*Fishery Technology* 63 (2026) : 1 - 9

# Screening and *in vitro* Characterization of Allergic Proteins of Kiddi Shrimp (*Parapenaeopsis stylifera*) by Peptide Mass Fingerprinting

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## Abstract

Allergy associated with consumption of shellfishes is a major cause of food induced anaphylaxis. Kiddi shrimp (*Parapenaeopsis stylifera*), locally known as 'karikkadi chemmeen' is a highly preferred variety. Allergens in raw and cooked extracts were subjected to identification by immunological methods using sera of shrimp allergic individuals and subjected to *in vitro* characterisation. Two protein fractions, namely hemocyanin (75 kDa) and tropomyosin (37 kDa), were identified as major allergens in the raw extract. In contrast. 37 kDa tropomyosin alone was identified as the major allergen in the cooked extract by 1D and 2DE immunoblotting. Both raw and cooked extracts exhibited higher IgE activity to sera from shrimp sensitive individuals. Tropomyosin of 37 kDa was recognized as the major allergen with the highest frequency of immunoreactivity. Mass spectrometry analysis of trypsin-digested tropomyosin was carried out using MALDI-TOF. The peptide mass fingerprint analysis showed strong similarity to other crustacean species and exhibited within sequence variation compared to others.

**Keywords**: Food allergen, kiddi shrimp, immunoblotting, tropomyosin

## Introduction

Food allergy is one of the major food safety issues in most developing countries. Immune system-mediated hypersensitive reactions triggered by the dietary intake of proteins are a major cause of food allergy (Sicherer & Sampson, 2010) and is the fastest growing allergic disorder (Gupta, Sheikh, Strachan, & Anderson, 2007). Among these, seafood allergies mediated by immunoglobulin E (IgE) is becoming increasingly common among both adults and children (Margaret, Jinap, & Faizal, 2015). The production and consumption of seafood have increased worldwide, leading to a higher incidence of adverse reactions. Adverse reactions encompass acute urticaria (allergic skin reactions with itchy, raised, red patches or wheals), atopic dermatitis (chronic, inflammatory skin reaction or eczema), asthma, gastrointestinal disorders, and anaphylaxis (Sicherer, 2011). Even though many shellfish species are allergenic (Burney et al., 2010), the prevalence associated with consumption of shrimp species is comparatively higher, particularly in developing countries (Lopata, O'hehir, & Lehrer, 2010). Chiang et al. (2007) have also reported a higher prevalence of shellfish allergy in Asian countries. In addition to dietary exposure, airborne allergens present in the shellfish processing environments, such as boiling, steaming or frying, can also elicit allergic symptoms in highly sensitive subjects.

The reported major allergen in a number of crustacean species is tropomyosin, a protein of myofibrillar origin ranging between 34 and 38 kDa (Lehrer, Ayuso, & Reese, 2003; Motoyama, Suma, Ishizaki, Nagashima, & Shiomi, 2007; Motoyama et al., 2008; Nakano, Yoshinuma, & Yamada, 2008). Subjects with shrimp allergy also show reactions to other crustaceans and molluscan groups. Specific linear and conformational epitopes present in allergic proteins can be identified by IgE (Ayuso et al., 2010). About 80% of shrimp allergic patients have the ability to generate IgE against this allergen (Daul, Slattery, Reese, & Lehrer, 1994). Other allergens include myosin light chain, arginine kinase, sarcoplasmic calcium binding proteins,

Received 27 September 2025; Revised 13 April 2026; Accepted 15 April 2026

Handling Editor: Dr. B. Madhusudana Rao

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hemocyanin, and amylase (Yu, Lin, Chiang, & Chow, 2003; Ayuso et al., 2008; Shiomi, Sato, Hamamoto, Mita, & Shimakura, 2008; Taylor, 2008).

