



Identification of allergic proteins of Flower tail shrimp (*Metapenaeus dobsonii*)

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Abstract Allergy to seafood mainly fish and shellfish has been reported. Tropomyosin is recognized as a major allergen in many crustaceans especially shrimps. A study was carried out to identify the IgE reactive proteins of allergic nature in Flower tail shrimp (*Metapenaeus dobsonii*), a frequently consumed shrimp in India mainly in dried form. Protein profiling and identification of IgE reactive proteins in raw and cooked extracts of Flower tail shrimp was carried out by SDS-PAGE and immunoblotting using sera of 13 patients having allergic reactions on consumption of shrimp and positive to skin prick test. The IgE binding ability was determined by ELISA and it was found higher in the case of cooked extracts ranging from 0.244 to 0.440 at 490 nm. The SDS-PAGE of raw extract revealed many protein bands between 205 and 6.5 KDa, while in the case of cooked extracts bands of 36, 20, 29 and 70 KDa were prominent. Heat resistant protein of 37 KDa, tropomyosin was showing a clear immune reaction both in the case of raw and cooked extracts was identified as the major allergen by all the patient sera. The minor proteins of raw extract identified by immune reaction are 50, 75 and 100 KDa. The identified allergen can be used for the diagnosis and management of shrimp allergy.

Keywords Food allergen · Flower tail shrimp · Immunoblotting · Tropomyosin

Introduction

In many of the developing countries, food allergy is considered as a major food safety concern. Food allergy is mainly associated with hypersensitive reaction mediated through immune system to the dietary intake of proteins (Sicherer and Sampson 2010). Eight kinds of food including soy, wheat, peanut, tree nuts, fish, shellfish, egg and milk have been identified as source of allergens and 90% of food allergies are due to these eight foods. In case of seafood, prawns and shrimps are the major contributors about 20–22% by volume of the world seafood market (FAO 2014). Shellfishes mainly crustaceans and molluscs are frequent elicitor of food hyper sensitive reactions which is mainly mediated through immunoglobulin E and these immune mediated reactions comprise acute urticaria, atopic dermatitis, asthma, gastrointestinal disorders including diarrhea, vomiting and life-threatening anaphylaxis (Sicherer 2011). Higher prevalence of shrimp allergic reactions is noticed in case of developing countries and coastal areas (Lopata et al. 2010). A large variety of shellfish species are reported as causative of allergy (Burney et al., 2010). In addition to ingestion of foods containing shrimp, inhalation or skin contact while cooking or working can also leads to development of immune reactions. Shrimp allergy is considered as a Type I allergy as the initial encounter with an allergen sensitizes the individual to future exposures.

Tropomyosin, a myofibrillar protein having molecular weight ranging from 34 to 38 KD is reported as a major allergen in many crustacean species (Lehrer et al. 2003;

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Motoyama et al. 2007, 2008; Nakano et al. 2008). It consists of two identical subunits with each in α -helix form coiled around one another in the coiled-coil formation (Shanti et al. 1993). It is also involved in cross reactivity with other proteins from invertebrates. Allergic proteins are having specific linear and conformational epitopes which can be recognized by IgE (Ayuso et al. 2010). Among the shrimp sensitized individuals, 80% are able to produce IgE against tropomyosin (Daul et al. 1994). However, tropomyosin from invertebrate source is only reported to be allergenic to humans (Reese et al. 1999). Other minor allergens reported in shrimp include myosin light chain (Ayuso et al. 2008), arginine kinase (Yu et al. 2003) and sarcoplasmic calcium binding proteins (Shioni et al. 2008).

Information regarding allergens associated with the commonly consuming shrimp species in Indian coast is lacking. Also in India, the number of people sensitive to shrimp is more in comparison to fish and there is frequent reporting of shrimp allergic reactions. In this background, *Metapenaeus dobsonii* commonly called as flower tail shrimp, which is a highly preferred shrimp locally was selected to identify the allergic proteins. Locally it is called as 'poovalan chemmeen' or 'thelly chemmeen'. It is a widely distributed small sized shrimp variety in both east and west coast of India and it is a major constituent of catch in trawls, traditional ring seines and mudbank fishery during monsoon season in Kerala (Chakraborty et al. 2014). Shrimp is reported as an excellent source of protein by Yanar and Celik (2006). There is a huge consumer demand for the dried form of this shrimp and also for different products made out of the dried shrimp. As the allergenic protein component and its molecular weight is varying in different shrimp species, it is highly important to understand the allergic component specific to species. Hence the main aim of this study is to evaluate the prevalent allergic protein of Flower tail shrimp that can result hypersensitive reactions in sensitive people.

