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ORIGINAL ARTICLE

Identification of Major and Minor Allergens of Mud Crab *(Scylla Serrata)*

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ABSTRAK

Ketam adalah sumber nutrien berharga iaitu protein dan lipid berfungsi yang diambil secara meluas. Walau bagaimanapun, prevalen alergi terhadap ketam telah meningkat beberapa tahun kebelakangan ini. Dalam usaha untuk lebih memahami alergi terhadap ketam, adalah penting untuk mengenal pasti alergen-alergen ketam. Tujuan kajian ini adalah untuk membandingkan protein pengikat-IgE pada ekstrak ketam lumpur (Scylla serrata) mentah dan yang dimasak. Ekstrak ketam lumpur mentah dah yang dimasak telah disediakan. Profil protein dan corak kereaktifan-IgE telah dikenalpasti dengan menggunakan elektroforesis gel natrium dodesil sulfat berpoliakrilamida (SDS-PAGE), diikuti dengan pemblotan imuno menggunakan serum daripada 21 orang pesakit yang positif dalam ujian cucuk kulit (SPT). Dalam SDS-PAGE, sebanyak 20 jalur protein (12 hingga 250 kDa) telah diperhatikan dalam ekstrak ketam mentah manakala ekstrak yang dimasak menunjukkan sedikit jalur. Jalur protein antara 40 hingga 250 kDa adalah sensitif terhadap penyahaslian haba dan tidak dapat diperhatikan lagi dalam ekstrak yang dimasak. Dalam eksperimen pemblotan imuno, ekstrak mentah dan yang dimasak masing-masing menunjukkan 11 dan 4 protein pengikat-IgE, dengan berat molekul antara 23 dan 250 kDa. Protein 36 kDa yang tahan haba, yang sepadan dengan tropomiosin ketam telah dikenal pasti sebagai alergen major pada kedua-dua ekstrak. Sebagai tambahan, protein 41 kDa yang sensitif haba yang dipercayai adalah arginine kinase telah dikenal pasti sebagai alergen major dalam ekstrak mentah. Alergen minor yang lain juga telah diperhatikan pada pelbagai berat molekul.

Kata kunci: Scylla serrata, pemblotan imuno, tropomiosin, arginina kinase

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ABSTRACT

Crab meat is a valuable source of proteins and functional lipids and it is widely consumed worldwide. However, the prevalence of crab allergy has increased over the past few years. In order to understand crab allergy better, it is necessary to identify crab allergens. The aim of the present study was to compare the IgE-binding proteins of raw and cooked extracts of mud crab (*Scylla serrata*). Raw and cooked extracts of the mud crab were prepared. Protein profiles and IgE reactivity patterns were identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting using sera from 21 skin prick test (SPT) positive patients. In SDS-PAGE, 20 protein bands (12 to 250 kDa) were observed in the raw extract while the cooked extract demonstrated fewer bands. Protein bands between 40 to 250 kDa were sensitive to heat denaturation and no longer observed in the cooked extract. In immunoblotting experiments, raw and cooked extracts demonstrated 11 and 4 IgE-binding proteins, respectively, with molecular weights of between 23 and 250 kDa. A heat-resistant 36 kDa protein, corresponding to crab tropomyosin was identified as the major allergen of both extracts. In addition, a 41 kDa heat-sensitive protein believed to be arginine kinase was shown to be a major allergen of the raw extract. Other minor allergens were also observed at various molecular weights.

Keywords: *Scylla serrata*, immunoblotting, tropomyosin, arginine kinase

INTRODUCTION

Crab is an example of shellfish which contributes an important source of proteins for humans. *Scylla serrata*, also known as mud crab is highly demanded as a protein food source by various communities in China, Vietnam, Singapore, Taiwan, Hong Kong and Malaysia (Keenan 1999). However, in Malaysia, the mud crab industry is still not making headway and is only carried out on a small scale by local fishermen. From the annual production, over 1.3 million tonnes were contributed by the local fishery industry (FAO 2010).

Consumption of crab may also cause seafood allergy (Abramovitch et al. 2013; Hsu & Li 2012; Ma et al. 2012; Huang et al. 2010). In Malaysia, the prevalence of shellfish allergy, one of which includes crab, was reported to be 44% among patients with allergic rhinitis and asthma (Shahnaz et al. 2001). Most crab species, provoking allergic reaction including the skin, gastrointestinal, respiratory and cardiovascular systems (Lopata & Lehrer 2009; Liang et al. 2008; Fontan et al. 2005). Exposure to crab allergens may occur through ingestion of crab diet and inhalation or skin contact while processing, cooking or working (Butt & Macdougall 2008).

