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IDENTIFICATION OF TROPOMYOSIN AS THE MAJOR ALLERGEN OF BLACK TIGER PRAWN (*PENAEUS MONODON*) BY AN ALLERGENOMIC APPROACH

Rosmilah Misnan[a*], Noor Asyikin Kamarazaman[a], Zailatul Hani Mohd Yadzir[b], Noormalin Abdullah[b], Mohd Faizal Bakhtiar[b], Shahnaz Murad[b]

[a]Department Of Biology, Faculty Of Science And Mathematics, Universiti Pendidikan Sultan Idris, 35900 Tanjong Malim, Perak, Malaysia
[b]Allergy And Immunology Research Centre, Institute For Medical Research, 50588 Kuala Lumpur, Malaysia

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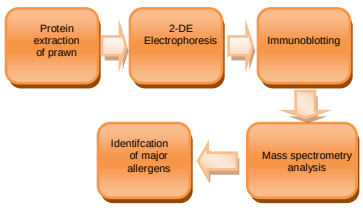
*Corresponding author
rosmilah@fsmt.upsi.edu.my

Graphical abstract



Abstract

Shellfish has been recognized as one of the leading causes of food allergy in both adults and children in Asia Pacific region. In Malaysia, black tiger prawn (*Penaeus monodon*) is among the most widely consumed species. Our previous studies have successfully identified several major allergens including a thermostable protein of 36 kDa. Thus the aim of this study was to identify the 36 kDa major allergen by an allergenomic approach. Protein extracts of raw prown were prepared and resolved by 2-dimensional electrophoresis (2-DE). Immunoblotting was then performed using sera from patients with prawn allergy. Selected spot from 2-DE was then excised, digested and analyzed by mass spectrometry. The 2-DE profile of the extract revealed approximately 100 protein spots between pH of ~4 to 10 and the size range between (<10 to 250 kDa). The 2-DE immunoblotting has detected numerous IgE-binding spots at 36 kDa. Mass spectrometry analysis of the major IgE-binding spot (spot 1a) has identified the 36 kDa spot as tropomyosin. Our findings indicated that tropomyosin play a major role in allergic reaction to black tiger prawn among local patients with prawn allergy, and should be included in diagnostics and therapeutic strategies of this allergy.

Keywords: Allergy, black tiger prawn, *Penaeus monodon*, proteomics

1.0 INTRODUCTION

Prawn has been widely identified as the largest shellfish group that can causes allergy [1-4]. In Malaysia, the prevalence of shellfish allergy including prawn allergy was reported to be 44% among patients with allergic rhinitis and asthma [5]. So far, allergens of 34 to 38 kDa identified as tropomyosin have been established as the major allergen of a number of prawn [3, 4, 6, 7]. Beside tropomyosin, arginine kinase with a molecular mass of 40 kDa [6-9] and sarcoplasmic calcium-binding protein (SCP) with a molecular mass of ~20 kDa [10] and myosin light chain [11] have also been reported as prawn allergens.

In Malaysia, black tiger prawn (*Penaeus monodon*) which is also locally known as 'udang harimau' is among the most widely consumed species [12]. This prawn is also considered as the most common allergenic prawn among local patients with atopic

diseases. Our previous studies on characterization of major allergens of this species of prawn have successfully identified several major allergens at molecular weights of 36, 41, 51 and 75 kDa by immunoblotting [13]. However, the biochemical characterization of the allergens has yet to be identified.

Thus, the aim of this study was to identify one of the major allergen, the heat-stable 36 kDa protein by an allergenomic approach. The findings from this study will be used for production of recombinant proteins with the biochemical and immunological properties of the natural allergen, with high specificity and sensitivity, which may directly contribute to advancements in diagnosis (in vivo and in vitro), management of allergic patients to the development of immunotherapy and to the standardization of allergenic test products as tools in molecular allergology.

2.0 MATERIALS AND METHODS

2.1 Preparation of Allergen Extracts for Proteomic analysis

Live *P. monodon* was obtained from an aquaculture centre in Selangor. Prawn proteins were extracted according to the procedures described by Zailatul Hani *et al*. [6]. Briefly, the prawn flesh was homogenized in purified water using a blender, followed by an overnight extraction at 4 °C under constant mixing. The homogenates was then centrifuged, sterile-filtered and then lyophilized. The lyophilized extracts were stored at -20 °C until use. Protein concentrations of the extract was determined by the total protein kit (Sigma, USA), according to the manufacturer's instructions.

2.2 Serum Samples

Stored sera (-20 °C) from 10 patients with allergy to *Penaeus monodon* were used in this study. Those sera were confirmed to have specific IgE reactivity to the prawn proteins in our previous study [13]. Serum from a non-allergic individual was used as a control.

