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RESEARCH ARTICLE

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CHARACTERIZATION OF MAJOR AND MINOR ALLERGENS OF SHARP-ROSTRUM PRAWN (PARAPENAEOPSISHARDWICKII)

ABSTRACT

Seafood allergy seems to be increasing in Asia as well as worldwide. Characterization of seafood allergens is important in order to understand the immune response to these allergens. To date, several prawn allergens have been identified including tropomyosin, arginine kinase, sarcoplasmic calcium-binding protein (SCP) and myosin light-chain (MLC).

Objective: The aim of this study was to identify the major and minor allergens of Parapenaeopsishardwickii (sharp-rostrum prawn), the most commonly consumed prawn in Malaysia.

Methods: The raw and cooked extracts were prepared from the prawn shell and meat. Both extracts were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes and analyzed by immunoblotting using sera from patients with allergy to prawns.

Results: In SDS-PAGE, the raw extract exhibited more protein bands than the cooked extract, ranging from molecular weights of 15 to 200 kDa. Immunoblotting of raw extract demonstrated numerous IgE-binding bands, whereas the cooked extract had fewer IgE-binding bands. The 36 kDa heat-resistant protein constitutes a major allergen, while several other heat-sensitive and heat-resistant proteins were recognized as potential minor allergens.

Conclusion: A heat-stable band of 36 kDa was identified as the major allergen of this species of prawn. This protein is believed to be tropomyosin, a well-documented major heat-stable allergen in prawn.

Keywords: prawn allergy, Parapenaeopsishardwickii, immunoblotting, tropomyosin

1.0 INTRODUCTION

Seafood allergy is a universal health care issue and is one of the most common forms of food allergies worldwide [1]. A large variety of seafood including prawns is used for human consumption. In 2008, more than 5 million tonnes of seafood was consumed worldwide [2]. Because of the increasing consumption of seafood, the occurrence of hypersensitivity reactions has also increased [3,4]. Allergic reactions to seafood often develop within minutes through ingestion [1, 5], inhalation or skin contact while cooking or working [5]. Typical symptoms include vomiting, abdominal pain and cramping, respiratory distress, throat swelling and diarrhea [6]. More severe life-threatening reactions may lead to asphyxia, vascular collapse, irregular heartbeat or myocardial infarction [6,7].

The interest in allergen identification and characterization is very important to further understand the immunopathologic mechanism of food allergy [8]. Tropomyosin is one of the seafood allergen that has been well-characterized [4,9,10]. This heat-resistant protein which is located in the muscle tissues has been identified as the major and cross reactive allergen in prawns [10, 11] including Penaeusaztecus (Pen a 1) [9], Penaeusmonodon (Pen m 1) [12] and Metapenaeusensis (Met e 1) [13]. Subsequently, a 40 kDa protein, known as arginine kinase was reported [14]. More recently, researchers have also identified sarcoplasmic calcium-binding protein (SCP) and myosin light-chain (MLC) as new prawn allergens [15, 16, 17].

Previous studies have suggested that the cooking process may affect the allergenicity of food [8]. Therefore the characterization of raw and cooked extracts is very important in the management of patients with prawn allergy. Local studies on prawn allergens are limited. Parapenaeopsishardwickii or sharp-rostrum prawn, locally named as ‘udangminyak’ is among the major commercial prawn species in Malaysia [2]. Thus, the objective of this study was to characterize the major and minor allergens of this species of prawn.

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2.0 METHODS

2.1 Prawn Extracts

Sharp-rostrum prawn was purchased from a fresh market in Port Klang, Selangor. The prawn was used to prepare raw and cooked extracts, following the methods as described by Daulet al. [9]. The shell and meat (1:1) were blended and homogenized in phosphate-buffered saline (PBS) pH 7.2, and extracted overnight at 4^{o}C under constant stirring. After centrifugation at 4200 rpm for 30 minutes at 4^{o}C, the supernatant was collected, re-centrifuged at 14000 rpm at 4^{o}C for 15 minutes, sterile-filtered using syringe filter 0.22 µm, lyophilized using a freeze dryer (Christ, Germany) and stored at -20^{o}C until use. For preparation of cooked extract, the prawn homogenates was first boiled on a hot plate at 100^{o}C for 15 minutes before extraction as mentioned above. The protein concentration was measured by the Lowry-Biuret method using a total protein kit (Sigma-Aldrich, UK).

