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Molecular Characterization and Comparative Analysis of Tropomyosin Gene Sequence from Orange Mud Crab, *Scylla olivacea* with other Crustaceans

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Abstract: Consumption of shellfish can yield multiple allergenic reactions in hypersensitive persons. Among shellfish, crab including mud crab can cause mild, moderate, severe and potentially life-threatening allergic reactions. Tropomyosin, a myofibrillar protein at 36 kDa has been recognized as the major allergen in orange mud crab *Scylla olivacea* by proteomics approach. However, report on the molecular characterization of tropomyosin from *S. olivacea* and comparative analysis with other crab and crustacean species are unavailable. Hence, this study aimed to clone, identify and compare the molecular structure of tropomyosin in *S. olivacea* and other crab and crustacean species. This study has first cloned the full-length tropomyosin cDNA from *S. olivacea*. The sequence was then compared with other custacean tropomyosin in the database. The tropomyosin cDNA of *S. olivacea* contained an open reading frame (ORF) of 855 bp which encoding a tropomyosin with 285 amino acids that shared high sequence identity with other crab tropomyosin. This includes 99% identity to the giant mud crab (*Scylla serrata*) and 97% identity to both Japanese blue crab (*Portunus trituberculatus*) and swimming crab (*Charybdis feriatus*). While, the similarity with other crustacean species was ranged between 90.14 to 95.74%. As a conclusion, this study demonstrated the sequence of amino acid in tropomyosin of crab and other crustacean species is highly conserved, which might contributing to highly allergenic cross-reactivity between crab and other crustacean species.

Keywords: *cDNA; Scylla olivacea; crustacean; tropomyosin; PCR*

1. Introduction

Shellfish, including crab is one of the eight groups of food that causes allergic reactions after ingestion by 10% of the world population [1, 2]. Allergic reactions after consumption of crab can produce multiple allergenic symptoms from mild and moderate to severe allergic reactions including anaphylactic shock [2]. Diagnosis of crab allergies are similar to other shellfish allergies which includes clinical history and symptoms evaluation, followed by the confirmation tests either by *in vitro* or *in vivo* tests to detect the specific allergen [3]. Consequently, specific allergens must be identified in a particular crab species in order to improve the diagnosis, therapeutic and management of crab allergy due to the crab biodiversity [3-4].

The major and cross-reactive crab allergen in many populations is a myofibrillar protein of 34 to 38 kDa known

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as tropomyosin, identified in many crab species such as *Portunus pelagicus*, *Charybdis feriatus*, and *Scylla paramamosain*, *Scylla tranquebarica*, *Scylla olivacea*, *Scylla serrata* and *Eriocheir sinensis* [5-12]. Tropomyosin is grouped in a highly conserved protein family with numerous isoforms found in the non-muscle and muscle cells of both invertebrate and vertebrate organisms [3-15]. In non-muscle cells, tropomyosin participated in the cell morphology and motility regulation [15], while in the muscle cells, tropomyosin and troponin formed a protein complex which involved in muscle contraction by cooperating with myosin and actin [16].

Tropomyosin presents as anα-helical coiled coil homo-dimeric protein at highly stable configuration in physiological state. Slow and fast isoforms of tropomyosin are generated depending on alternate splicing mechanisms [15]. Crab contains all the putatively slow isoforms, while the putatively fast isoforms comprise of tropomyosins from lobsters, prawns and shrimps [14]. In crab species, the slow isoform was divided into two froms; the slow tonic and slow twitch [17]. The characterization of tropomyosin isoforms is required to further appreciate on the molecular variance amongst the dissimilar isoforms and their comparative allergenicity [14].

Beside tropomyosin, a phosphotransferase at 40 kDa, the arginine kinase, is the second cross-reactive allergen of different crab species [5-7, 18]. The other allergen, the 20-kDa sarcoplasmic calcium-binding protein is identified in *S. paramamosain* [19]. Recently, the first novel crab allergen, pyruvate kinase at 56 kDa was reported as a major allergen in *C. feriatus* in Taiwan population [20].