2.3 Two-dimension Electrophoresis (2-DE)

The lyophilized extract of the prawn was first purified using a clean-up kit (Biorad, USA). The protein sample was then resuspended overnight in rehydration buffer at room temperature. In brief, 100 µg of the sample was applied to 7 cm of immobilized pH 3-10 non-linear gradient strip (Biorad, USA). The first dimensional was performed using IEF cell (BioRad, USA) to separate the proteins by charge with 4 steps: 100 V for 1 minute, 250 V for 30 minutes, 4000V for 2 hours and 4000 V for 10 000 v-hr. Prior to running the 2nd dimension (SDS-PAGE), the strips were equilibrate in two equilibration buffers of DTT and iodoacetamide, respectively. The strip containing the focused protein fractions was then further separated on 12.5% polyacrylamide gel with 5% stacking gel using Mini Protean 3 apparatus (BioRad, USA). Protein spot was then stained with Coomassie brilliant blue R250 and analyzed by an imaging densitometer (BioRad, USA) and PDQuest Software (BioRad, USA).

2.4 Immunoblotting

The IgE-binding spots of the prawn were detected by immunoblotting using sera from 10 patients as mentioned above. Briefly, the separated protein spots were electrophoretically transferred from unstained 2-DE gel to a 0.45 mm pore size nitrocellulose membrane using a Mini Transblot System (BioRad, USA). The nitrocellulose blot with protein spots (between pH ~4 to 10) was cut, washed, blocked and then incubated overnight with individual patient's serum as the primary antibody and biotinylated goat antihuman IgE (KPL, UK) as the secondary antibody. The IgE-binding spots were detected by streptavidin-conjugated alkaline phosphatase (BioRad, USA) and alkaline phosphatase conjugate substrate kit (Biorad, USA).

2.5 Mass Spectrometry Analysis

The coomassie-blue stained protein spots corresponding to those recognized by the above sera were excised, destained, digested by trypsin, and the resulting peptide fragments were analyzed using 4800 Proteomics Analyzer by First Base Laboratories Sdn Bhd, Malaysia. The resulting peptides spectra were searched against the Ludwig NR Database using Matrix Science's Mascot search engine.

3.0 RESULTS AND DISCUSSION

3.1 2-DE profiles and Immunoblots

Figure 1a shows the 2-DE gel profile of *P. monodon* proteins fractionated the 36 kDa band to several distinct protein spots between isoelectric point (*pI*) of approximately 4 to 10. Immunoblotting of the 2-DE gels using sera from 10 different patients showed that several spots at 36 kDa were able to bind to IgE-antibodies. However, only spot 1a (pI ~4.9) was recognized as the major IgE-reactive spot, as detected by more than 50% of the sera (Figure 1b and Table 1). None of the proteins showed reactivity with control serum (data not shown).

3.2 Mass Spectrometry Analysis

The spot 1a were analyzed after tryptic digestion by MALDI-TOF. As shown in Table 2, four peptide fragments were identical to tropomyosin from numerous closely related species of prawn. The observed MW and pI values of the spot were

consistent with the predicted values of the prawn tropomyosin.