Materials and methods

Chemicals used

Bovine serum albumin (BSA), O-phnylenediamene dihydrochloride and wide range protein marker for SDS PAGE were procured from Sigma Aldrich, Spruce Street, St. Louis, USA. Precision plus protein dual color standard as protein marker in SDS PAGE for immunoblotting, tween-20, tris glycine transfer buffer and tris buffered saline (TBS) from Bio-Rad, California, USA were used for analysis. Horse radish peroxidase (HRP) conjugated goat anti- human IgE antibody was from Thermo Fisher Scientific, Waltham, MA USA. Opti-4CN substrate kit from

Bio-Rad, California, USA was used for visualization of membranes after immunoblotting. All other chemicals including coomassie blue R-250 and acetic acid were of highest analytical grade from Merck, Mumbai, India.

Proximate and protein composition

The proximate composition analysis of shrimp meat was performed as per AOAC method (AOAC International 2000). The protein composition of shrimp meat is expressed in terms of sarcoplasmic protein, non protein nitrogen, myofibrillar protein, alkali soluble protein and stroma protein as per Hashimoto et al. (1979).

Preparation of shrimp extracts

Fresh flower tail shrimp (*Metapenaeus dobsonii*) samples having an average weight of 13.58 ± 0.42 g and average length of 3.83 ± 0.22 cm were collected from Kalamukku landing centre of Cochin, Kerala. The sample was immediately iced at 1:1 ratio (shrimp to ice) and transferred to the laboratory in an insulated box. Then the shrimps were beheaded, peeled and collected the meat. The meat was homogenized and stored at -20°C for further analysis.

Shrimp extracts were prepared as per Motoyama et al. (2006). For the preparation of raw shrimp extract, the homogenized meat was added with four volumes of 0.01 M Phosphate buffered saline, PBS (Containing 0.137 M NaCl, 0.0027 M KCl, 0.01 M Na_2HPO_4 and 0.0018 M KH_2PO_4 , pH 7.2) and homogenized at 10,000 rpm for 10 min. Then centrifuged at 8000 rpm for 20 min and collected the supernatant as raw extract. In order to prepare crude cooked extract, homogenized meat with four volumes of 0.01 M Phosphate buffered saline was boiled for 15 min and cooled immediately using ice. Centrifuged at 8000 rpm for 20 min and collected the supernatant as crude cooked extract. Protein concentrations of the extracts were determined by Biuret method (Gornall et al. 1949) using bovine serum albumin as standard. The prepared extracts were freeze dried and stored at -20°C .

Human sera of sensitized individuals

Details of 13 patients with a history of immediate hypersensitive reaction and positive skin prick test to shrimp was collected from the record of Mary Queens hospital, allergy and asthma research centre, Cochin, Kerala. The details are given in Table 1. This study was approved by the Institutional Ethical Committee and blood samples were drawn from the patients with their consent for research purpose. The withdrawn blood is kept in a centrifuge tube without

Table 1 Clinical characteristics of shrimp allergic subjects

Sera no.	Sex	Age	Skin prick test	Food material	Symptoms	Family history
P1	M	31	+	Shrimp, crab	Breathlessness, diarrhea, vomiting, oral allergy syndrome, anaphylaxis	No, sibling is allergic
P2	F	41	+	Shrimp	Urticaria, oral allergy symptom	No
P3	M	20	+	Shrimp, crab	Bulging of eyes, oral allergy symptom, urticaria	Yes
P4	F	32	+	Shrimp, crab	Asthmatic, swelling of face and hands, urticaria, vomiting	No
P5	M	56	+	Shrimp, crab	Urticaria, breathing problems	No
P6	F	52	+	Crab, clam	Urticaria, breathing problems	No
P7	F	43	+	Crab, clam	Urticaria, diarrhea	No
P8	M	29	+	Shrimp, crab	Urticaria, oral allergy syndrome	No
P9	F	58	+	Shrimp, crab	Urticaria, oral allergy syndrome	No
P10	F	36	+	Shrimp, crab	Urticaria, breathing problems, oral allergy syndrome	Yes
P11	F	21	+	Shrimp, crab	Urticaria, oral allergy syndrome	No
P12	M	31	+	Shrimp, crab	Urticaria, breathing problems, oral allergy syndrome	No
P13	F	21	+	Shrimp, crab	Breathlessness, urticaria	No, sibling is allergic