As the identification and characterisation of clinically relevant seafood allergens is incomplete, there is a limitation in understanding their role in the immunopathogenic mechanisms responsible for hypersensitive reactions. It is also important to accurately diagnose food allergy in order to avoid false positive diagnosis, which can lead to unnecessary dietary restrictions and ultimately nutritional deficiency. The specificity of commercial extracts used in food allergy diagnosis can vary from local species-specific extracts; hence, extracts from local shrimp species can help in more accurate diagnosis (Piboonpocanun, Boonchoo, Pariyaprasert, Visitsunthorn, & Jirapongsananuruk, 2010). It is known that many people are more hypersensitive to shrimp than to fish, particularly in India, and severe allergic responses among shrimp consumers are regular reported. At the same time, information regarding clinically relevant shrimp allergens along the Indian coast is limited. Kiddi shrimp (*P. stylifera*), locally known as 'karikkadi chemmeen' is a highly preferred variety in Kerala, India, and is abundant along the coasts of Gujarat to Kerala, forming one of the main species in the inshore fishery. The allergic proteins of this shrimp have not yet been reported, and this study aims to identify and characterise these proteins using peptide mass finger printing.

## Materials and Methods

Kiddi shrimps (*P. stylifera*) of 17.5 ± 0.56 g weight and 7.36 ± 0.33 cm length were collected in a highly fresh condition from Kalamukku landing centre of Cochin, Kerala, iced (1:1 ratio), and immediately transported to the laboratory. The meat was homogenized and stored at -20 ºC. Meat was extracted in 0.01 M phosphate-buffered saline (PBS, pH 7.2) as per Motoyama, Ishizaki, Nagashima, and Shiomi (2006) in raw and cooked form and the protein content was determined (Gornall, Bardawill, & David, 1948). The extracts were freeze-dried and stored at -20 ºC for further analysis. Sera collected from 13 individuals hypersensitive to shrimp were used for screening allergic proteins. The symptoms and history of shrimp sensitive individuals were described previously (Laly, Sankar, & Panda, 2019a).

The IgE reactivity of shrimp extracts was determined by Enzyme linked immunosorbent assay (ELISA) (Ishikawa, Shimakura, Nagashima, & Shiomi, 1997), and the method was detailed in Laly et al. (2019a). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as per Laemmli (1970) using Mini Protean 3 apparatus (Bio-Rad, California, USA). The separated proteins were subjected to one-dimensional immunoblotting as per the method detailed in Laly et al. (2019a). Two-dimensional gel electrophoresis (2-DE) and immunoblotting were carried out using pooled sera from shrimp allergic patients (Pillai et al., 2018; Laly et al., 2019b).

Purification of tropomyosin was performed with slight modification to the method described by Huang and Ochiai (2005) at a temperature of 4 ºC. The method is discussed in detail in Laly, Kumar, Sankar, and Panda (2022). Trypsin digested peptides were extracted and subjected to peptide mass fingerprint analysis by MALDI-TOF (Matrix assisted laser desorption ionization and Time-of flight) using an UltrafleXtreme (Bruker Daltonics Germany) at the Proteomics facility, MBU, IISC, Bangalore, with support from Bioinnovations, Mumbai. Analysis was carried out using Flex Analysis 3.1 and searched using Matrix Science (Mascot) search engine.

In silico mapping of IgE epitopes was carried out using the AlgPred server, as described by Saha and Raghava (2006). A phylogenetic tree was constructed using MEGA X software (Kumar, Stecher, Li, Knyaz, & Tamura, 2018) by the neighbour-joining method (Saitou & Nei, 1987) and its robustness was tested using the bootstrap method with 1000 replications (Felsenstein, 1985).

The amino acid composition of purified tropomyosin was analysed using High Performance Liquid Chromatography (Shimadzu Prominence, Japan) (Ishida, Fujita, & Asai, 1981). The method has been previously reported by Laly, Sankar, and Panda (2020).