To date, several clinically relevant allergens of crab have been identified (Abdul Rahman et al. 2011; Liang et al. 2008; Lehrer et al. 2003).

Tropomyosin, a 34-38 kDa protein has been demonstrated to be the major allergen for crabs (Rosmilah et al. 2012; Shriver & Yang 2011; Abdul Rahman et al. 2011; Liang et al. 2008; Lehrer et al. 2003) and is believed to be a pan-allergen of invertebrates including arthropods and mollusks (Suma et al. 2007; Lehrer et al. 2003; Ayuso et al. 2002; Chu et al. 2000). Apart from tropomyosin, arginine kinase was also identified in mud crab (Yu et al. 2013; Liu et al. 2012; Shen et al. 2011). Tropomyosins are a family of closely related proteins present in muscle and non-muscle cells and, in association with the troponin complex, they play a central role in the actin-linked calcium regulatory system of muscle contraction in all invertebrates and vertebrates (Reese et al. 1999). Arginine kinase is an enzyme that reversibly catalyzes the transfer of phosphate between ATP and various phosphogens in all invertebrates (Abdel Rahman et al. 2011).

Several additional new allergens of crab were identified as sarcoplasmic calcium-binding protein (20 kDa), troponin (23 kDa), -actine (42 kDa), smooth endoplasmic reticulum Ca2+ATPase (113 kDa) (Abdel Rahman et al. 2011) and myosin light chains (Abramovitch et al. 2013).

Since, there is scarcity of information on characterization of allergens of this species of crab, the present study was conducted to compare the IgE-binding proteins of raw and cooked extracts of the mud crab (*Scylla serrata*) among the local population with crab allergy.

MATERIALS AND METHODS

PREPARATION OF CRAB EXTRACT

Live mud crab was purchased from an aquaculture centre in Port Klang, Selangor. Raw and cooked mud crab extracts were prepared according to procedures described by Rosmilah et al. (2012). Briefly, the crab meat was washed with purified water and then homogenized in phosphate buffered saline (PBS) with pH 7.2 for 3 mins. The homogenates were then extracted overnight at 4°C under a constant mixing using a shaker, followed by centrifugation at 4°C at 4500 rpm for 30 mins and at 14,000 rpm at 4°C for another 30 mins. The clear supernatant was filtered using syringe filter 0.20 µm. Finally, the supernatant was transferred to 1.5 ml eppendorf tubes before lyophilizing using freeze dryer and then stored at -20°C until use. For the cooked extract, the homogenates were boiled for 10 mins before extraction.

PATIENTS' SERA

Sera of 21 patients with history of mud crab allergy and positive skin prick test to mud crab extract were used in this study. Skin Prick Test (SPT) was performed by medical officers at Allergy Clinic of Kuala Lumpur General Hospital. Serum from a non-allergic subject was used as a negative control. Ethical approval was obtained from the Medical Research and Ethics Committee (MREC), Ministry of Health, Malaysia.

SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

SDS-PAGE was performed according to the procedure of Rosmilah et al. (2012). Mud crab extracts were heated at 97˚C for 4 mins in a denaturing Laemmli buffer before resolving into 12.5% separating gel with a 5% stacking gel by using a Mini Protean 3 apparatus (BioRad, USA) at 120mA for 45 mins. Precision Plus Protein Dual Color Standard (BioRad, USA) was used as a marker. The gel was then stained with Coomassie brilliant blue (CBB) R-250. The molecular weight of each protein was estimated by comparing the protein's gel position to the protein markers (Biorad, USA) in the same gel using an imaging densitometer (BioRad, USA).

IMMUNOBLOTTING

Immunoblotting technique was carried out as described by Rosmilah et al. (2012) using sera from 21 patients as described above. Protein samples subjected to SDS-PAGE electrophoresis were transferred onto nitrocellulose membrane using a Mini Transblot System (BioRad, USA) at 100 V for 70 mins. Each membrane was stained with Ponceau S (Sigma, USA) to verify transfer of the separated proteins. Then, the membrane was cut into 3 mm width followed by washing them with TTBS (containing 0.05% Tween 20). Non-specific protein sites were blocked with 5% of non-fat milk in TBS acting as a blocking buffer. Then, the strips were incubated in the test sera at 4˚C overnight with a shaker. The IgE-binding on the strips were detected by incubation with biotinylated goat antihuman IgE (Kirkergaard and Perry Laboratories, UK) and streptavidin-conjugated alkaline phosphatase (BioRad, USA) that encourage labelling the antibody for 30 mins at room temperature. Finally, an Alkaline Phosphatase Conjugate Substrate Kit (BioRad, USA) was used to detect the IgE binding proteins by incubating the strips with the substrate for 10 mins at room temperature. Each plate or set of strips contained a blank (no serum) and a negative control (normal serum).

