

COMPARISON OF ALLERGENIC PROTEINS OF SEA SNAIL (*CERITHIDEA OBTUSA*) AND FRESHWATER SNAIL (*POMACEA CANALICULATA*)

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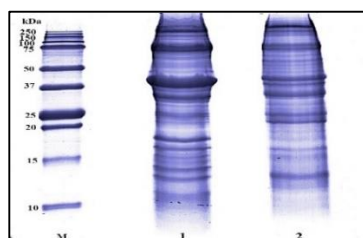
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Graphical abstract



Abstract

In Malaysian and certain Asian countries, snail has high market demand and popular to the local people as food. However, snail is also frequently reported as one of the worst food allergens, dominated by severe symptoms such as asthma and anaphylactic shock. Thus, the aims of this study is to determine the allergenicity of two species of edible snails; the local sea snail, *Cerithidea obtusa* and the freshwater snail *Pomacea canaliculata*. Snail extracts were prepared from the snail flesh and analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine their protein profiling. Allergenic proteins were detected by immunoblotting test using sera from 10 snail-allergic patients. The snails contain 31 to 34 protein fractions between 11 to >250 kDa. The prominent bands were seen at 33, 42, 74 and 250 kDa. Immunoblotting detected 15 and 16 allergenic proteins in *C. obtusa* and *P. canaliculata*, respectively. Three protein fractions at 30, 33 and 42 kDa were identified as the major allergens of *C. obtusa*, while six major allergens at 30, 33, 42, 74, 124 and 218 kDa were detected in *P. canaliculata*. Various minor allergens were also detected in both snails. This study indicated that numerous proteins of *C. obtusa* and *P. canaliculata* were allergenic. Thus, combined allergen extracts of both snails are essential to be included in diagnosis of snail allergy among local allergic patients.

Keywords: *Cerithidea obtusa*, *Pomacea canaliculata*, snail, allergen, SDS-PAGE, immunoblotting

Abstrak

Di Malaysia dan beberapa negara Asia, siput mempunyai permintaan pasaran yang tinggi dan popular kepada penduduk tempatan sebagai makanan. Walau bagaimanapun, siput juga sering dilaporkan sebagai antara bahan alergi makanan yang paling teruk, dikuasai oleh gejala yang teruk seperti asma dan renjatan anafilaksis. Oleh itu, matlamat kajian ini adalah untuk menentukan alergeniti dua spesies siput yang boleh dimakan; siput laut tempatan, *Cerithidea obtusa* dan siput air tawar, *Pomacea canaliculata*. Ekstrak siput telah disediakan daripada daging siput dan dianalisis menggunakan elektroforesis gel natrium dodesil sulfat- berpoliakrimalida (SDS-PAGE) untuk menentukan profil protein. Protein alergenik dikesan oleh ujian pembloatan imuno menggunakan serum daripada 10 pesakit alergi siput. Siput mengandungi 31-34 fraksi protein antara 11 hingga > 250 kDa. Jalur utama berada pada 33, 42, 74 dan 250 kDa. Pembloatan imuno mengesan masing-

masing 15 dan 16 protein alergenik pada *C. obtusa* dan *P. canaliculata*. Tiga fraksi protein pada 30, 33 dan 42 kDa telah dikenal pasti sebagai alergen utama pada *C. obtusa*, manakala enam alergen utama pada 30, 33, 42, 74, 124 dan 218 kDa dikesan pada *P. canaliculata*. Pelbagai alergen minor juga dikesan dalam kedua-dua jenis siput. Kajian ini menunjukkan bahawa pelbagai protein *C. obtusa* dan *P. canaliculata* adalah alergenik. Oleh itu, ekstrak alergen gabungan kedua-dua siput adalah penting untuk dimasukkan ke dalam diagnosis alergi siput di kalangan pesakit alergi tempatan.

Kata kunci: *Cerithidea obtusa*, *Pomacea canaliculata*, siput, alergen, SDS-PAGE, pemblotan imuno

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1.0 INTRODUCTION

Snail flesh is a popular food among Asian, European, North American and Australian [1, 2, 3]. In Europe such as France and Italy, heavy consumers of the delicacy particularly *Helix Aspersa*, *Eobania vermiculata* and *Helix pomatia* species were reported [1,4]. Snail is also used for therapeutic purposes, such as *Bellamyia bengalensis* is commonly used in India as a traditional medicine against rheumatism [5], while sea snail (*Cerithide obtusa*) and freshwater snail (*Pomacea canaliculata*) were used in Indonesia as traditional medicines to treat some illness including fever, wounds and itching [2].