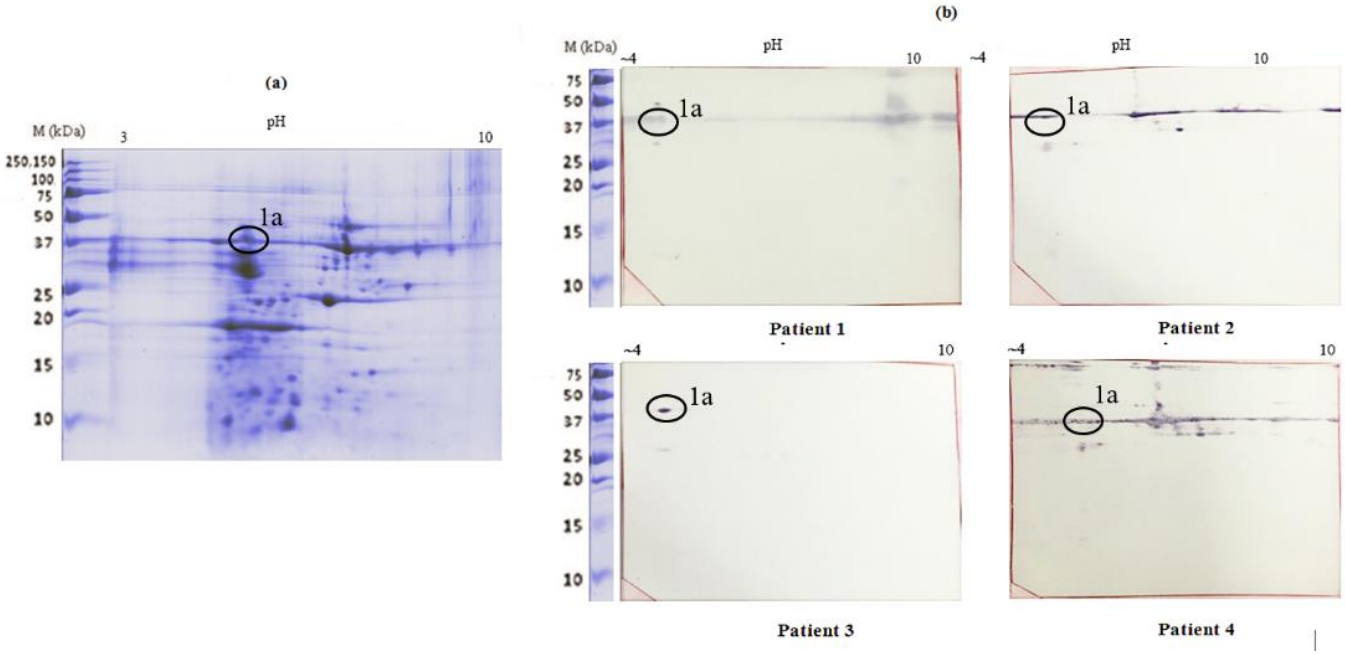


Figure 1 Coomassie blue stained of 2-DE profile of raw prawn (a) and immunoblot profiles of four patients' sera (b). The circle represents the IgE-binding spot at the molecular size of 36 kDa. M, molecular mass markers in kiloDalton (kDa)

Table 1 Immmunoblotting result of black tiger prawn using sera from 10 patients' sera

Spot	Size (kD/pI)	Patients										Frequency (%)
		1	2	3	4	5	6	7	8	9	10	
1a	36, ~4.9	X	X	X	X	X	X	X	X			8 (80%)

Table 2 MASCOT search results summary of black tiger prawn from MS/MS spectra of peptides from spot 1a

Spot	Observed MW, pI	Theroritical MW, pI	Protein Identification	Accession No./Organism	% Coverage MS/MS Match Peptide
1a	36000, ~4.9	32794, 4.73	Tropomyosin	tr | C0LU07 *(Procambarus clarkia)*	13% (4) IQLLEEDLER IVELEEELR IVELEEELRVVGNNLK LAEASQAADESER
		32830 4.72	Tropomyosin	sp | A1KYZ2 (*Penaeus monodon*), tr | A2V731 | (*Penaeus japonicus*), tr | D2KMW0 (*Fenneropenaeus chinensis*)	
		32830, 4.72	Tropomyosin (Pen a 1)	tr | Q3Y8M6 *(Farfantepenaeus aztecus)*	
		32830, 4.72	Tropomyosin (Lit v 1)	tr | B4YAH6 *(Litopenaeus vannamei)*	
		32826, 4.73	Tropomyosin	tr | D3XNR9 *(Macrobrachium rosenbergii)*	
		31685, 4.66	Tropomyosin	tr | D3XNS0 *(Fenneropenaeus merguiensis)*	

Currently, seven allergens in numerous species of prawn including tropomyosin, arginine kinase, myosin light chain, sarcoplasmic calcium binding protein, triose phosphate isomerase, aldolase, and titin were identified as prawn allergens [3, 4, 6-11]. Our previous study has identified several major allergens of *P. monodon* at the molecular mass of 36 kDa, 42 kDa, 49 kDa and 75 kDa by IgE-immunoblots [13]. The 36 kDa has the highest binding capacities, therefore was selected in this current study to be further identified.

The immunoblots of 2-DE map obtained with different sera showed a remarkable heterogeneity in recognition of the prawn allergens. The variation could be due to genetic variation, environmental factors and different exposure to allergens [14], which reflecting different patterns of allergen sensitization and allergy symptoms [15].

MALDI-TOF analysis of the peptide fragments isolated from the digested spot of 36 kDa identifies the spot as tropomyosin. The spot strongly matched with tropomyosin from numerous species of prawn includes *Penaeus monodon* (black tiger prawn), *Penaeus japonicus* (Kuruma prawn), *Fenneropenaeus chinensis* (fleshy prawn), *Litopenaeus vannamei* (Pacific white shrimp) and *Macrobrachium rosenbergii* (giant freshwater prawn). This finding was consistent with other reports which described tropomyosin as the major and cross-reactive allergens among various species of crustaceans, mollusks, mites, insects and other invertebrates [7,16,17].

Tropomyosin, a highly water soluble and heat stable protein with a molecular weight of 34 to 38 kDa is a highly conserved actin-binding proteins exist in muscle and non-muscle cells of all vertebrates and invertebrates, and plays a central role in muscle contraction [2,6].

4.0 CONCLUSION

We have identified the heat-resistant major allergens of 36 kDa as prawn tropomyosin. This data could provide general insights in the properties of epitopes being responsible for eliciting allergic reaction in patients with prawn sensitization. Hence, we recommended that the allergen should be included for diagnostic and therapeutic strategies of prawn allergy.

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