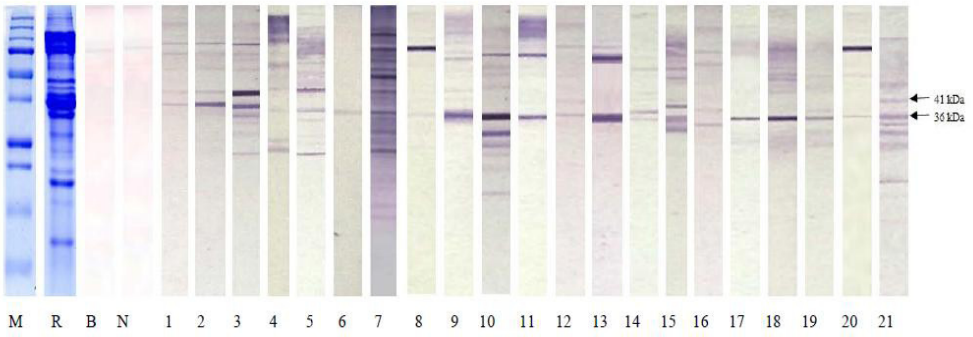
RESULTS

SDS-PAGE OF MUD CRAB EXTRACTS

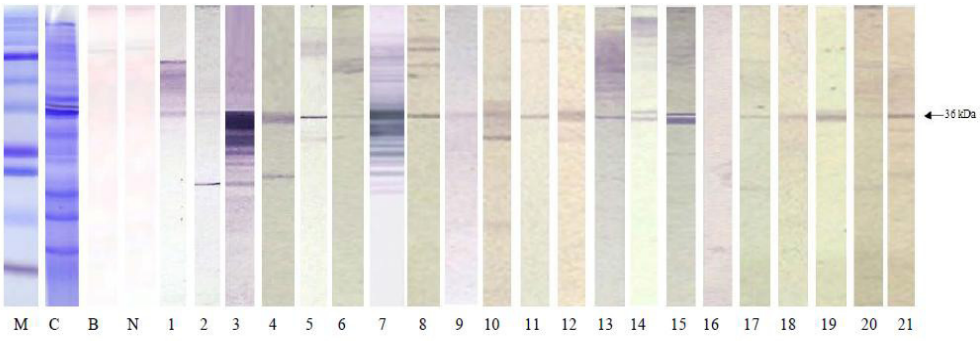
SDS-PAGE of raw mud crab extract revealed 20 protein bands with molecular masses ranging from 12 to 250 kDa. Both raw and cooked extracts afforded a prominent heat-stable protein band of 36 kDa. Protein bands between 40 to 250 kDa were absent in cooked extract compared to raw extract due to heat denaturation. Hence, they were identified as heat-sensitive proteins.

REACTIVITY OF IGE IN PATIENTS' SERA WITH MUD CRAB EXTRACTS

Immunoblotting experiment using 21 sera from mud crab allergic patients demonstrated several IgE-binding proteins at various molecular masses between 23 to 250 kDa (Figure 1). Generally, more IgE-binding was



(a)



(b)

Figure 1: SDS-PAGE and immunoblotting profiles of raw (a) and cooked (b) extracts of mud crab. Lane M indicates molecular weight markers in kiloDalton (kDa). Lane B and N represent a blank and a negative control of a normal serum, respectively. Lane 1 through 21 represents immunoblotting results of sera from 21 patients with mud crab allergy.

detected in the immunoblotting of raw extract compared to cooked extract. A heat-resistant 36 kDa protein was identified as the major allergen of mud crab with frequencies of 95% in both extracts. In addition, a heat-sensitive protein of 41 kDa has also been recognized as a major allergen in raw extract. Other heat-sensitive proteins of 25, 28, 65, 75 and 95 kDa were also recognized as potential minor allergens (frequencies of more than 25%) only in raw extract. IgE-binding profiles of all sera tested were summarized in Table 1. No IgE-binding protein was detected in the negative control and blank.

DISCUSSION

Previous studies reported the prevalence of shellfish allergy to be common among local population in Malaysia with allergic rhinitis and asthma (Shahnaz et al. 2001).