## Results and Discussion

The protein content of kiddi shrimp extracts was estimated, revealing that the cooked extract (2.27 ± 0.3 g $100g^{-1}$) had less protein content than the raw extract (5.54 ± 0.8 g $100g^{-1}$), likely due to degradation of thermally sensitive proteins during cooking. The average protein content in *P. stylifera* was estimated to be 17.62 g $100g^{-1}$. The soluble protein constitutes about 31.44% of the total proteins, while heat-stable proteins accounted for 13%. Notably, the heat-stable

proteins formed about 41% of the total soluble protein.

IgE binding of raw and heated extracts of kiddi shrimp is shown in Fig. 1A. IgE binding activity of raw and cooked extract using pooled allergic patient sera showed a significant difference ($p < 0.05$) compared to that of control sera. This indicates that both raw and cooked extracts exhibit allergenic potential, with higher IgE activity observed in shrimp sensitive sera compared to controls, suggesting the presence of allergic protein components. Extracts from both raw and boiled shrimp, *Penaeus vannamei*, retained an IgE-binding protein at 38 kDa (Liu et al., 2010). A marked increase in IgE activity following cooking has been reported in crustaceans such as *P. monodon*, *P. merguiensis*, *Scylla serrata*, and *Portunus pelagicus* (Abramovitch, Lopata, O'Hehir, & Rolland, 2017). A wide range of IgE activity to different crustacean extracts in raw and heated form, along with variation between subjects (Kamath et al., 2014), suggests the need to include both raw and heated shellfish extracts in diagnosis. Hence, the accuracy of identifying crustacean-sensitized individuals at potential risk can be improved.

SDS-PAGE profile of kiddi shrimp extracts is shown in Fig. 1B. Complex multiple bands were observed in raw extract. The raw extract showed a complex protein pattern. The prominent bands found in the cooked extract were 75, 36, 29 kDa etc. The number of protein bands was greatly reduced after heat treatment. The reduction or loss in the number of bands in the cooked extracts can be associated with damage to secondary and tertiary protein structures (Samson et al., 2004). Davis and Williams (2008) reported loss of secondary and tertiary structures of proteins at temperatures above 55 °C while retaining their primary structure. The number of studies focusing on thermal effects of seafood protein is limited (Paschke & Besler, 2002). Heat-resistant protein bands at 18, 20, 36, and ~150 kDa were reported in the case of black tiger prawn and king prawn by Sahabudin et al. (2011).

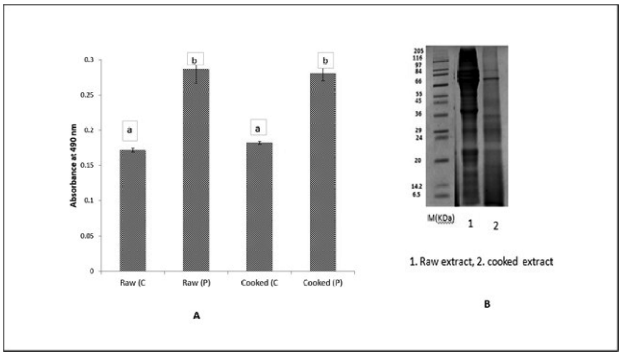


Fig. 1. (A) ELISA analysis of IgE activity of patient and control sera of raw and cooked extracts of kiddi shrimp (*P. stylifera*); (B) SDS PAGE profile of raw and cooked extracts

IgE-binding proteins in *P. stylifera* were identified in raw and cooked extracts by immunoblotting and are shown in Fig. 2A and 2B. Many IgE-reactive proteins were observed in both extracts of kiddi shrimp. Protein bands between 20 to 150 kDa of raw extract showed IgE binding, except the 100 kDa band. In the cooked extract, protein bands between 20 to 150 kDa showed IgE binding, except the 25 kDa band. The major allergen identified in both raw and cooked extract was tropomyosin of 37 kDa. The second major allergen in the raw extract was hemocyanin of 75 kDa. No IgE binding pattern was observed with control sera.