2.2. Sera

Sera from 31 patients with a history of prawn allergy and tested by positive skin prick test (SPT) to raw extract of sharp-rostrum prawn were used in this study. The SPT was performed at the Allergy Clinic, Hospital Kuala Lumpur by a medical officer. 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 protein components of both crude extracts (raw and cooked) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), as described by Daulet al. [9] with slight modification. The extracts were heated in SDS reducing buffer at 97^{o}C for 4 minutes. A Precision Plus Protein Standards (BioRad, USA) was used as molecular weight markers. The samples were then fractionated by SDS-PAGE using a 12% separating gel with a 5% stacking gel using a Mini Protean 3 Apparatus (BioRad, USA) at 120 mA for 45 minutes. After SDS-PAGE, the protein bands were stained with Coomassie Brilliant Blue R-250 (BioRad, USA) and analyzed using an imaging densitometer instrument (BioRad, USA).

2.4 Immunoblotting

Immunobloting for detection of IgE-binding proteins of raw extract was performed using sera from 31 patients with prawn allergy, as mentioned above. To reveal the effect of boiling on the allergenic properties of the prawn allergen, 10 sera were further tested by immunoblotting of cooked extract. In brief, after electrophoresis, the separated prawn proteins were transferred onto nitrocellulose membrane 0.45 µm using a Mini Transblot System (BioRad, USA) at 100V for 70 minutes. The membrane were then cut into strips and washed with Tween-20 Tris-buffered-saline (TTBS) (BioRad, USA) before blocked for 2 hours with 5% non-fat milk in Tris-buffered saline (TBS) (BioRad, USA). The blocked strips were incubated overnight with each individual patient's serum at 4 °C under constant mixing. After washing with TTBS, the strips were incubated with biotinylated goat anti-human IgE(Kirkergaard and Perry Laboratories, UK) for 30 minutes, followed by incubation in streptavidin-conjugated alkaline phosphatase (BioRad, USA) for 30 minutes. Colour detection was then carried out using Alkaline Phosphate Conjugate Substrate Kit (BioRad, USA). Immunoblotting results were analysed using an imaging densitometer analyzer (BioRad, USA). Each set of strips contained a blank (no serum) and a negative control (serum from a non-allergic individual).

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3.0 RESULTS

3.1 SDS-PAGE of Prawn Extracts

The SDS-PAGE gels of raw and cooked prawn extracts are presented in Figure 1. The protein profile of raw extract reveal approximately 14 protein bands with molecular weights ranging from 15 to 200 kDa, while the cooked extract demonstrated fewer protein bands. Several protein bands between 40 to 75 kDa were not detected in the cooked extract. However, the detection of an 18 kDa band was enhanced in the cooked extract. A 36 kDa protein band which was equivalent in size as prawn tropomyosin was demonstrated in both raw and cooked extracts.

3.2 Immublotting

The immunoblot experiment revealed the presence of several allergenic proteins as shown in Figure 1. Figure 1(A) and Table 1 shows the immunoblots of raw extract using 31 subjects while Figure 1(B) and Table 2 shows the immunoblots of both raw and cooked extracts using 10 subjects.

Immunoblotting of raw and cooked extracts identified numerous IgE-binding proteins at various molecular weights between 15 to 150 kDa and 18 to 100 kDa, respectively. In this study, a protein at 36 kDa showed the highest frequency of IgE-binding proteins in both raw and cooked extracts. In addition, proteins of 49, 65 and 75 kDa have also been detected as potential minor allergens in the raw extract by 45, 42 and 45% subjects, respectively. In addition, three potential minor allergens at 34, 41 and 100 kDa were also detected in immunoblotting of raw extract by 32% of the subjects.

Certain differences were detected between raw and cooked extracts in their allergenic profiles. Immunoblotting results showed that all heat-sensitive IgE-binding proteins were not detected in immunoblotting of cooked extract, while several heat-resistant IgE-binding bands particularly the major allergen of 36 kDa retained its specific IgE-binding capacity in the cooked extract. No binding was observed in the negative control and blank strips.

4.0 DISCUSSION

Prawn is one of the most highly allergenic seafood responsible for allergic reactions in both children and adults [17, 18]. In spite of the high prevalence of prawn allergy, there is limited information about the proteins involved in the induction of such allergic reactions among our local patients.

SDS-PAGE demonstrated that both raw and cooked extracts have different protein profiles. Several protein bands between 40 to 75 kDa which were present in raw extract were no longer visible in the cooked extract. Pomset al. [19] suggested that high temperatures such as those reached during cooking or grilling, tend to break the disulfide bridges of proteins, modifying their secondary and tertiary structures and their capacity to bind antibodies. In this study, several bands of 20, 36 and a high molecular weight band of ~100 kDa were found to be heat-resistant. Other studies also reported the similar results of heat-resistant protein bands [8, 15]. The existence of seafood allergens that are resistant to heat treatment has been previously documented [7, 11]. It was reported that thermal processing could modify the protein structure [20] and could lead to the formation of neo-allergens or loss of some original antigenicity [21].

