

RESEARCH NOTE

IDENTIFICATION OF TROPOMYOSIN AS MAJOR ALLERGEN OF WHITE SQUID (*LOLIGO EDULIS*) BY TWO-DIMENSIONAL IMMUNOBLOTTING AND MASS SPECTROMETRY

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Abstract. IgE-mediated allergic reaction to squid is one of the most frequent molluscan shellfish allergies. Previously, we have detected a 36 kDa protein as the major allergen of *Loligo edulis* (white squid) by immunoblotting using sera from patients with squid allergy. The aim of this present study was to further identify this major allergen using a proteomics approach. The major allergen was identified by a combination of two-dimensional electrophoresis (2-DE), immunoblotting, mass spectrometry and bioinformatics tools. The 2-DE gel fractionated the cooked white squid proteins to more than 50 different protein spots between 10 to 38 kDa and isoelectric point (pI) from 3.0 to 10.0. A highly reactive protein spot of a molecular mass of 36 kDa and pI of 4.55 was observed in all of the patients' serum samples tested. Mass spectrometry analysis led to identification of this allergen as tropomyosin. This finding can contribute to advancement in component-based diagnosis, management of squid allergic patients, to the development of immunotherapy and to the standardization of allergenic test products as tools in molecular allergology.

Keywords: *Loligo edulis*, tropomyosin, allergen, 2-DE, immunoblotting, mass spectrometry

INTRODUCTION

Loligo edulis, known as the white squid, is classified in the phylum Mollusca, class Cephalopoda, order Teuthida and

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family Loliginidae. White squid are one of the popular edible shellfish in Malaysia; approximately 69,000 tons are caught along the Peninsular Malaysia annually (DOF, 2009). However, an IgE-mediated allergic reaction to squid is one of the most frequent shellfish allergies among local patients with allergic rhinitis and asthma (Shahnaz *et al*, 2001). Hypersensitivity to squid may induce symptoms of food allergy in sensitized individuals such as

urticaria, angioedema, asthma, rhinitis, gastrointestinal distress and anaphylaxis (Sampson, 1993; Miyazawa *et al*, 1996).

To date, there are only few reports on the identification of squid allergens. Miyazawa *et al* (1996) first reported a 38 kDa heat stable protein, designated as TOD p 1, as the major allergen of Pacific squid/Pacific flying squid (*Todarodes pacificus*) and identified it as tropomyosin based on nucleotide and amino acid sequence comparison. No allergens from white squid (*Loligo edulis*) have yet been fully characterized, although tropomyosin has been detected and implicated (Leung *et al*, 1996; Yadzir *et al*, 2010). In our previous study, the major allergens of white squid (*Loligo edulis*) were detected using one-dimensional (1-D) immunoblotting (Yadzir *et al*, 2010). However, 1-D immunoblotting does not explain sensitization to individual allergens because IgE reactive bands may contain more than one protein. Further, the technique allows only detection and not the identification of IgE binding components (Barea *et al*, 1999).

Two-dimensional electrophoresis (2-DE) is a high resolution separation technique that allows simultaneous identification of the several IgE-binding components of a source when coupled with immunoblotting (Barea *et al*, 1999). The technique has been used previously to identify the IgE-binding proteins of shrimp (Yu *et al*, 2003), crab (Abdel Rahman *et al*, 2011) and fish (Misnan *et al*, 2008). In the present study, 2-DE in conjunction with immunoblotting was employed to elucidate the allergen spectra of white squid (*Loligo edulis*). Mass spectrometry of IgE reactive spots most frequently recognized by patients' sera was performed to identify the individual allergens responsible for the allergic sensitization.

MATERIALS AND METHODS

Preparation of allergen extracts for proteomic analysis

White squid was obtained from the local market. Extracts was prepared by washing squid in distilled water, followed by homogenization in phosphate buffered saline (PBS), pH 7.2 (1:10 weight/volume) using a Waring blender. The homogenate was boiled for 30 minutes. Protein was extracted overnight by means of agitation at 4°C. The homogenate was centrifuged at 6,196g for 30 minutes at 4°C and then at 19,277g for 15 minutes at 4°C. The clear supernatant then was filtered using a sterile 0.45 μ m syringe filter. The lyophilized extracts were stored at -20°C until use. The protein content of the extracts was determined using Total Protein Kit (Sigma-Aldrich, St Louis, MO).

Serum samples

Stored sera (-20°C) from patients with positive skin prick test (SPT) and immunoblotting to white squid were used in this study (Yadzir *et al*, 2010).