Crustaceans are normally reported to contain heat-stable and thermally susceptible proteins (Gill et al., 2009). The allergenicity of crustaceans in both raw and cooked form is significant, as they are consumed in both forms. Also, the variation in IgE-binding pattern and profile with respect to individual patient sera indicates variation in IgE-binding epitopes to which the allergen binds. Tropomyosin of 34 to 36 kDa has been reported as a major allergen in invertebrates such as prawn, crab, cockroach, and house dust mites (Shimakura,

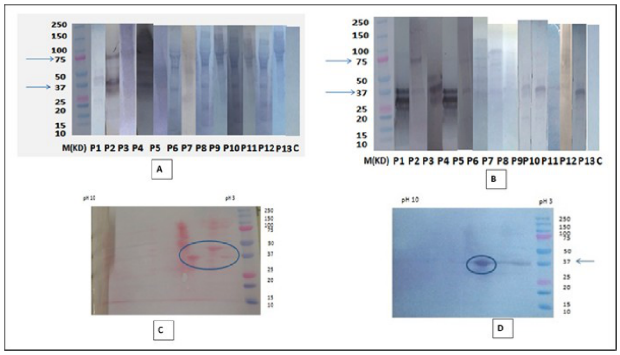


Fig. 2. Identification of allergens in raw (A) and cooked (B) extracts of *P. stylifera* by one-dimensional immunoblotting. M- Marker; P1-P13 – Patient sera; C – Control sera. Two-dimensional gel electrophoresis (C) and immunoblot analysis (D) of cooked extract of kiddi shrimp using pooled patient sera; M – Molecular weight marker

Tonomura, Hamada, Nagashima, & Shiomi, 2005). Hemocyanin of 75 kDa was reported as an allergen in black tiger shrimp by Sahabudin et al. (2011). Allergic proteins reported in different shrimp species are compiled by Woo and Bahna (2011). These include tropomyosin in northern sea shrimp (Cra c 1, Cra c 5), whiteleg shrimp (Lit v 1. Lit v 2, Lit v 4), and black tiger shrimp (Pen m 1, Pen m 2, Pen m 4); arginine kinase in northern sea shrimp (Cra c 2); myosin light chain in brine shrimp (Art fr 5); and sarcoplasmic binding protein in northern sea shrimp (Cra c 4) and narrow-clawed crayfish (Pon I 4).

Frequency of specific IgE-binding in raw and cooked form is shown in Table 1. In the raw extract, maximum frequency of IgE binding was identified at 37 kDa (84.6%), followed by 75 kDa (53.8%). In