Immunoblotting of raw sharp-rostrum prawn extract detected several major IgE-binding proteins between 15 to 150 kDa. A 36 kDa heat-resistant protein was identified as the major allergen of raw extract. This band was also recognized by all sera in immunoblotting of cooked extract, suggesting a highly heat stable protein which might correspond to prawn tropomyosin. Tropomyosin (34-38 kDa) has been well-documented as major allergen in invertebrates such as crab, squid, cockroach and house dust mites [4, 10, 22, 23]. This protein

has been well-documented as a heat-resistant allergen [13, 24]. It was previously thought that subjects with prawn allergy would develop hypersensitivity reactions to all prawn due to the common major allergen, tropomyosin [10, 25, 26]. However, immunoblotting of raw extract demonstrated that only 81% of our subjects exhibited IgE-binding to this 36 kDa protein.

Our study also indicated several heat-sensitive and heat-resistant proteins as minor allergens. A minor allergen of 41 kDa was detected only in immunoblotting of raw extract by 32% of the subjects. This protein was found to be sensitive to high temperature, hence was not detected in immunoblotting of cooked extract. Previously, several studies have identified a 40 kDa allergen as arginine kinase in Penaeusmonodon (Pen m 2) and Litopenaeusvannamei (Lit v 2) [14, 27]. It is therefore possible that the 41 kDa protein identified in this study, could be arginine kinase. Arginine kinase is a phosphagen kinase that plays a key role in energy metabolism in invertebrates [27]. It has also identified as a pan-allergen in other invertebrates such as moth, cockroach, and lobster [7, 15].

In addition, the presence of minor allergens at 49, 65 and 75 kDa was also detected in immunoblotting of raw extract by more than 40% of the subjects. Based on SDS-PAGE analysis, these bands were also found to be sensitive to boiling process, therefore were detected only in immunoblotting of raw extract. Although these allergens have not yet been identified, there are some studies reported IgE-binding proteins of similar molecular weights [25, 26]. In a previous study, we found that the 49 and 75 kDa proteins to be major allergens of two local prawns (Penaeusmonodon and Penaeuslatisulcatus) [28].

In this study, the cooked extract showed enhancement of band intensity of an 18 kDa protein. This finding is similar with other studies that reported the enhancement of 16.5 to 20 kDa bands in the boiled extract [8]. William [20] reported that the enhancement might be due to a lot of basic charged proteins that exposed due to unfolding, which could result in increased interactions with the coomassie blue dye molecules. However, our immunoblotting experiments showed that this protein was only detected in one subject, suggesting that it may not be an important allergen among local patients with prawn allergy.

In summary, the boiling process has modified the protein profile of sharp-rostrum prawn, but retained its allergenicity as both heat-resistant and heat-sensitive proteins were found to be allergenic. However, our study demonstrated a highly heat-stable protein at 36 kDa which may correspond to prawn tropomyosin as major allergen of this species of prawn. With this information, new approachs towards the understanding and management of prawn allergy among local patients could be developed.

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Subjects	Molecular Weight of allergenic protein (kDa)											
	15	**18**	**25**	**28**	**34**	**36**	**41**	**49**	**65**	**75**	**100**	**150**
1						√						
2						√		√	√	√		
3						√						
4										√	√	
5	√									√	√	
6						√						
7										√		
8										√		
9						√						
10						√		√	√			
11						√		√	√			
12						√	√			√	√	
13										√	√	√
14						√	√					
15						√		√				
16						√						
17						√	√	√				
18					√	√	√	√	√	√		
19					√	√	√	√				
20							√	√	√	√		
21					√	√			√			
22			√	√	√	√	√	√	√	√	√	
23				√	√	√	√	√	√	√	√	
24						√		√	√	√	√	
25						√		√	√		√	
26			√			√	√	√	√	√	√	
27			√	√	√	√	√	√	√	√	√	
28					√	√						
29		√	√		√	√			√			
30					√	√						
31					√	√						
Frequency (%)	3	3	13	10	32	**81 ***	32	45	42	45	32	3

√ IgE-binding protein, * Major allergen

Table 1: The frequency of IgE-binding proteins of raw extract of sharp-rostrum prawn by immunoblotting analysis using sera from 31 patients with prawn allergy.

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	R	C	R	C	R	C	R	C	R	C	R	C	R	C	R	C	R	C	R	C
22			√		√		√	√	√	√	√		√		√		√		√	
23					√		√		√	√	√		√		√		√		√	
24									√	√			√		√		√		√	
25									√	√			√		√				√	
26			√						√	√	√		√		√		√		√	
27			√		√		√		√	√	√		√		√		√		√	
28							√		√	√										
29	√	√	√				√	√	√	√					√					
30							√		√	√										
31							√	√	√	√										

√ IgE-binding protein

Table 2: The comparison of IgE-binding proteins of raw (R) and cooked (C) extracts of sharp-rostrum prawn by immunoblotting analysis using sera from 10 patients with prawn allergy.

