of protein structure as the result of proteolytic degradation by the activity of acidic protease at acidic pH [46, 48].

Seafood proteins between molecular weights of 40 to 90 kDa were commonly described as thermo-sensitive proteins [15, 16, 21, 22]. This study also indicated that the bands within that range were also sensitive to the sun-drying process and low pH. While the 33 and ~100 kDa bands were recognized as the most stable protein bands in all treated snails as they were preserved after the treatments except for the fried and pickled snails which demonstrated faint and smearing bands at the regions. We believed the 33 kDa band was tropomyosin, a highly heat-stable major allergen in shellfish [21, 22]. Tropomyosin has very stable alpha-helical coils in its secondary protein structure. Thus, it is highly resistant to extreme pH, enzymatic digestion, and other food treatments [26, 30-32]. While the 100 kDa protein might be a fraction of myosin protein at 205 kDa [55].

3.2 Major Allergens of *P. canaliculata*

As demonstrated in Figure 2, immunoblotting of raw snails revealed 20 IgE-binding proteins between 17 to 240 kDa. A major allergen is defined as an IgE-binding protein that was detected by more than 50% of the patients' sera [21, 29]. Therefore, two major allergens at 33 and 42 kDa that were detected by 80 and 60% sera, respectively were identified as the major allergens of this snail in this study.

In general, compared to the raw snail, all the treated snails have a lesser number of IgE-binding proteins. Among the thermal-treated snails, the IgE-binding of boiled snails was retained in the majority of the tested sera (Figure 3). Conversely, a decrease in the protein bands recognition was seen in the roasted snail, while the fried had eliminated most of the IgE-reactive bands but retained some IgE-binding capability which was seen as weak smears. These results were following other reports which demonstrated that thermal treatments including boiling, roasting or frying would alter the conformation of protein structures shape, which possibly modify or destroy the IgE-binding epitopes [21, 30]. However, some reports also revealed the generation of neo-allergens in heat-treated shellfish due to exposure to IgE-binding epitopes or the formation of new epitopes [17, 22]. Thus, the allergenicity based on the number of IgE-binding bands in decreasing order of thermal stability was raw > boiled > roasted = fried.

The 33 kDa major allergen was detected as highly thermostable as it still appeared in all thermal-treated snails. While, the 42 kDa major allergen was found to be thermolabile as it could not retain its IgE-binding ability in immunoblotting of all thermally treated snails, except in one serum (Subject No. 5) which appeared as a smeared band.

Similarly, immunoblotting results of the non-thermal treated snails; the salted, dried and pickled extracts also demonstrated lesser IgE-binding bands than the raw snail, as presented in Figure 4. Both salted and dried snails have more ability to retain their allergenic bands (13 bands) compared to the pickled snail (8 bands), indicating the allergenic stability according to the number of IgE-binding bands in decreasing order was: raw > salted > dried > pickled. Similar to the thermal treated extracts, all the non-thermal treated extracts also exhibited smeared bands between 33 to 124 kDa in salted and dried snails, and 29 to 49 kDa in pickled snails.

For the salted and dried snails, the lesser IgE-binding bands compared to the raw snail indicated the decrease of immunoreactivity of the allergenic proteins, probably because of the proteolysis activity, which was triggered by the sun-drying process at moderate temperatures in the dried snail and high salt in salted snail. As mentioned earlier, in the sun-dried snails, moderate heat was applied to dry the snail muscles, while in the salted snail, high salt concentrations were applied. Both treatments may cause protein degradation.

Meanwhile, the pickled snail has the lowest IgE-binding bands compared to the raw and other non-thermal-treated snails. Pickling is an acid treatment that can reduce the snail allergenicity through allergenic structure alteration after the vinegar treatments [46, 48]. In addition, acid treatments can also decrease the allergenic response of snail by increasing protein digestion through reducing the gastric pH in the stomach [47], as reported in some foods such as shrimp [46, 48], peanuts [49, 50], lentils and eggs [47].

This study demonstrated that salting and drying have no major impact on the allergenicity of some thermostable protein bands, comprising the 33 kDa major allergen. However, pickling revealed a decrease in recognition of the 33 kDa band to a weak IgE-binding smear. On the contrary, the other major allergen at 42 kDa was emained in the immunoblotting of salted and dried snails but disappeared in the immunoblots of the pickled snails.

Thus, these results revealed various patterns of modification on the allergenic profile of *P. canaliculata* after being subjected to selected thermal and non-thermal treatments. We suggested the allergenic stability degree based on the number of allergenic proteins, major allergens and band intensities in order: raw > salted > dried > boiled > roasted > fried > pickled.