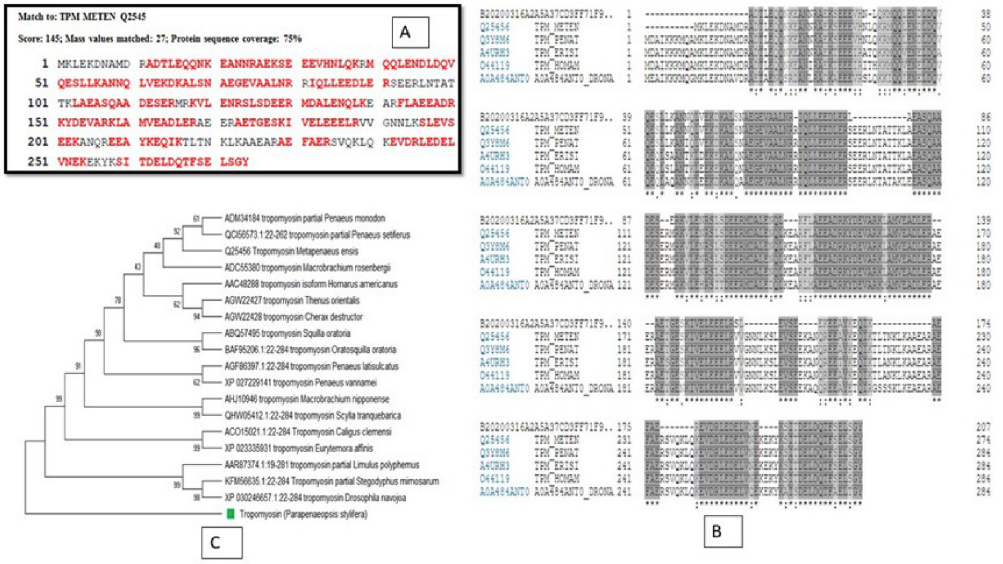


Fig. 3. (A) Peptide mass fingerprint analysis of tropomyosin from *P. stylifera* matching with *M. ensis*. The matched peptides shown in bold red; (B) Alignment of amino acid sequence of *P. stylifera* tropomyosin (B20200316) with Greasyback shrimp (*M. ensis*) Q25456; Brown shrimp (*P. aztecus*) Q3Y8M6; Chinese mitten crab (*Eriocheir sinensis*) A4URH3; American lobster (*Homarus americanus*) O44119; Fruit fly (*Drosophila navojoa*) A0A484ANT0; (C) Phylogenetic tree based on amino acid sequences of tropomyosin from *P. stylifera*

Table 1. Frequency of specific IgE-binding with kiddi shrimp (*P. stylifera*) allergens identified by one-dimensional immunoblotting analysis

| Sl. No. | Protein fraction (kDa) | Frequency (%) in raw shrimp extract | Frequency (%) in cooked shrimp extract |
|---|---|---|---|
| 1 | 15 | Nd | Nd |
| 2 | 20 | 15.4% | 7.7% |
| 3 | 25 | 23.1% | Nd |
| 4 | 37 | 84.6% | 100% |
| 5 | 50 | 38.5% | 15.4% |
| 6 | 75 | 53.8% | 30.7% |
| 7 | 100 | Nd | 15.4% |
| 8 | 150 | 38.46% | 7.7% |
| 9 | 250 | Nd | Nd |

the cooked extract, tropomyosin of 37 kDa yielded the maximum frequency of IgE binding. Other proteins with lower frequency of IgE binding are considered minor allergens. All minor allergens except 25 kDa were observed to withstand heat treatment.

The cooked protein extract of kiddi shrimp was subjected to 2D gel electrophoresis followed by immunoblotting with pooled sera of shrimp-sensitive individuals (Fig. 2C and 2D). Protein spots in the range of 25 to 150 kDa were observed. Upon 2-D immunoblotting by pooled shrimp allergic sera, a prominent IgE binding spot was observed at 37 kDa (Fig. 2D), identified as tropomyosin. Yadzir, Misnan, Bakhtiar, Abdullah, and Murad (2015) identified two IgE-binding proteins at 37 and 42 kDa proteins as major allergens in carpet clam (*Paphia textile*) using 2D immunoblotting with pooled patient sera. Similarly, tropomyosin and arginine kinase were identified as major allergenic components of *P. pelagicus* (blue swimming crab) by Rosmilah, Shahnaz, Zailatul, Noormalin, and Normilah (2012).

Table 2. De nova sequencing of tryptic peptides of tropomyosin from *P. stylifera*