EDTA, allowed to stand for one hour, centrifuged at 2500 rpm for 20 min. The obtained supernatant, sera containing IgE antibodies were stored at -20°C . Sera from three healthy volunteers having no adverse reaction to food materials after ingestion were pooled, stored at -20°C and used as control sera for the studies.

ELISA

Enzyme linked immunosorbent assay (ELISA) was carried out as per previously reported method of Ishikawa et al. (1997) using 96 wells flat bottomed polystyrene microtitre plates. Initially, the plates were coated with 100 μl of extracts diluted with 0.05 M carbonate buffer (Containing 0.0345 M NaHCO_3 and 0.0151 M Na_2CO_3 , pH 9.5) at 1:1000. Then it was subjected to immunoreactions with 100 μl of serum from patient as well as control, diluted at 1:50 with 0.1% BSA in PBS. Then added 100 μl of HRP conjugated goat anti- human IgE antibody diluted at 1:2500 with 0.1% BSA in PBS. A substrate solution of 0.1% O-phenylenediamine dihydrochloride and 0.03% hydrogen peroxide in 0.05 M phosphate-citrate buffer, pH 5 (Containing 0.2 M Na_2HPO_4 and 0.1 M citric acid) was used to carry out the enzymic reaction for resulting colour development which can be measured at 490 nm using microplate reader (r-biopharm, Darmstadt, Germany). All the analyses were carried out in triplicate and the results were given as mean $\pm$ standard error.

SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as reported by Laemmli (1970) using Mini Protean 3 Apparatus (Bio-Rad, California, USA). It was carried out with a 12% polyacrylamide separating gel and a stacking gel of 4% at 150 V for 50–60 min. The shrimp extracts were mixed with Lamelli buffer in the ratio of 1:4, heated at 95°C for 4 min, cooled and loaded for running along with a wide range protein marker as a reference. After completion of running the gels were stained with coomassie blue R-250 and destained with acetic acid solution.

Immunoblotting

After electrophoresis, the separated proteins were electrophoretically transferred to a 0.45 μm nitrocellulose membrane using tris glycine transfer buffer in a Mini Trans-Blot System (Bio-Rad, California, USA) at 80 V for 120 min. The membrane was washed with 0.05% tween-20 in TBS and blocked using 3% BSA (w/v) in TBS. After washing, the blocked membrane was reacted with individual patient's sera (diluted at 1:50 in blocking buffer) at 4°C overnight. Followed by the membrane was subjected to reaction with HRP conjugated goat anti- human IgE antibody diluted at 1:500 at 37°C for 1 h. Then the membrane was subjected to incubation with colorimetric HRP substrate using Opti-4CN substrate kit.

Results and discussion

Proximate and protein composition

The proximate composition and composition of protein in terms of sarcoplasmic, myofibrillar, alkali soluble and stroma were given in Table 2. The results obtained are comparable with the reports of Shamsundar and Prakash (1994). High content of proteins make the shrimp meat as an important source of amino acids. The nutritional value, sensory qualities and shelf life of shrimp can depend upon the proximate composition of particular species which is affected by many factors like growth stage, feed and season (Karakoltsidis et al. 1995).

Myofibrillar protein content in the range of 80–82% was reported by Shamsundar and Prakash (1994), while myofibrillar protein content in the range of 76.63% was observed in the present study (Table 2). Sarcoplasmic protein content is the second major protein component in the shrimp meat. As per Hashimoto et al. (1979) and Sriket et al. (2007), the major constituents of fish meat are myofibrillar and sarcoplasmic proteins. The content of non protein nitrogen in prawn meat is 17% of total nitrogen.