However, snails can also trigger adverse reactions including metal toxicity and allergic reactions [1,2,4-6]. Although snail allergy is generally less common than other shellfish allergy, the clinical symptoms of this allergy is frequently dominated by severe asthma [7,8]. It can also cause more serious and fatal allergic reactions due to anaphylactic shock [1,7]. Cross-reactivity between snail and house dust mites is well recognized [7-9], as snail allergy is frequently associated with allergy to dust mites and this may account for the high frequency of asthma and rhinitis seen as symptoms (9). Allergy to snail is also associated with allergy to crustacean and other molluscs such as abalone and limpet which can also include severe or fatal reactions (9,10). Some studies also reported a worsen effect of immunotherapy in patients with multiple snail and mite allergy [4,7].

Tropomyosin, a protein which presents in all eukaryotic cells and plays a significant role in contractile activity and regulation of cell morphology and motility is reported as the most important allergen in several species of snails [1,11-13]. Tropomyosin is a well-documented major allergen of various invertebrates including crustaceans and molluscs [1,14]. Snail species frequently reported as causing allergic reactions in American and European countries include *Helix aspersa*, *Helix pomatia*, *Helix terrestris*, *Cerithide virgate*, *Theba pisana* and *Bolinus brandaris* [1,13,14].

In contrary, different species of snails were used as a source of food and medicine in Malaysian and other Asian populations [3]. It was reported that allergic

reactions to particular shellfish capable to reflect regional consumption of that specific species [10].

Cerithide obtusa or obtuse horn shell is a local mangrove or sea snail belongs to the Family Potamididae commonly consumed by local populations in coastal areas [15]. This snail has distributed in Indo-West Pacific, from Madagascar and India to eastern Indonesia, north to the Philippines and south to northern Queensland. Therefore, this snail serves commonly as food in Southeast Asia including Malaysia [15].

Similarly, *Pomacea canaliculata* (golden apple snail), a freshwater snail commonly found in rice field areas and wetlands is also being collected as an exotic food by local population and offered in certain restaurants [16]. This species belongs to the Family Ampullariidae which is originally from Argentina, Bolivia, Brazil, Paraguay and Uruguay. Currently this snail is also found in many Asian countries including Philippines, Japan, Korea, Taiwan, Vietnam, Indonesia and Malaysia [15]. This species was initially introduced in Malaysia in 1992 in Sabah, then has spread to the Peninsular Malaysia [16].

Therefore, the aims of this study is to determine and compare the allergenicity of *C. obtusa* and *P. canaliculata* among local patients with snail allergy.

2.0 METHODOLOGY

2.1 Preparation of Allergen Extracts

C. obtusa and *P. canaliculata* were purchased from a local fresh market. Protein extracts of both snails were prepared using the standard methods of allergen extraction [17]. In brief, the raw extracts were prepared by homogenization of the flesh in phosphate-buffered saline (PBS), pH 7.2 (1:10 weight/volume) using a blender, followed by an overnight extraction at 4°C. The homogenates were centrifuged at 14000 rpm for 15 min at 4°C. After centrifugation, the clear supernatant was filtered using a sterile 0.22 µm syringe filter. Extracts were then lyophilized in a freeze dryer. The lyophilized extracts were stored at -20°C until use. The total protein content in the extract was determined using the Bio-

Rad Protein Assay (BioRad, CA, USA), following the manufacturer's instructions.

2.2 Human Sera

Sera were obtained from 20 snail-allergic patients from Allergy Clinic, Kuala Lumpur General Hospital. Written informed consent was obtained from each patient. All the patient sera had been shown to have positive reactions in skin prick test to snail extracts and/or elevate IgE specific for a commercial snail by ImmunoCAP test (Phadia, Uppsala, Sweden). In the present study, serum from a healthy volunteer was used as a negative control. All sera were stored at -20°C until used. This study 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 SDS-PAGE was performed to determine the protein profiles of both snails on a Mini Protean 3 System (BioRad, CA, USA) according to the manufacturer's instructions, and following the method described by Zailatul *et al.* [17]. Briefly, the snail proteins were dissolved in a sample buffer, heated at 97°C for 3 minutes and subjected to 12.5% separating gel and 5% stacking gels. Precision plus protein standards (Bio-Rad, CA, USA) were run along with samples as a reference. After running, the gel was stained with Coomassie Brilliant Blue R-250. The molecular weight of the protein bands were estimated by comparing the bands with the marker used using an Imaging Densitometer and Quantity One Software (BioRad, CA, USA).