Gel electrophoresis

Protein extract was mixed with a rehydration buffer containing 8 M urea, 50 mM DTT, 4% chaps, 0.2% carrier ampholyte pH 3-10, 0.0002% bromophenol blue. For the 7 cm immobilized non-linear pH 3-10 gradient strip (BioRad, Hercules, CA), 50 μ g of squid extract was applied for rehydration overnight (12-14 hours). Isoelectric focusing was performed at 20°C using Protean IEF Cell apparatus (BioRad, Hercules, CA) with the following voltage/time gradient: 100 V for 1 minute, 250 V for 30 minutes, 4,000 V for 2 hours and 4,000 V for 10,000 V-hours (V-hr). Before transferring the IPG strip onto the second dimension electrophoresis step, the strip was equilibrated sequentially for 10 minutes

in a buffer containing 65 mM dithiothreitol and then 135 mM iodoacetamide in 125 mM Tris-HCl, pH 6.8, 6 M urea, 2% SDS, 30% glycerol and 0.01% bromophenol blue. After equilibration, the strip then was placed on 12% polyacrylamide separating gel and sealed in place using ReadyPrep Overlay Agarose (BioRad, Hercules, CA). Proteins were separated using Mini Protean 3 apparatus (BioRad, Hercules, CA) for 45 minutes or until the bromophenol blue dye reached the bottom of the gel. Protein spots profile was visualized with Coomassie brilliant blue (CBB) R-250 dye.

2 DE immunoblotting

For specific IgE immunodetection, proteins on the 2-D gel were electrotransferred from the gel to a nitrocellulose membrane using Mini Transblot System (BioRad, Hercules, CA) at 100 V for 70 minutes. The non-specific sites were blocked with 5% non-fat milk in Tris-buffered saline (TBS). Following washing with TBS containing 0.05% Tween 20 (TTBS), the membrane was incubated with individual patient's serum overnight at 4°C. IgE-binding proteins were detected using biotinylated goat antihuman IgE antibody (Kirkegaard and Perry Laboratories, Gaithersburg, UK) diluted 1:1,000 in TBS with 5% non-fat milk, followed by incubation with streptavidin-conjugated alkaline phosphatase (BioRad, Hercules, CA) for 30 minutes at room temperature. Finally, Alkaline Phosphatase Conjugate Substrate Kit (BioRad, USA) was used for color development. Serum from a non-allergic subject was used as negative control.

Identification of the major allergenic spots

The Coomassie-stained protein spots corresponding to those recognized by the patients' sera were manually excised

and destained with 50% (v/v) acetonitrile containing 50 mM ammonium bicarbonate. The spots then were dried in 100% acetonitrile. These protein spots were analyzed using mass spectrometry at Adelaide Proteomics Centre and Hanson Institute, Australia. In brief, the protein spot were subjected to tryptic digestion using Mass Prep Robotic Workstation followed by reverse-phase LC separation of the peptides and analysis by electrospray-IonTrap mass spectrometry. The resulting spectra were matched to protein sequence database using the MS/MS search program from Matrix Science (Mascot, www.matrix-science.com) and Vertebrates taxonomy using the NCBI database. Search results were tabulated and scores assigned allowing identification of proteins in the digests.

RESULTS

2-DE profiles and immunoblots

Coomassie blue staining of the separated protein components showed ~50 distinct spots, with molecular weights of 10 to 38 kDa and pI ranging from 3.0 to 10.0 (Fig 1a), whereas immunoblotting showed a relatively simple IgE-reactive spots. The most reactive spot with a molecular weight of 36 kDa and a pI of 4.55 was observed in all of the serum samples (Fig 1b). None of the proteins showed reactivity with control serum (data not shown).

Mass spectrometry

In the peptide mass fingerprint analysis, the excised protein band showed the highest correlation with tropomyosin from neon flying squid *Ommastrephes bartramii* (accession no. gi|83715934) with peptide sequence coverage of 78%, combined ion score (threshold score/cut) of 1629/41 and 28 unique peptides.