| Residue numbers of matched regions | Mass observed | Mass expected | Mass calculated | Peptide sequence |
|---|---|---|---|---|
| 12 – 20 | 1046.6220 | 1045.6147 | 1045.5040 | ADTLEQQNK |
| 21 – 25 | 603.3680 | 602.3607 | 602.2772 | EANNR |
| 21 – 28 | 931.5030 | 930.4957 | 930.4519 | EANNRAEK |
| 29 – 38 | 1212.7280 | 1211.7207 | 1211.5782 | SEEEVHNLQK |
| 40 – 56 | 2031.2110 | 2030.2037 | 2029.9990 | MQQLENDLDQVQESLLK |
| 57 – 64 | 915.5950 | 914.5877 | 914.4821 | ANNQLVEK |
| 57 – 66 | 1158.7470 | 1157.7397 | 1157.6040 | ANNQLVEKDK |
| 67 – 80 | 1414.9080 | 1413.9007 | 1413.7212 | ALSNAEGEVAALNR |
| 82 – 91 | 1257.8260 | 1256.8187 | 1256.6612 | IQLLEEDLER |
| 103 – 115 | 1376.7980 | 1375.7907 | 1375.6215 | LAEASQAADESER |
| 118 – 123 | 758.5310 | 757.5237 | 757.4446 | KVLENR |
| 119 – 123 | 630.4360 | 629.4287 | 629.3497 | VLENR |
| 124 – 130 | 835.4780 | 834.4707 | 834.3719 | SLSDEER |
| 131 – 139 | 1061.6510 | 1060.6437 | 1060.5223 | MDALENQLK |
| 143 – 150 | 950.5630 | 949.5557 | 949.4505 | FLAEEADR |
| 143 – 151 | 1078.6690 | 1077.6617 | 1077.5454 | FLAEEADRK |
| 152 – 157 | 752.4440 | 751.4367 | 751.3501 | YDEVAR |
| 152 – 158 | 880.5470 | 879.5397 | 879.4450 | YDEVARK |
| 158 – 168 | 1274.8390 | 1273.8317 | 1273.6700 | KLAMVEADLER |
| 159 – 168 | 1146.7240 | 1145.7167 | 1145.5750 | LAMVEADLER |
| 173 – 179 | 721.3930 | 720.3857 | 720.3290 | AETGESK |
| 180 – 188 | 1129.7430 | 1128.7357 | 1128.6026 | IVELEEELR |
| 196 – 203 | 920.5630 | 919.5557 | 919.4498 | SLEVSEEK |
| 208 – 216 | 1137.7010 | 1136.6937 | 1136.5713 | EEAYKEQIK |
| 229 – 234 | 722.4260 | 721.4187 | 721.3395 | AEFAER |
| 242 – 254 | 1587.9690 | 1586.9617 | 1586.7787 | EVDRLEDELVNEK |
| 259 – 274 | 1804.9950 | 1803.9877 | 1803.8051 | SITDELDQTFSELSGY |

Table 3. IgE epitopes of *P. stylifera* tropomyosin

| Sl. No. | IgE epitope | Sequence matched | Position |
|---|---|---|---|
| 1 | AQLLAEEADRKYD | LKFLAEEADRKYD | 112 |
| 2 | EKYKSITDELDQTFS | VNEKSITDELDQTFS | 188 |
| 3 | ESKIVELEEELRVVG | ESKIVELEEELRSLE | 144 |
| 4 | FLAEEADRK | FLAEEADRK | 114 |
| 5 | MQQLENDLDQVQESLLK | MQQLENDLDQVQESLLK | 28 |
| 6 | QKLQKEVDRLEDELV | EFAEREVDRLEDELV | 174 |
| 7 | RIQLLEEDLERSEER | RIQLLEEDLERLAEA | 68 |
| 8 | RSLSDEERMDALENQ | RSLSDEERMDALENQ | 97 |
| 9 | VAALNRRIQLLEEDL | EVAALNRIQLLEEDL | 62 |
| 10 | VDRLEDELVNEKEKY | VDRLEDELVNEKSIT | 180 |

*In vitro* characterization of purified tropomyosin of kiddi shrimp was carried out. MALDI-TOF MS assists in the identification of species-specific peptides within the allergic protein fraction. Potential peptide markers can be detected in the allergen, which may be useful in routine techniques for rapid and accurate immunological assays. The de nova sequenced *P. stylifera* tropomyosin is shown in Table 2. It showed maximum similarity with tropomyosin from *Metapenaeus ensis* (protein score 145 and sequence coverage of 75%). The matched peptides are shown in Fig. 3A. Tropomyosin from other related edible crustaceans showed protein scores ranging from 91 to 116, with sequence coverage of 62 to 71% and the number of matched peptides ranged from 21 to 27.