2.4 Immunoblotting

In immunoblotting, the unstained snail proteins separated by SDS-PAGE were first electro-transferred from the gel to a nitrocellulose membrane using the Mini Transblot System apparatus (BioRad, CA, USA) at 100 volt for 70 minutes, as described in the manufacturer's manual. Transfer of the proteins to the membrane was confirmed by staining with Ponceau S. The membrane was then washed with TTBS, a Tween-tris-buffered saline (tris buffered saline, pH 7.4, containing 0.05% Tween 20) and blocked with 10% skimmed milk in TBS at 4°C for two hours. After washing with TTBS, the membrane was incubated with individual patient serum (diluted 1:500) at 4°C overnight and biotinylated goat-antihuman IgE (KPL, Maryland, USA) at 4°C for 30 minutes (diluted 1:10 000). For detection of IgE-binding protein, the strips were incubated in streptavidin-conjugated alkaline phosphatase and Alkaline Phosphate Conjugate Substrate Kit (BioRad, CA, USA). The molecular weight of the IgE-binding proteins was estimated by comparing to the marker used.

3.0 RESULTS AND DISCUSSION

3.1 Comparison of Protein Profiles of *C. obtusa* and *P. canaliculata*

Figure 1 shows the protein profiles of *C. obtusa* and *P. canaliculata* extracts. Both snails displayed a complex protein pattern with the majority of visible protein bands within the molecular weight between 11 to >250 kDa. The molecular weight of the fractions were estimated using a precise and wide range protein marker mixture between molecular weight of 10 to 250 kDa as most of IgE-binding proteins reported in literatures are between this range [14,17,18]. *C. obtusa* has more protein bands, approximately 34 bands, higher than *P. canaliculata* which comprises only 31 bands. Both extracts have prominent protein bands at 33, 42, 74 and 250 kDa. The 33 and 42 kDa bands might be corresponded to tropomyosin and arginine kinase or actin, respectively.

Overall, both snails have almost similar protein profiles but with different intensities, except for some bands at low molecular weight regions between 12 to 25 kDa which were more remarkably seen in the extract of *C. obtusa*. The protein profile patterns of both snails were in accordance with numerous studies which reported several prominent bands particularly the 34 to 38 kDa and 40 to 42 kDa in SDS-PAGE gels of various molluscan shellfish [1, 17, 18].

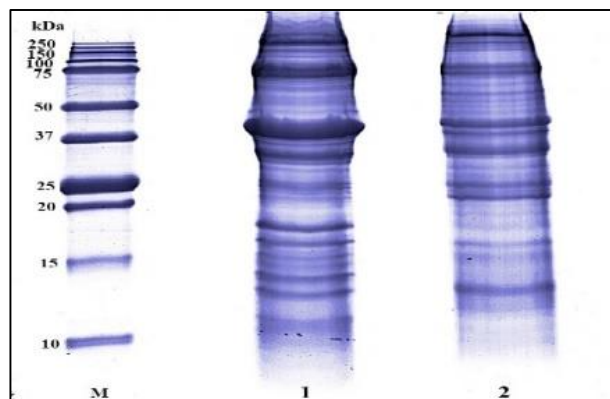


Figure 1 Comparison of SDS-PAGE profiles of extract of *C. obtusa* (lane 1) and *P. canaliculata* (lane 2). Lane M is molecular mass markers in kiloDalton (kDa)

3.2 Comparison of Allergenic Proteins of *C. obtusa* and *P. canaliculata*

Figure 2 indicates the immunoblotting results of *C. obtusa* and *P. canaliculata*. Immunoblotting detected 15 and 16 IgE-binding proteins in *C. obtusa* and *P. canaliculata*, respectively, between 18 to 240 kDa. The frequency of each allergenic proteins was summarized in Table 1.