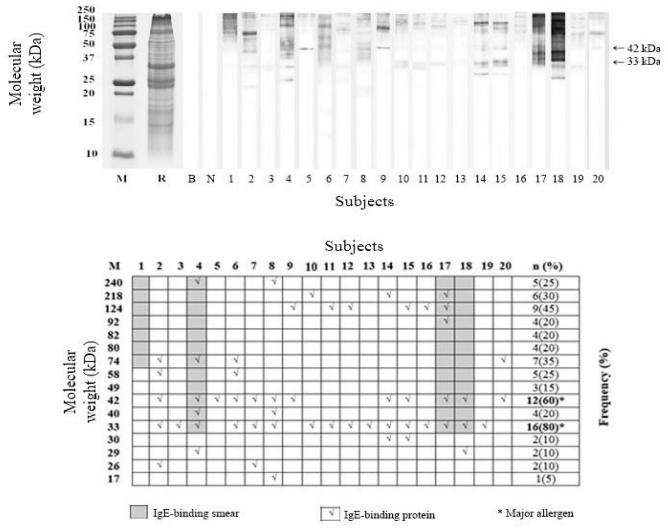

Figure 2 Immunoblotting results of raw extract of *Pomacea canaliculata* using 20 sera from snail-allergic patients (lane 1-20) and their respective IgE-binding protein analysis. Lane M, molecular weight markers; lane B, blank; N, negative control. Arrows indicate the major allergens

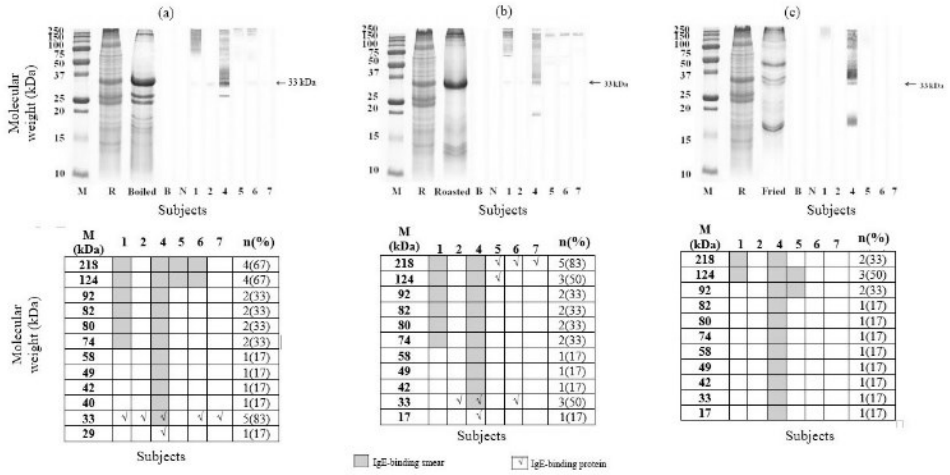

Figure 3 Immunoblotting results of boiled (a), roasted (b) and fried (c) extracts of *Pomacea canaliculata* using the same sera from 6 snail-allergic patients (lane 1-7) and their respective IgE-binding protein analysis. Lane M, molecular weight markers; lane B, blank; N, negative control. Arrows indicate the heat-stable major allergen of 33 kDa

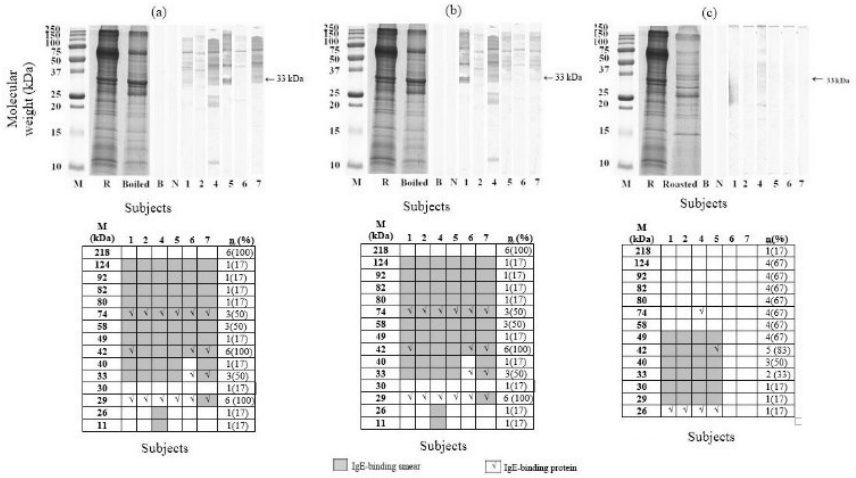

Figure 4 Immunoblotting results of salted (a), dried (b) and pickled (c) extracts of *Pomacea canaliculata* using the same sera from 6 snail-allergic patients (lane 1-7) and their respective IgE-binding protein analysis. Lane M, molecular weight markers; lane B, blank; N, negative control. Arrows indicate the heat-stable major allergen of 33 kDa