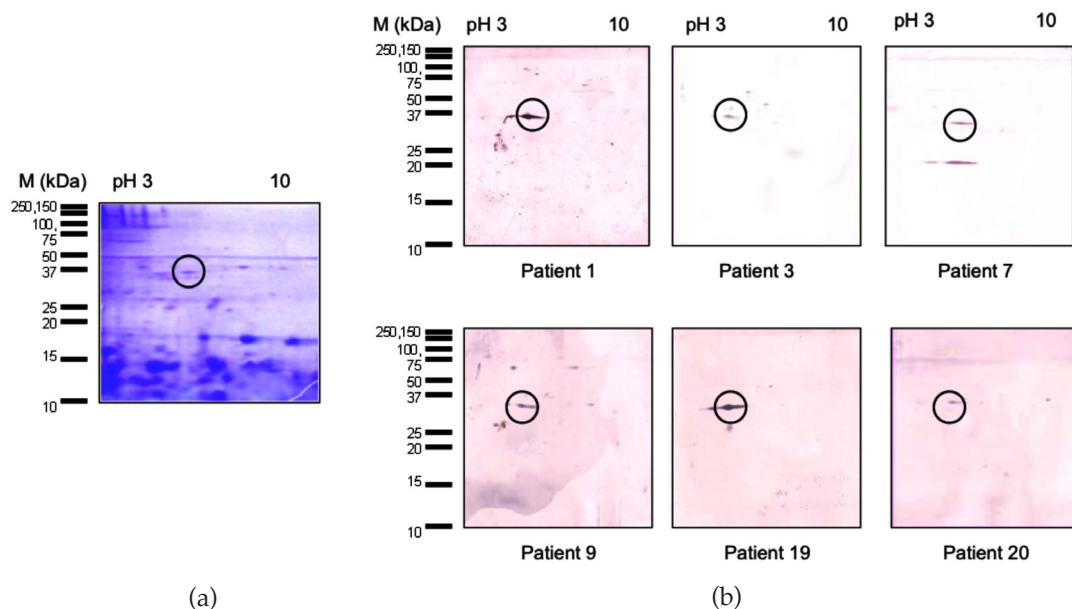


Fig 1—Two dimensional electrophoresis and immunoblot analysis of cooked white squid (*Loligo edulis*) protein extract. (a) Coomassie blue stained blot. (b) Immunoblot with individual patients' sera. The circle shows the spot analyzed by LC/MS/MS.

DISCUSSION

During the last decade, purified native or recombinant allergens have shown promise as a safe and reliable diagnosis and therapy of allergic disorders (AAAAI, 1997). For this purpose, identification of the relevant allergens with appropriate techniques is a crucial step (Chardin *et al*, 2002). The introduction of proteomic techniques has paved a way to identify and characterize the important allergenic proteins in complex biological mixtures (Peng and Gygi, 2001). In this study, we evaluated a proteomic strategy for characterizing a 36 kDa allergen of white squid (*Loligo edulis*). Characterizing this 36 kDa allergen is therefore an important step towards understanding, reliable diagnosis and safe therapy of squid allergic disorders. Moreover, it has been proposed that only a few allergens may

be sufficient to detect the vast majority of allergen-specific sensitizations owing to the predominance of some allergens and the high degree of allergen cross-reactivity (Valenta *et al*, 1999; Kazemi-Shirazi *et al*, 2002). There are even concerns that immunotherapy with natural extracts that consist of mixture of allergenic components to which the patient is not sensitized may in fact induce new sensitization that may worsen the symptoms rather than reducing them (Kazemi-Shirazi *et al*, 2002).

In a previous study by 1-D immunoblotting, a 36 kDa protein fraction was IgE reactive to more than 50% of patient's sera in both raw and cooked extracts (Yadzir *et al*, 2010). In this study, we used cooked extract only for 2-D immunoblot analysis. We did not investigate raw squid extract further, as in this country it is often eaten cooked. The majority of immune responses will be derived to heat treated

squid allergens. In addition, in reports on squid allergy, patients develop immediate hypersensitivity reactions after eating squid or inhaling vapors while cooking squid (Miyake *et al*, 1987; Carrillo *et al*, 1992; Witteman *et al*, 1994). The signal caused by specific IgE-bound allergens might reflect altered epitopes on allergens that cause an increase in IgE affinity. It has been suggested previously that thermal processing may alter epitopes of allergens leading to enhanced antibody affinities (Jirapongsananuruk *et al*, 2008; Piboonpocanun *et al*, 2010).