The amino acid sequence alignment of *P. stylifera* tropomyosin with other species is shown in Fig. 3B. Sequence identity among available tropomyosin sequences was evaluated using the UniProt database. Shrimp species showed the highest sequence identity (63.1%), followed by lobster species (62 and 59.7%), while crabs showed sequence identities of 60.5 and 58%. A phylogenetic tree was constructed (Fig. 3C) and a comparison was performed among crustacean and fruit fly tropomyosins. The analysis revealed a close association of *P. stylifera* tropomyosin with other shrimp species, compared to lobsters, crabs, and squilla, while the fruit fly formed a sister branch. AlgPred analysis mapped IgE epitopes in *P. stylifera* tropomyosin (Table 3), and prediction using the Support Vector Machine (SVM) method, based on amino acid and dipeptide composition, indicated that it is potentially allergenic. Sequences 4, 5, and 8 showed complete matching, with regions corresponding to Pen a 1 (brown shrimp, *P. aztecus*) at positions 133-148 being fully conserved in *P. stylifera* tropomyosin, while minor substitutions indicated potential cross reactivity.

Table 4. Amino acid profile of purified tropomyosin form *P. stylifera*

| Amino acid | Percentage of total amino acids |
|---|---|
| Arginine (Arg) | 11.84±0.14 |
| Histidine (His) | 2.12±0.01 |
| Isoleucine (Ile) | 2.20±0.01 |
| Leucine (Leu) | 9.82±0.05 |
| Phenyl alanine (Phe) | 1.77±0.01 |
| Threonine (Thr) | 2.18±0.01 |
| Valine (Val) | 2.56±0.01 |
| Methionine (Met) | 2.04±0.02 |
| Lysine (Lys) | 22.26±0.13 |
| Tyrosine (Tyr) | 1.04±0.01 |
| Total essential amino acids | 57.83±0.4 |
| Alanine (Ala) | 6.83±0.07 |
| Aspartic acid (Asp) | 6.74±0.04 |
| Glycine (Gly) | 3.27±0.03 |
| Glutamic acid (Glu) | 20.66±0.15 |
| Proline (Pro) | 0.23±0.00 |
| Serine (Ser) | 4.22±0.02 |
| Cysteine (Cys) | 0.22±0.00 |
| Total non-essential amino acids | 42.17±0.33 |
| EAA/NEAA | 1.37 |

Amino acid profiling (Table 4) showed lysine as the most abundant amino acid in tropomyosin from kiddi shrimp, followed by glutamic acid. Several studies have reported that shrimp tropomyosin consist of approximately 284 amino acids without disulfide bonds, and is rich in lysine, arginine, and other amino acids with active side chains (Byun et al., 2000; Chu, Wong, & Leung, 2000; Rajagopal, Ganesh, & Rao, 2000; Ayuso, Reese, Leong-Kee, Plante, & Lehrer, 2002).

The study identified and characterized the major allergen, tropomyosin of 37 kDa in kiddi shrimp, *P. stylifera.* The highest frequency of IgE binding in the raw extract was observed at 37 kDa tropomyosin (84.6%) and 75 kDa hemocyanin (53.8%), while in the cooked extract, the 37 kDa band (100%) showed the highest frequency by 1D immunoblotting. In 2D immunoblotting of cooked extract, a prominent IgE binding spot was also observed at 37 kDa. Other minor allergens identified included proteins at 20, 25, 50, 100, and 150 kDa. *In vivo* characterization of major allergic protein, tropomyosin by peptide mass fingerprinting revealed significant protein scores ranging from 91 to 145 at a 95% confidence level, with sequence coverage between 62 and 75%. IgE epitope mapping further indicated the cross-reactivity with different shrimp species. The study provides clear information on the major allergen and its cross-reactivity in this locally important and commonly consumed species in India.



## Acknowledgement

This research work was carried out with the support of Indian Council of Agricultural Research, New Delhi, India. The authors are sincerely thankful to the Director, Indian Council of Agriculture Research-Central Institute of Fisheries Technology (ICAR-CIFT), Cochin, for providing the necessary facilities and support to complete the study.

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