IgE binding ability of raw and heated extracts

Allergens are often having the following characteristic or ability to sensitize a genetically predisposed individual by triggering the production of IgE antibodies, also to bind with the particular IgE antibodies, and eliciting allergic reactions. IgE binding ability of raw and heated extracts were carried out by ELISA method using sera from thirteen shrimp allergic patients and a pooled control sera from three healthy individuals and the results are given in Fig. 1. All the shrimp allergic patient's sera showed IgE binding ability to both raw and cooked extracts of flower tail

Table 2 Proximate composition and protein composition of Flower tail shrimp (*Metapenaeus dobsonii*)

Moisture (%)	79.86 ± 1.21
Crude protein (%)	16.87 ± 0.96
Crude fat (%)	1.12 ± 0.01
Ash (%)	1.69 ± 0.04
Sarcoplasmic protein (as % of total protein)	16.82 ± 1.33
Myofibrillar protein (as % of total protein)	76.67 ± 1.65
Alkali soluble protein (as % of total protein)	1.52 ± 0.04
Stroma protein (as % of total protein)	1.02 ± 0.02
Non protein N ₂ (%)	4.9 ± 0.2
Raw extract (g/ 100 g)	5.61 ± 0.46
Cooked extract (g/ 100 g)	3.4 ± 0.4

Values are mean ± SD

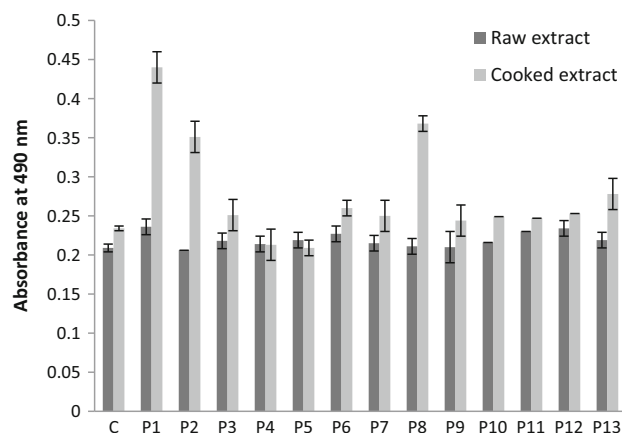


Fig. 1 ELISA analysis of IgE activity of patient and control sera on raw and cooked extracts of Flower tail shrimp (*Metapenaeus dobsonii*)

shrimp, with a higher binding ability to cooked extracts in comparison to raw extracts. The results indicate the definite high reactivity of 85% of patient sera towards the cooked extracts. The response was comparatively less in case of patient sera 4 and 5.

In case of raw and cooked extracts, the IgE binding ability was ranging from 0.21 to 0.24 and 0.244 to 0.44 respectively at absorbance of 490 nm. There were slight differences in the reactivity of sera of patients. The sera of P1 were highly reactive compared to others. The positive reactivity towards the shrimp extracts indicate the presence of cross reactive major allergen in crustaceans, tropomyosin as reported previously in different species of crustaceans and molluscs (Chuo et al. 2000; Motoyama et al. 2006).

SDS PAGE analysis of shrimp extracts

The extracts of sarcoplasmic protein, myofibrillar protein, raw and cooked of *M. dobsonii* were analysed by SDS PAGE and given in Fig. 2. In case of myofibrillar protein a clear prominent band of 36 KDa was visible. The raw extract contained several protein bands between 205 and 6.5 KDa. While in the case of cooked extract, many of the protein bands between 205 and 45 KDa were not detected. The disappearance of protein bands can be related to the effects of heat on the structure of proteins. These losses or reductions of protein bands might be due to changes in secondary or tertiary structure especially at temperature above 55 °C (Samson et al. 2004a, b). Heat denaturation disrupted the tertiary structure of globular proteins and lead to random coiled aggregation and insolubility (Peng and Gygi 2001). Sathe et al. (2005) reported that alterations in protein structure due to food processing methods can lead to changes in epitope by means of destruction,