In a recent study, tropomyosin from *Ommastrephes bartramii* was strongly matched to this 36 kDa protein as the most abundant. Tropomyosin is a 34 to 38 kDa protein that is highly water soluble and heat stable (Daul *et al*, 1994). Tropomyosin is a highly conserved actin-binding protein that exists in muscle and non-muscle cells of all species of vertebrates and invertebrates. Tropomyosin is also a pan-allergen since a specific IgE cross-reacts with a broad range of invertebrates, such as crustaceans (shrimp, crab and lobster), mollusks (squid, octopus, cuttlefish, mussel, scallop, snail and oyster), arachnids (house dust mites) and insects (cockroaches and midges) (Shanti *et al*, 1993; Leung *et al*, 1996; Santos *et al*, 1999; Reese *et al*, 1999; Chu *et al*, 2000; Mikita and Padlan, 2007). In contrast, tropomyosin of vertebrates, including cattle, chicken and other animals, appear to be non-allergenic (Reese *et al*, 1999; Mikita and Padlan, 2007). Cross-reactivity to tropomyosin has been reported between mollusks species (Lopata *et al*, 1997), between mollusks and crustaceans (Daul *et al*, 1993; Leung *et al*, 1996; Leung and Chu, 1998a; Reese *et al*, 1999) and between mollusks and insects or mites (Koshte *et al*, 1989; Van Ree *et al*, 1996; Pajno *et al*, 2002; Azofra and Lombardero, 2003).

Analysis of putative allergens with proteomic strategies is a promising avenue for rapid identification leading to insights into possible clinical applications for diagnosis and therapy.

ACKNOWLEDGEMENTS

The authors wish to thank the Director General of Health Malaysia for his permission to publish this paper. This work was supported by a grant (JPP-IMR 09-020) from Ministry of Health, Malaysia.

REFERENCES

- Abdel Rahman AM, Kamath SD, Lopata AL, Robinson JJ, Helleur RJ. Biomolecular characterization of allergenic proteins in snow crab (*Chionoecetes opilio*) and de novo sequencing of the second allergen arginine kinase using tandem mass spectrometry. *J Proteomics* 2011; 74: 231-4.
- American Academy of Allergy Asthma and Immunology (AAAAI). The use of standardized allergen extracts. *J Allergy Clin Immunol* 1997; 99: 583-6.
- Azofra J, Lombardero M. Limpet anaphylaxis: Cross-reactivity between limpet and house-dust mite *Dermatophagoides pteronyssinus*. *Allergy* 2003; 58: 146-9.
- Barea PL, Calvo E, Rodriguez JA, *et al*. Characterization of *Candida albicans* antigenic determinants by two-dimensional polyacrylamide gel electrophoresis and enhanced chemiluminescence. *FEMS Immunol Med Microbiol* 1999; 23: 343-54.
- Carrillo T, Castillo R, Caminero J, *et al*. Squid hypersensitivity: A clinical and immunologic study. *Ann Allergy* 1992; 68: 483-7.
- Chardin H, Raulf-Heimsoth M, Chen Z, *et al*. Interest of two-dimensional electrophoresis analysis for the characterization of the individual sensitization to latex allergen. *Int Arch Allergy Immunol* 2002; 128: 195-203.
- Chu KH, Wong SH, Leung PSC. Tropomyosin is the major mollusk allergen: Reverse

