## RESEARCH NOTE

# IDENTIFICATION OF IgE-BINDING PROTEINS OF RAW AND COOKED EXTRACTS OF *LOLIGO EDULIS* (WHITE SQUID)

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**Abstract.** Allergy to different classes of mollusks, including squid, which are members of the class Cephalopods has been reported. Tropomyosin, a major muscle protein, is the only well-recognized allergen in squid. The aim of this study was to characterize IgE-binding proteins of local *Loligo edulis* (white squid) consumed in Malaysia. Protein profiles and IgE-binding proteins were detected by sodium dodecyl sulfate-polyacrylamide gel-electrophoresis (SDS-PAGE) and immunoblotting using sera from 23 patients with positive skin prick test to raw squid extract. SDS-PAGE of the raw extract exhibited 21 protein bands (10-170 kDa) but those ranging from 19 to 29 kDa and 41 to 94 kDa were not found in the cooked extract. Immunoblotting of raw extract demonstrated 16 IgE-binding bands, ranging from 13 to 170 kDa. A heat-resistant 36 kDa protein, corresponding to squid tropomyosin, was identified as the major allergen of both extracts. In addition, a 50 kDa heat-sensitive protein was shown to be a major allergen of the raw extract. Our findings indicate that the allergen extract used for diagnosis of squid allergy should contain both the 36 kDa and 50 kDa proteins.

**Key words:** *Loligo edulis,* allergy, IgE-binding proteins, SDS-PAGE

## INTRODUCTION

Shellfish is a well known cause of allergic reactions to food in hypersensitive individuals (Lee and Park, 2004). Shellfish is a broad term for all aquatic animals that have a shell or shell-like exoskeleton (Lu *et al*, 2007). The generic term "shellfish" includes those sea animals belonging to phylum Arthropods, Class Crustaceans (shrimp, prawn, lobster, crab and crayfish), and to phylum Mollusks, class Gastropods (abalone and snail), class Bivalvia (clam, cockle, mussel, scallop and oyster) and class Cephalopods (octopus, cuttlefish and squid) (Daul *et al*, 1993). Mollusks, including squid, play an important role in human nutrition and the world economy (Wild and Lehrer, 2005). However, IgE-mediated allergic reaction to squid is one

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of the most frequent molluscan shellfish allergies (Castillo *et al,* 1994). Sensitization to squid may involve the skin, gastrointestinal, respiratory and cardiovascular systems (Sampson, 1993; Miyazawa *et al,* 1996).

The major allergen of molluscan shellfish is tropomyosin, a muscle protein. The term major allergen is used to define proteins that elicit IgE binding in the sera of half or more of patients with allergies to the specific source (Metcalfe *et al,* 1996). Tropomyosin is a 34 to 38 kDa protein that is highly water soluble and heat stable (Daul *et al,* 1994). Tropomyosin can be found in both muscle and many non-muscle cells in animals. In muscle cells, tropomyosin is associated with thin filaments in muscle and plays a role in the contractile activity of muscle cells (Smille, 1982). In non-muscle cells, tropomyosin is found in microfilaments but its function is less well understood (Smille, 1982).

Tropomyosin is now recognized as a pan-allergen among 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; Reese *et al,*1999; Santos *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). Miyazawa *et al* (1996) identified the major allergen of squid *Todarodes pacificus* as a protein at 38 kDa (Tod p 1) a marked amino acid sequence homology with tropomyosin from the planorbid blood fluke (*Biomphalaria glabrata*).

In Malaysia, squids are the most important edible mollusks; approximately 69,000 tons are caught along the Peninsular Malaysia annually (DOF, 2009). *Loligo edulis* (white squid) is one of the most frequently consumed squids in Malaysia. Previous research has shown that the prevalence of shellfish allergy is common among patients with allergic rhinitis and asthma (Shahnaz *et al,* 2001), but very little is known about the allergenic proteins of local squid. The aim of this study was to characterize the IgE-binding proteins of *Loligo edulis* in local patients with squid allergy.