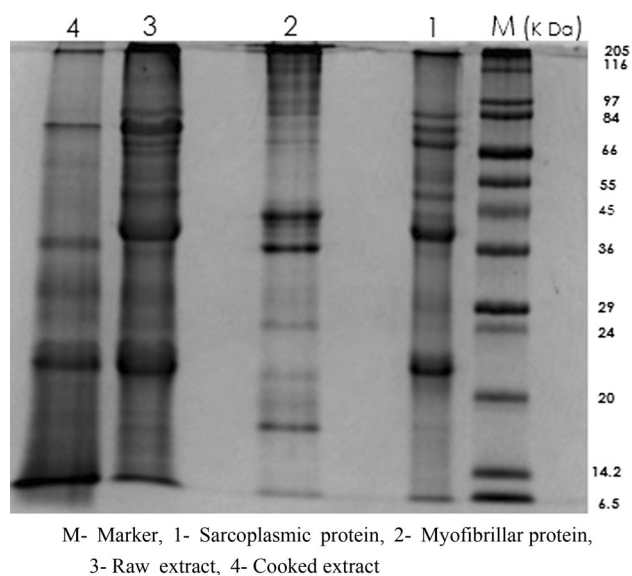


Fig. 2 Analysis of protein extracts by SDS-PAGE

modification, masking or unmasking and can finally affect the allergenicity of proteins. The bands appeared in the cooked extract were of heat resistant, mainly the bands of 36, 20, 29 and 70 KDa. These proteins were previously reported as allergens in case of different species of crustaceans and molluscs (Emoto et al. 2009; Leung et al. 1996).

Identification of Allergen by Immunoblotting

Immunoblotting of raw and cooked extracts of *M. dobsonii* is given in Fig. 3a, b. Multiple IgE binding proteins were identified in case of raw extracts and the number of IgE binding proteins was more in the case of raw extracts compared to cooked extracts. Each patient sera used in the study had a pattern of individual IgE binding. It is reported by several previous studies that crustaceans are allergic both in the raw and cooked form and it is significant as it is consumed in both forms (Samson et al. 2004a, b). In the case of immunoblotting of raw protein extracts, 9 IgE

Fig. 3 Analysis of raw (a) and cooked (b) extracts of *Metapenaeus dobsonii* by immunoblotting