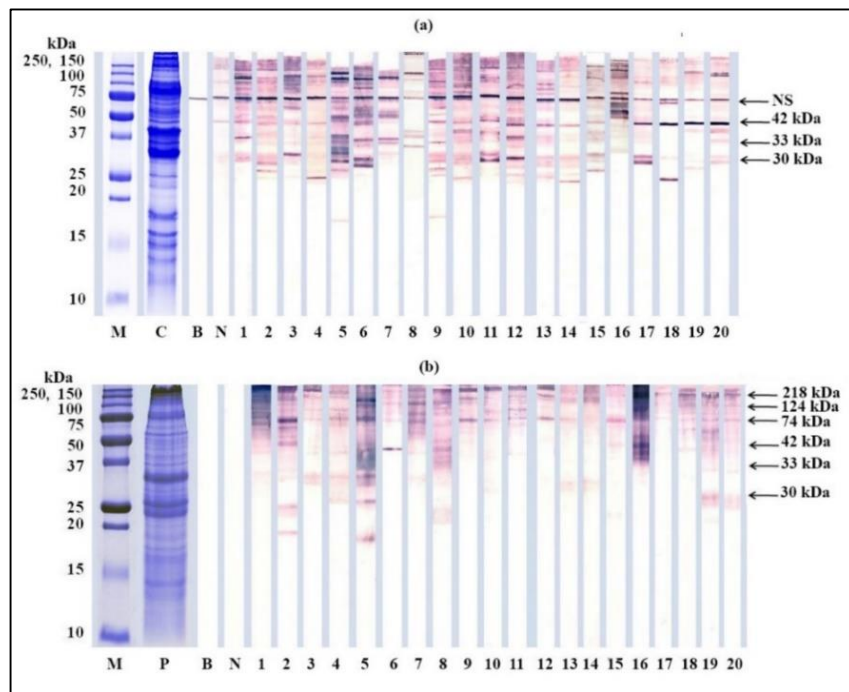


Figure 2 Immunoblotting results of *C. obtusa* (a) and *P. canaliculata* (b) using sera from 20 snail-allergic patients (lane 1 to 20). Lane M is molecular mass markers in kiloDalton (kDa); lane C and P are protein profiles of *C. obtusa* and *P. canaliculata*, respectively; lane B is blank and lane N is immunoblots using a negative control serum

Table 1 The frequency of IgE binding proteins of *C. obtusa* and *P. canaliculata* using sera from 20 snail-allergic patients

		<i>C. obtusa</i>																				n	%
M (kDa)		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		
240																						9	45
218																						9	45
124																						8	45
92																						5	25
82																						5	25
74																						5	25
58																						4	20
49																						7	30
42																						18	90*
40																						6	25
33																						14	70*
32																						9	45
30																						15	70*
28																						9	45
18																						2	10

		<i>P. canaliculata</i>																				n	%
M (kDa)		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		
240																						9	45
218																						14	70*
124																						11	55*
92																						6	30
82																						9	45
74																						16	80*
58																						5	25
49																						3	15
42																						13	65*
40																						2	10
33																						10	50*
32																						2	10
30																						12	60*
28																						2	8
25																						2	8
18																						2	8

IgE-binding Protein * Major allergens

Interestingly, immunoblotting tests indicated that almost half of the snail proteins in both snails were capable to bind to IgE antibodies from snail allergic patients. Although majority of the IgE-binding proteins of both snails were detected at similar molecular weights, the frequencies of IgE-binding were varied. Thus, these findings suggested the presence of common epitopes between the snail allergens. Interestingly, protein at 25 kDa were only found as allergenic in *P. canaliculata*, which suggesting the existence of species-specific epitopes in *P. canaliculata*. It should be noted that both snails are closely related, but belonging to different taxonomic classification as *C. obtusa* and *P. canaliculata* are grouped under family Potamididae and Ampullariidae, respectively [15]. It was reported that organisms with a close taxonomic relationship will probably have proteins with homologous sequences which possibly responsible for high degree of cross-reactivity between different species of organisms [19].

In addition, all 20 sera used in this study presented highly heterogeneous IgE-binding patterns towards snail proteins. These results showed that allergic immune responses to snail allergens are differ between individuals, clearly due to variations of individuals' immune responses towards allergens. It was reported that the development of allergic diseases depends upon both genetic predisposition and the environmental factors [20].

Allergenic proteins are designated as a major allergen if majority (at least 50%) of tested patients have IgE responses to the particular protein [21]. Our study revealed that *C. obtusa* and *P. canaliculata* have different set of major allergens. Three proteins at 30, 33 and 42 kDa of *C. obtusa* were able to bind to IgE antibodies of at least half of the tested sera, and thus were identified as the major allergens for *C. obtusa*. In contrast, *P. canaliculata* has six major allergens at 30, 33, 42, 74, 124 and 218 kDa. This results showed that *P. canaliculata* which contained more major allergens might probably has higher potential to elicit allergic responses than *C. obtusa*.