## MATERIALS AND METHODS

### Preparation of allergen extracts

White squid was obtained from the local market. Cooked and raw extracts of the squid were prepared as described by Miyazawa *et al* (1996) with minor modification. Raw extract was prepared by washing squid in purified water, followed by homogenization in phosphate buffered saline (PBS), pH 7.2 (1:10 weight / volume) using a Waring blender. The homogenate then was extracted overnight at 4ºC with constant mixing using an orbital shaker, followed by centrifugation at 4ºC at 6,196*g* for 30 minutes and 19,277*g* for 15 minutes. The clear supernatant was then filtered using a sterile 0.45 μm syringe filter. The lyophilized extracts were stored at -20ºC until use. For preparation of cooked extract, the squid was boiled in purified water for 30 minutes, and processed as described above. The protein content of the

extracts was estimated using Total Protein Kit (Sigma, NY).

**Patients' sera**

Sera of 23 patients with a history of squid allergy and a positive skin prick test (SPT) to raw squid extract were used in this study. This study was approved by the Medical Research and Ethics Committee (MREC), Ministry of Health Malaysia.

**Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)**

Protein profiles of the squid extracts were determined by SDS-PAGE using the method described by Miyazawa *et al* (1996) with minor modification. In brief, the squid samples and prestained molecular weight markers (Biorad, CA) were resolved in a 12% separating gel with a 5% stacking gel using a Mini Protean 3 Apparatus (BioRad, CA) at 120 mA for 45 minutes. After electrophoresis, gels were stained with Coomassie brilliant blue R-250 dye. Protein sizes were estimated by comparing the protein bands with molecular weight markers using an Imaging Densitometer GS800 and Quantity One Software (BioRad, CA).

**IgE-immunoblotting**

Immunoblotting was performed to identify the IgE-binding components of raw and cooked white squid extracts using patient's sera. The resolved proteins were electrophoretically transferred from unstained SDS-PAGE gel onto a 0.45 μm pore size nitrocellulose membrane using a Mini Transblot System (BioRad, USA) at 100 V for 70 minutes. Membrane then was stained with Ponceau S dye (Sigma, USA) to verify transfer of the separated proteins. Strips of 3 mm in width were cut from the membrane, washed with Tris-buffered saline (TBS) containing 0.05% Tween 20 (TTBS) and then incubated with blocking buffer containing 5% non-fat milk in TBS. The strips were incubated overnight with individual patient's serum at 4˚ C. Bound IgE on the strips was detected by incubating with biotinylated goat antihuman IgE antibody (Kirkergaard and Perry Laboratories, UK) followed by incubation with streptavidin-conjugated alkaline phosphatase (BioRad, CA) for 30 minutes at room temperature. Finally, Alkaline Phosphatase Conjugate Substrate Kit (BioRad, CA) was used to detect the bound IgE. Serum from a non-allergic subject was used as negative control.

## RESULTS

**SDS-PAGE of squid extracts**

SDS-PAGE of raw white squid extract revealed at least 21 protein bands with molecular masses ranging from 10 to 170 kDa (Fig 1). Smaller numbers of bands were detected in the cooked extract as several protein bands between 19-29 kDa and 41 to 94 kDa were sensitive to heat denaturation and thus were no longer detected in the gel. However, there was an enhancement of band intensity of 170 kDa in the cooked extract. An intense stained band at 36 kDa which is most likely to be squid tropomyosin, was detected in both extracts.