- transcriptase polymerase chain reaction, expression and IgE reactivity. *Mar Biotechnol* 2000; 2: 499-509.
- Daul C, Slattery M, Reese G, Lehrer SB. Identification of the major brown shrimp (*Penaeus aztecus*) as the muscle protein tropomyosin. *Int Arch of Allergy Immunol* 1994; 105: 49-55.
- Department of Fisheries (DOF) Malaysia, 2009. [Cited 2010 Aug 10]. Available from: URL: <http://www.dof.gov.my> (2 July 2009).
- Jirapongsananuruk O, Sripramong C, Pacharn P, et al. Specific allergy to *Penaeus monodon* (seawater shrimp) or *Macrobrachium rosenbergii* (freshwater shrimp) in shrimp-allergic children. *Clin Exp Allergy* 2008; 38: 1038-47.
- Kazemi-Shirazi L, Niederberger V, Linhart B, Lidholm J, Kraft D, Valenta R. Recombinant marker allergens: diagnostic gatekeepers for the treatment of allergy. *Int Arch Allergy Immunol* 2002; 127: 259-68.
- Koshte VL, Kagen SL, Aalberse RC. Cross-reactivity of IgE antibodies to caddis fly with Arthropoda and Mollusca. *J Allergy Clin Immunol* 1989; 84: 174-83.
- Leung PSC, Chow WK, Duffey S, Kwan HS, Gershwin ME, Chu KH. IgE reactivity against a cross-reactive allergen in crustacean and mollusca: Evidence for tropomyosin as the common allergen. *J Allergy Clin Immunol* 1996; 98: 954-61.
- Leung PSC, Chu KH. Molecular and immunological characterization of shellfish allergens. In: Le Gal Y, Halvorson HO, eds. *New developments in marine biotechnology*. New York: Plenum Press, 1998; 155-64.
- Lopata AL, Zinn C, Potter PC. Characteristics of hypersensitivity reactions and identification of a unique 49 kDa IgE-binding protein (Hal-m-1) in abalone (*Haliotis midae*). *J Allergy Clin Immunol* 1997; 100: 642-8.
- Mikita CP, Padlan EA. Why is there a greater incidence of allergy to the tropomyosin of certain animals than to that of others? *Med Hypotheses* 2007; 69: 1070-3.
- Misnan R, Murad S, Taylor G, et al. Identification of parvalbumin and other proteins as major allergens of Indian Scad (*Decapterus russelli*, Ruppell). *Asian Pac J Allergy Immunol* 2008; 26: 191-8.
- Miyake T, Kawamori J, Yoshida T. Detection of specific IgE antibodies to squid by radioimmunoassay with squid extract for scratch testing: a case of anaphylactic shock induced by squid. *Jpn J Pediatr Allergy Clin Immunol* 1987; 1: 14-7.
- Miyazawa H, Fukamachi H, Inagaki Y, et al. Identification of the first major allergen of a squid (*Todarodes pacificus*). *J Allergy Clin Immunol* 1996; 98: 948-53.
- Pajno GB, La Gutta S, Barberio G, Canonica GW, Passalacqua G. Harmful effect of immunotherapy in children with combined snail and mite allergy. *J Allergy Clin Immunol* 2002; 109: 627-9.
- Peng J, Gygi SP. Proteomics: the move to mixtures. *J Mass Spectrom* 2001; 36: 1083-91.
- Piboonpocanun S, Boonchoo S, Pariyaprasert W, Visitsunthorn N, Jirapongsananuruk O. Determination of storage conditions for shrimp extracts: analysis of specific IgE-allergen profiles. *Asian Pac J Allergy Immunol* 2010; 28:47-52.
- Reese G, Ayuso R, Lehrer SB. Tropomyosin: An invertebrate pan-allergen. *Int Arch Allergy Immunol* 1999; 119: 247-58.
- Sampson HA. Adverse reactions to foods. In: Middleton EJr, Reed CE, Ellis EF, Adkinson NF, Yunginger JW, Busse WW, eds. *Allergy: principles and practice*. St. Louis: C.V. Mosby, 1993: 1661-86.
- Santos AB, Chapman MD, Aalberse RC, et al. Cockroach allergens and asthma in Brazil: Identification of tropomyosin as a major allergen with potential cross-reactivity with mite and shrimp allergens. *J Allergy Clin Immunol* 1999; 104: 329-37.
- Shahnaz M, Gendeh BS, Nasuruddin A. Skin test reactivity to inhalant and food allergens in patients with allergic rhinitis. *Int Med Res J* 2001; 5: 69-73.

- Shanti KN, Martin BM, Nagpal S, Metcalfe DD, Subba Rao PV. Identification of tropomyosin as the major shrimp allergen and characterization of its IgE-binding epitopes. *J Immunol* 1993; 151: 5354-63.
- Valenta R, Lidholm J, Niederberger V, Hayek B, Kraft D, Grönlund H. The recombinant allergen-based concept of component-resolved diagnostics and immunotherapy (CRD and CRIT). *Clin Exp Allergy* 1999; 29: 896-904.
- Van Ree R, Anonicelli L, Akkerdas JH, *et al.* Asthma after consumption of snails in house dust mite allergic patients: A case of IgE cross reactivity. *Allergy* 1996; 51: 387-93.
- Witteaman AM, Akkerdaas JH, van Leeuwen J, van der Zee JS. Identification of a cross-reactive allergen (presumably tropomyosin) in shrimp, mite and insects. *Int Arch Allergy Immunol* 1994; 105: 56-61.
- Yadzir MZH, Misnan R, Abdullah N, Bakhtiar F, Arip M, Murad S. Identification of IgE-binding proteins of raw and cooked extracts of *Loligo edulis* (white squid). *Southeast Asian J Trop Med Public Health* 2010; 41: 653-9.
- Yu CJ, Lin YF, Chiang BL, Chow LP. Proteomics and immunological analysis of a novel shrimp allergen, Pen m 2. *J Immunol* 2003; 170: 445-53.