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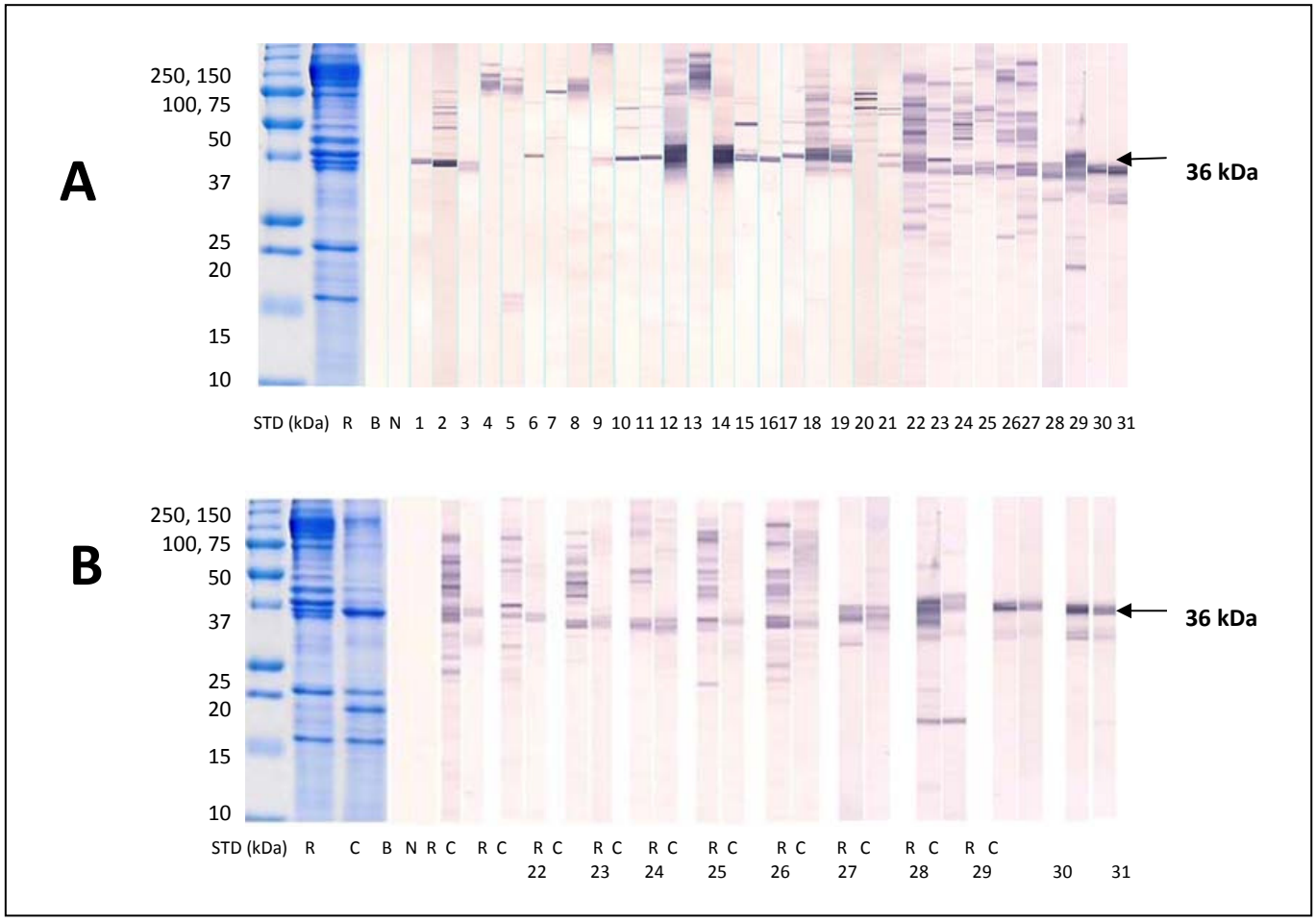

Fig 1: Protein profiles and IgE-binding patterns of raw (A) and cooked (B) extracts of sharp-rostrum prawn. Lane STD is molecular weight markers in kiloDalton (kDa). Lane R and C are SDS-PAGE profiles or immunoblotting profiles of raw and cooked extracts, respectively. Lanes 1 to 31 are immunoblotting profiles of 31 subjects with prawn allergy. B and N are blank and negative control, respectively. Arrows indicate the position of the major allergen at 36 kDa.

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