**IgE-immunoblots of patients' sera to squid extracts**

Fig 1 shows the IgE-binding patterns of raw and cooked extracts of white squid using immunoblotting. All tested sera demonstrated IgE-binding to raw extract with various protein profiles ranging from 13 to 170 kDa. On the other hand, immunoblotting of the cooked extract detected only one IgE-binding band at 36 kDa. Nineteen sera (83%) showed IgE-reactivity to both raw and cooked extracts (36 kDa protein band), while the rest showed IgE-binding only to raw extract. SDS-PAGE gel showed enhancement of band intensity of

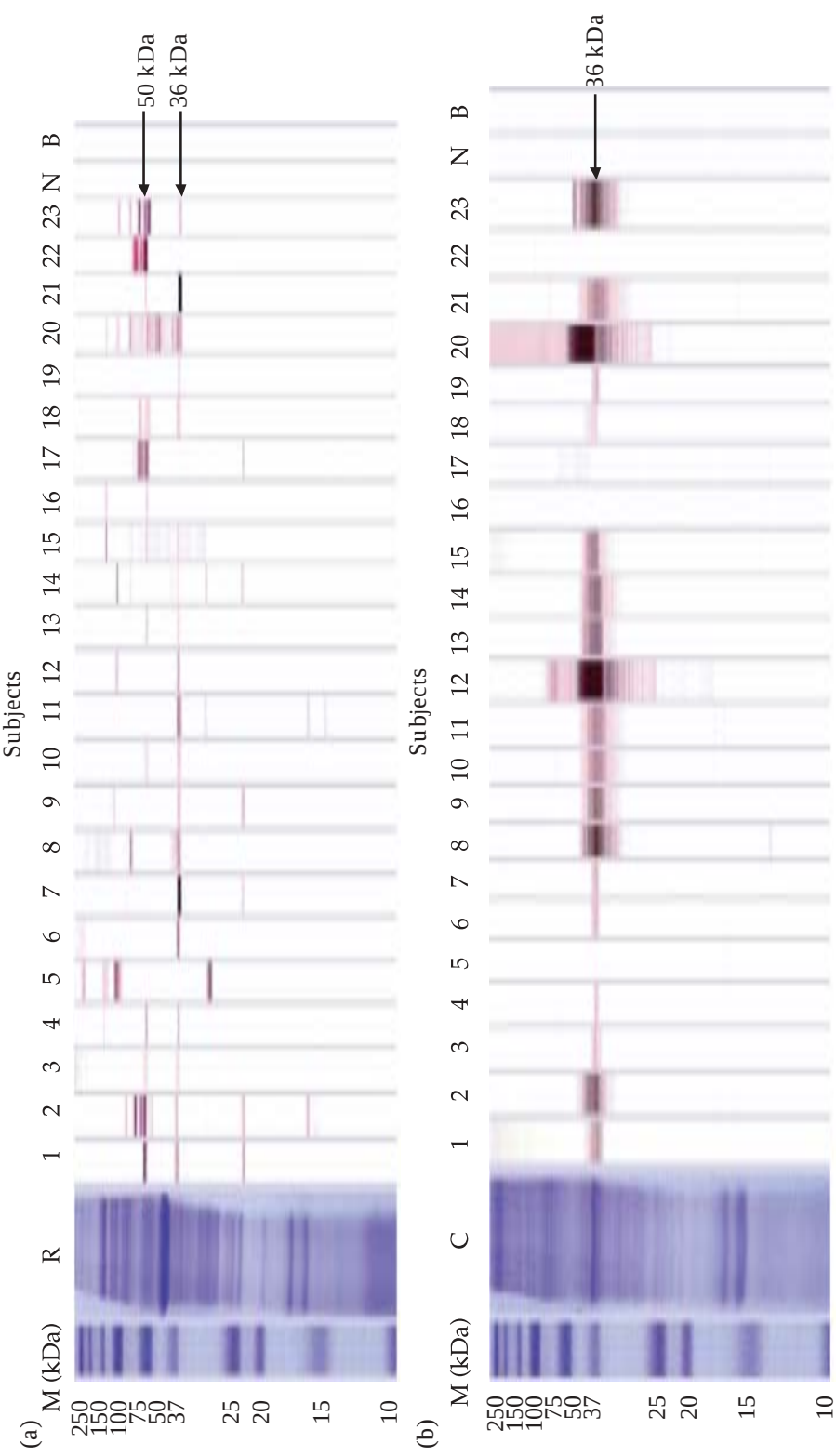


Fig 1–SDS-PAGE and immunoblotting profiles of raw (a) and cooked extracts (b) of *Loligo edulis* (white squid) using sera from 23 patients with white squid allergy. IgE-binding proteins were detected by Western blotting using biotinylated goat antihuman IgE antibody and strepavidin-conjugated alkaline phosphatase. Lane M, molecular mass markers; lane R, Coomassie blue staining of the raw extract; lane C, Coomassie blue staining of the cooked extract; lanes 1-23, immunoblots showing binding of IgE from different serum samples; lane N, immunoblot using serum from a non-allergic individual; and lane B, blank.

a high molecular weight protein (~170 kDa) in the cooked extract, only in one serum, suggesting a minor role in local squid allergy. In addition, a heat-labile protein of 50 kDa was identified as the major allergen of raw squid with the frequency of 61%, with several heat-labile proteins as minor allergens.

## DISCUSSION

A ~36 kDa heat-resistant allergen found in oyster (*Cra g* 1) (Ishikawa *et al,* 1997, 1998a; Leung and Chu, 2001), abalone (*Hal m* 2) (Lopata *et al,* 1997), whelk (*Buc u* 1) (Lee and Park, 2004), mussel (*Per v* 1) (Chu *et al,* 2000), clam (*Ens m* 1) (Jimenez *et al,* 2005), scallop (*Chl n* 1) (Chu *et al,* 2000; Lu *et al,* 2004), octopus (*Oct v* 1) (Ishikawa *et al,* 2001) and squid (*Tod p* 1) (Miyazawa *et al,* 1996) has been identified as tropomyosin. Similarly, our study also has identified a 36 kDa protein as the major allergen of white squid in both raw and cooked extracts. It is possible that this major allergen is probably tropomyosin, the well-known mollusk major allergen.