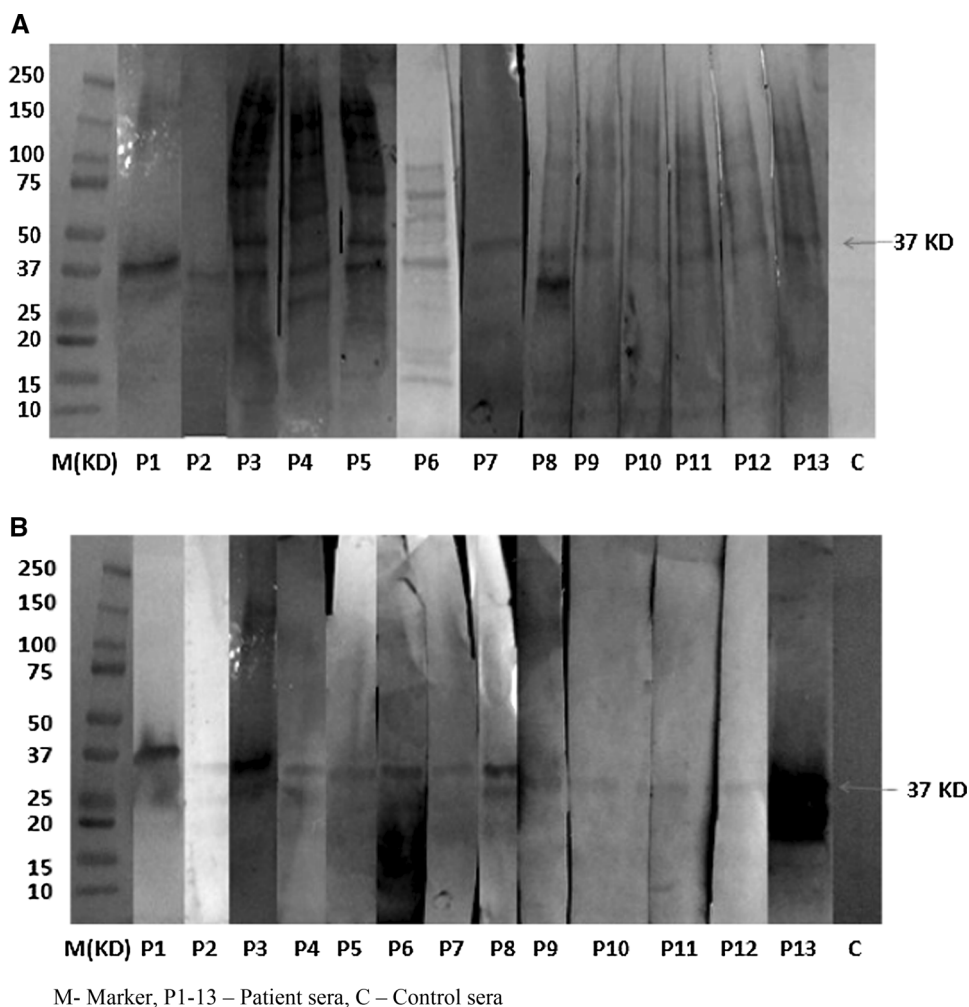


Table 3 Frequency of specific IgE-binding with Flower tail shrimp (*Metapenaeus dobsonii*) allergens identified by immunoblotting analysis

Sl no.	Protein fraction (KD)	Frequency (%) in raw shrimp extract	Frequency (%) in cooked shrimp extract
1	15	7.7	Nd
2	20	7.7	Nd
3	25	15.4	Nd
4	37	100	100%
5	50	30.8	Nd
6	75	30.8	Nd
7	100	46.2	Nd
8	150	23.1	Nd
9	250	23.1	Nd

binding proteins with molecular weight ranging from 15 to 250 KDa were identified. While in the case of cooked extracts, a clear IgE binding was observed at 37 KDa. From the IgE Immunoblotting result, it can be concluded that tropomyosin of 37 KDa is the sole and major protein with IgE binding activity in case of flower tail shrimp (*M. dobsonii*). Frequency of specific IgE-binding with allergens of Flower tail shrimp is given in Table 3. As the IgE binding pattern of different patient sera was showing variation, it can be suggested that patient's sera did not react to the same IgE-binding epitopes and it can finally result difference in allergic symptoms (Gill et al. 2009). The highest frequency of 100% IgE-binding was observed in case of 37KDa protein both in the case of raw and cooked extracts. Major allergens are identified based on the frequency of recognition by serum IgE antibodies; those allergens with a frequency of recognition greater than 50% can be designated as a major allergen. While the 100 KDa proteins were detected by 46.2% of patients; 50 and 75 KDa proteins bound IgE from 30.8% of patient sera. The 15, 20, 25, 150 and 250 KDa IgE binding proteins were recognized by 7.7–23.1% of the patients. All these minor allergens were detected only in case of raw extracts as they are heat sensitive in nature.

The study confirms the previous reports of tropomyosin as the major allergen in many crustaceans. Different studies report that muscle protein, tropomyosin of 34–39 KDa as the major allergen in shellfishes and more than 80% of sensitized individuals produce IgE reactive to this allergen (Shanti et al. 1993; Ayuso et al. 2002; Lopata et al. 2010). The minor blots found above or below of 37 KDa can be isoforms of tropomyosin or other proteins with cross reactive character. Tropomyosin of 34 to 36 KDa was previously reported as allergic protein of invertebrates like cockroaches and house dust mites (Shimakura et al. 2005; Liang et al. 2008). It is reported that tropomyosin is more

allergenic in boiled extracts, as it is highly heat-stable and the cooking process can alter the epitopes of the allergen (Yadzir et al. 2012). The altered epitope of allergen can result more increased IgE affinity.

Conclusion

The study has identified one major allergen and other minor allergens in flower tail shrimp (*Metapenaeus dobsonii*) which can have major roles in shrimp allergy among local shrimp allergy patients. The major allergen identified by immunoblotting is tropomyosin of 37 KDa which showed clear IgE reactive band both in the case of raw and cooked extracts by all the patient sera. The multiple IgE reactive bands in the raw extract were heat labile minor allergens. The major allergen, tropomyosin can be purified and characterized, which can be used in diagnosis and management of shrimp allergy.

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