Surprisingly, our study indicated that the major allergens of 42 kDa have the highest frequency of IgE binding (90% in *C. obtusa* and 80% in *P. canaliculata*), and thus were declared as the most important major allergens in both snails. Unfortunately, the nature of these proteins were not further characterized in this study. It should be noted that, aside from tropomyosin, there are other major allergens have been identified in shellfish at various molecular weights. Arginine kinase at 40 kDa has been characterized as a major allergen in shrimp, crab and other invertebrates [1, 17, 18, 22]. More recent, actin at 42 kDa was also identified as the major allergens in shrimp, crab and molluscan shellfish [23]. Arginine kinase, which has regulatory and transport properties is abundant in crustaceans muscle [17, 18], while actin is an important contractile protein which involved in the contraction of muscle cells in eukaryotic cells [23]. However, we were unable to verify whether the 42 kD

major allergen in both snails are arginine kinase, actin or other proteins.

In this study, 70 and 50% of the tested sera recognized the 33 kDa bands in *C. obtusa* and *P. canaliculata*, respectively. This finding showed that both bands are predominant in allergic responses towards both snail species. This size is consistent with tropomyosin. Thus, we strongly believed that both bands might be types of tropomyosins, the well-known pan-allergens in crustaceans, mites, insects, mollusks and other invertebrates [22–24], which involved in the highly cross-reactivity reactions among these organisms [25, 26]. Remarkably, our result is also in accordance with other reports which suggested tropomyosin is only involved in snail allergy as a minor allergen [13, 25]. Tropomyosin was also indicated to have no significant influence in provoking snail allergy in some cases [13, 25].

The 30 kDa protein is rarely described as a major allergen in shellfish. However, our study found that this band was recognized by 70% and 60% of the tested sera in immunoblotting of *C. obtusa* and *P. canaliculata*, respectively. Therefore, this band was also identified as one of the important major allergens of both snails. To our knowledge, there have been only one report on shellfish allergens have identified a 28 kDa band (close with the molecular weight of 30 kDa), as triose phosphate isomerase, an important allergen in shrimp *Crangon crangon* [24]. However, whether our 30 kDa major allergen is homologous to the 28 kDa was still unknown.

Our study also identified three higher molecular weight bands at 74, 124 and 218 kDa as major allergens, but only in immunoblotting of *P. canaliculata*. So far, there have been only one reports has identified a high molecular weight band at ~100 kDa on mollusc as paramyosin, a novel allergen other than tropomyosin in abalone *Haliotis discus discus* [25]. Therefore, we strongly believed that the 124 kDa allergenic protein in *P. canaliculata* was paramyosin. However, the 218 kDa band might be considered as an oligomer. It was reported that various food allergens might naturally form high molecular weight oligomers between 150 to 200 kDa, either as a dimer or trimer. This oligomers might have additional epitopes to elicit a higher potential of allergic reactions than monomers [26].

This study also indicated that most of the sera tested had minor IgE-binding to other proteins at various molecular weights with numerous frequencies. Similarly, there are numerous reports have identified minor allergens in molluscan shellfish with molecular weights of 10 to 250 kD, but are not well-characterized [1, 17, 18, 23]. These minor allergens were recognized by a limited number of patients, suggesting that these minor allergens might not a clinically relevant allergens among patients tested. It was suggested that minor allergens do not cause clinical symptoms [27], or they are only relevant for certain allergic patients [28]. This study also indicated a non-specific band was also seen in all immunoblot strips of *C. obtusa* at 75 kDa. This probably due to interpolating of

the blot membrane with the primary antibody and the secondary antibodies [29].

4.0 CONCLUSION

In summary, our study is the first in Asian countries to determine the snail allergenicity. We found that both snails, *C. obtusa* and apple snail, *P. canaliculata* capable to react with IgE antibodies of local snail-allergic patients. The allergenicity of *P. canaliculata* was found to be higher than the sea snail, *C. obtusa*, in term of their number of major allergens. The molecular weights of the allergenic bands in both snail species are almost similar to the crustaceans and other mollusc allergens. However, more detailed identification and characterization of these allergens have not yet been performed in this study. In addition, immunoblotting using sera from more snail-allergic patients is necessary to confirm this findings. As conclusion, since snail allergy is frequently associated with mite allergy, it may be worthwhile to include measurement of IgE to both species of snails which can facilitate the diagnosis and immunotherapy of allergic patients.

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