Studies on the effect of thermal processing on the allergenicity of molluscan shellfish are unavailable. However, several studies on various food proteins reported that thermal processing can lead to the formation of new antigenic structures or loss of some of the original antigenicity (Bernhisel-Broadbent *et al,* 1992; Urisu *et al,* 1997; Paschke and Besler, 2002; Samson *et al,* 2004). Immunoblotting of the cooked extract revealed more intense and high yielding IgE-binding of the 36 kDa protein. This suggests that this protein is highly heat stable and boiling altered the epitopes of the allergen, resulting in an increased IgE affinity as has been shown in shrimp (*Penaues indicus*) allergens (Nagpal *et al,* 1989).

Non-tropomyosin allergens have been identified in a number of molluscan shellfish species, including whelk (Leung *et al,* 1996; Leung and Chu 1998a, b; Lee and Park, 2004), limpet (Morikawa *et al,* 1990; Maeda *et al,* 1991; Azofra and Lombardero, 2003), cuttlefish (Lin *et al,* 1993), oyster, scallop, octopus and squid (Leung *et al,* 1996; Leung and Chu, 1998b). Some of these allergens have been proposed to be hemocyanin (Morikawa *et al,* 1990; Maeda *et al,* 1991), myosin heavy chain (Martins *et al,* 2005) and amylase (Azofra and Lombardero, 2003). Our study has identified an additional major allergen at 50 kDa similar to that reported by Leung *et al* (1996). Cooking may reduce allergenicity of some food allergens and may induce the appearance of higher molecular weight antigens that are not allergenic (Porcel *et al,* 2001). As observed in our study, these major allergens and several additional minor allergens were detected only in the raw squid extract. Moreover, 4 patients (sera 5, 16, 17 and 22) without specific IgE to the 36 kDa had only IgE-binding proteins in the raw squid extract.

In summary, our study has identified two major allergens with different properties which may play an important role in squid allergy among local patients. One was a heat-resistant 36 kDa protein which may correspond to squid tropomyosin, and the other was a heat-labile 50 kDa protein. Our findings suggest that allergen extract used for the diagnosis of squid hypersensitivity should contain both allergens.

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