As observed in SDS-PAGE gels, mud crab extracts contain various heat-stabile and heat-labile proteins ranging from 12 to 250 kDa. The raw crab extract revealed 20 protein bands while the cooked extract yielded smaller number of bands compared to raw extract due to denaturation of several protein bands in the range of 40 to 250 kDa. The disappearance of bands

Table 1 : Immunoblotting results of raw (a) and cooked (b) extracts of mud crab using sera from 21 patients with allergy to mud crab

M (kDa)	Subjects																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	%
23							▲			▲												9
25			▲	▲	▲		▲			▲											▲	28
28			▲		▲		▲			▲					▲		▲				▲	33
36	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲		▲	▲	▲	▲	▲	95*
41	▲	▲	▲	▲	▲		▲		▲	▲		▲			▲	▲					▲	57*
50							▲								▲						▲	14
65							▲					▲	▲	▲				▲		▲		28
75	▲			▲			▲	▲	▲		▲					▲				▲		38
95	▲	▲	▲				▲			▲					▲	▲					▲	38
150							▲															4
250							▲															4

(a)

M (kDa)	Subjects																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	%
23		▲	▲	▲			▲										▲				▲	28
25			▲				▲															9
28			▲				▲			▲					▲							19
36	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲		▲	▲	▲	▲	▲	95*
41																						
50																						
65																						
75																						
95																						
150																						
250																						

(b)

M = Molecular weight in kiloDalton (kDa), ▲ = IgE-binding protein, * = Major allergen

in cooked extract may be due to loss of some protein structures. Previous studies showed heat denaturation of globular proteins may disrupt their tertiary structure and lead to random-coiled aggregation and insolubility (Peng & Gygi 2001). Therefore, alteration in protein structure by food processing may lead to epitope destruction, modification, masking or unmasking thereby decreasing, increasing or having no effect on allergenicity (Sathe et al. 2005). In the present study, we detected several heat-resistant and heat sensitive proteins ranging from 23 to 250 kDa as IgE-reactive proteins.

Many food allergens are resistant to denaturation, which allows them to retain the ability to elicit allergic reactions under conditions such as heating and enzymatic degradation. Thus, proteins that are resistant to high heat or digestive conditions may enter the gastrointestinal tract with unaltered epitopes and cause allergic reactions (Shriver & Yang 2011). This is observed in tropomyosin, a 34 to 36 kDa heat-resistant major allergen in numerous invertebrates, including crabs (Rosmilah et al. 2012; Shriver & Yang 2011; Abdul Rahman et al. 2011; Liang et al. 2008; Lehrer et al. 2003). Interestingly, in this study, we found that 95% of patients exhibited IgE-binding proteins at 36 kDa in both extracts. Thus, it is possible that our 36 kDa may be due tropomyosin. Several sera demonstrated the enhancement of IgE-binding intensity to the tropomyosin protein (36 kDa) in immunoblotting of cooked extract indicating increased allergenicity. This finding was similar to other reports which suggested that tropomyosin was more allergenic in

boiled forms (Rosmilah et al. 2012; Yadzir et al. 2010; Liu et al. 2010a; Besler et al. 2001; Nagpal et al. 1989), probably because boiled tropomyosin may have undergone protein-protein interactions such as aggregation during the thermal treatment to cause enhanced IgE binding. Therefore, the boiled tropomyosin seems to be more effective in diagnosing of seafood allergy (Liu et al. 2010b).

Aside from tropomyosin, arginine kinase with molecular weight of 40 kDa has also been identified as a significant allergen in crabs (Shen et al. 2011; Abdel Rahman et al. 2011; Yu et al. 2003; Hendrickx et al. 1998). Notably, enzymes including arginine kinase are sensitive to high temperature and will lead to denaturation (Hendrickx et al. 1998). In the present study, we found that 57% of our subjects had binding to the 41 kDa band, which may be arginine kinase. However, the mechanism of allergic sensitization to arginine kinase is unknown (Yu et al. 2003). Although, it was reported that purified arginine kinase may bind IgE antibodies in *in vitro* tests and induce allergic reactions in sensitized patients, the clinical relevance and the functional relationship between arginine kinase activity and allergenicity may require further investigation (Yu et al. 2003). Moreover, other heat-sensitive proteins at 25, 28, 65, 75 and 95 kDa were detected as potential minor allergens in raw extract. In the present study, minor allergens were also observed at various molecular masses. However, more detailed identification and characterization of these proteins need to be performed.

CONCLUSION

The results of the present study showed that mud crab has two major allergens, a heat-resistant protein of 36 kDa and a heat-sensitive protein of 41 kDa. Further studies using allergenomic approaches may be carried out to identify the biochemical properties of these major allergens.

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