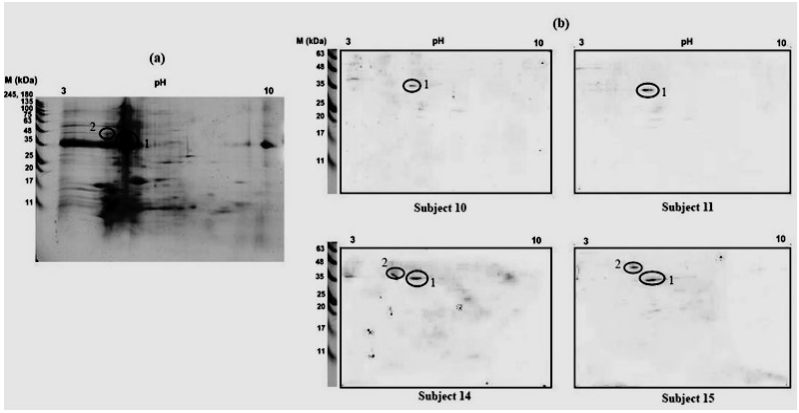

Figure 5 2-DE gel (a) and immunoblot results (b) of *P. canaliculata* using 4 sera (Patients 10 to 15). Lane M, molecular weight markers. The major allergenic spots of major allergens of 33 kDa (Spot 1) and 42 kDa (Spot 2) were circled

Table 1 Result of Mass spectrometry analysis of *P. canaliculate*

Spot No.	MW (kDa), pI Observed/predicted	Protein Identification	Organism	Accession No.	Residue numbers of matched regions	Coverage of protein sequence
1	33/32.6 kDa, 5.0/4.59	Tropomyosin	*Biomphalaria glabrata*	sp P42636	92-100, 101-105, 134-149	10%
2	42/40 kDa, 4.5/5.76	Cytoplasmic Actin	*Heliocidaris tuberculata*	gi 2289975 gb AB663031	82-99, 225-240	9%

Identification of *P. canaliculata* by Mass Spectrometry

This study used 2-Dimensional Electrophoresis (2-DE) to further resolve the major allergen bands of 33 and 42 kDa for mass-spectrometry analysis. Figure 5 (a) shows a 2-DE map of *P. canaliculata* proteins. Approximately more than 50 individual protein spots between 10 to 240 kDa and isoelectric point (pI) from 3.0 to 10.0 were visualized with coommasie blue staining. These results showed that the major allergens of 33 and 42 kDa were further fractionated to more than one spot by 2-DE.

Meanwhile, Figure 5 (b) shows the results of a 2-Dimensional (2-DE) immunoblot of *P. canaliculata* using four sera from patients with strong major allergen bands in immunoblotting of raw *P. canaliculata* as detected in Figure 2. Each serum showed a variety of patterns of IgE-binding spots and detected at least one IgE-binding spot. The most abundant IgE spot was labelled as Spot 1 at 33 kDa with pI 5.0, as detected by all sera (100%). Meanwhile, Spot 2 represented the major reactive spot of 42 kDa major allergen with pI of 4.5 as detected by half of the tested sera (50%).

MALDI-TOF result identified the 33 kDa major allergenic spot of *P. canaliculata* as tropomyosin, which showed a correlation with tropomyosin from *Biomphalaria glabrata*, with peptide sequence coverage of 10% (Table 1). Tropomyosin is a well-known major and cross-reactive allergenic protein in various species of shellfish [2, 12], including snails [9, 10, 13, 14]. In accordance with our findings, other reports also documented 32 to 38 kDa proteins as water soluble and highly thermostable to any processing methods due to the stability of their secondary structure (coiled-coil alpha helix stabilization) [17, 23, 25]. It was interesting to note that the immunoblotting result of one of the tested sera (patient no. 4) demonstrated a minor enhancement of band intensities of the majority of bands, probably because of the Maillard reaction [27, 28]. Tropomyosin which contains a high amount of lysine might freely be reacted with reducing sugar components and is responsible for the Maillard reaction in shellfish, causing the enhancement of IgE-binding intensities [27, 28].

Meanwhile, the 42 kDa major allergen was found to be unstable to all thermal and acid treatments. MALDI-TOF analysis identified this band as actin (Table 1). This band was found to be identical to actin from *Heliocidaris tuberculata* which corresponded to 9% sequence coverage. Previously, actin, a vital cytoskeleton component related to tropomyosin in muscle contraction [52-55] has been identified as a thermostable major allergen in crustaceans and molluscans [54-55] including snails [54].

4.0 CONCLUSION

This study indicated *P. canaliculata* has numerous allergenic proteins with different stability profiles. Two major allergens were identified in *P. canaliculata,* known as tropomyosin and actin. The tropomyosin was stable in all thermal and non-thermal treatments tested, while actin was only stable in salting and drying treatments. The allergenic stability of *P. canaliculata* based on the number of allergenic bands, major allergens and band intensities in decreasing order was: raw > salted > dried > boiled > roasted > fried > pickled. Consequently, thermal and non-thermal treatments mainly fried and pickled can be applied to minimize snail allergenicity. Our findings are essential in improving the diagnosis and management strategies of snail-allergic patients worldwide.

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Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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