Scylla olivacea, the orange mud crab is frequently found in mangrove forests and estuaries environments in the Indo-Pacific regions [21-24]. This species is considered as the important local mangrove crab that is popularly consumed and exported to countries around the world [24]. In Malaysia, the productions of this crab are practiced in the north Peninsular Malaysia particularly in Penang, Kedah and Perlis [22]. Tropomyosin was acknowledged as the major allergen in *S. olivacea* by proteomics approach (unpublished data). However, reports on the molecular characterization of tropomyosin from *S. olivacea* and comparative analysis with other crab and shellfish species are unavailable. Hence, this study cloned, identified and compared the molecular structure of tropomyosin in *S. olivacea* and other crustaceans species.

2. Materials and Methods

Sample Preparation

The live specimen of adult *S. olivacea* used in this study was obtained from Merbok River Kedah, Malaysia. The muscle tissues were immediately harvested from the crab and completely submerged into RNAlater RNA Stabilization Reagent (Qiagen, USA). The submerged tissues were refrigerated overnight at 4°C and stored at –80°C freezer until use.

Extraction of Total RNA and Synthesis of cDNA

The total RNA from *S. olivacea* was extracted using RNeasy Plus Mini Kit (Qiagen, USA). The quantity and quality of total RNA were determined using a QIAxpert Spectrophotometer (Qiagen, USA). The cDNA template for PCR was generated from total RNA (250 ng) using QuantiNova Reverse Transcription Kit (Qiagen, USA). The reaction mixture was incubated in PCR machine for 5 minutes at 42 °C and hold at 4 °C. The reverse transcription reaction was performed for 3 minutes primer annealing step at 25°C, the reverse transcript at 45°C for 10 minutes and then was inactivated at 85°C. Samples of all cDNA were diluted and stored at –20°C freezer for further experiment.

Primer Design

The primer sequences were designed using NCBI/Primer-Blast under default parameters. The specificity of PCR amplicons was confirmed by single band of target size by electrophoresis on 1.5 % agarose gel and single melt peak in melting curve generated from five points of 10X dilution of pooled cDNA. The primer sequences are listed as follows:

TR-F: 5’-ATGGACGCAATCAAGAAGAAGATG-3’

TR-R: 5’-TTAGTAGCCAGACAGTTCGCTG-3’

Cloning of Full-length Tropomyosin cDNA from *S. olivacea*

The full-length tropomyosin cDNA from *S. olivacea* was cloned. The *E. coli* host for cloning (DH5α) and expression (BL21 (DE3) as well as plasmid vector (pRSET A) for cloning of recombinant tropomyosin were used. The tropomyosin fragments amplified from *S. olivacea* and plasmid vector weree digested with specific restriction enzymes (BamHI and EcoRI), and ligated together into cloning E. coli hosts. The result of tropomyosin sequence was then compared with other shellfish in the database.

3. Results and Discussion

The complete coding sequence of tropomyosin cDNA of *S. olivacea* comprised a 855 bp open reading frame, encompassing of 284 amino acid residues (Figure 1). The deduced amino acid sequence of the cDNA cloned was highly comparable to the tropomyosins from various species of crustacean particularly from crabs, crayfish, prawn and lobster (Figure 2). It shared high sequence identity with other

shellfish tropomyosin in the range of 90.49 (cambarid freshwater crayfish, *Procambarus clarkii)* to 98.69% (orange mud crab, *Scylla olivacea*) (Table 1).

(98.59%), *Charybdis feriata* (98.48%), Chinese mitten crab, *Eriocheir sinensis* (98.24%), Japanese blue crab, *Portunus trituberculatus* (98.24%), horsehair crab, *Erimacrus isenbeckii* (97.54%), red king crab, *Paralithodes camtschaticus* (94.01%) and snow crab, *Chionoecetes opilio* (93.31%). It has the lowest shared identity with flower crab, *Portunus pelagicus* with the percentage of only 90.46%. Therefore, this study indicated that the sequence of amino acid in tropomyosin is highly preserved among various crab species, which might contributing to highly allergenic cross-reactivity between crab species. This is not surprising as all crab are phylogenetically related [1, 14, 24]. All crab species compared are belong to the same order, Decapoda, under Family Portunidae, except for *E. sinensis* that was grouped under Family Varunidae, *E. isenbeckii* under Family Cheiragonidae, *P. camtschaticus* under Family Lithodidae and *C. opilio* under Family Oregoniidae [27].

Meanwhile, when compared with crayfish (*Procambarus clarkii*), the percentage amino acid sequence of *S. olivacea* tropomyosin similarity was 90.49%, but surprisingly, the identity much higher (95.74%) when compared to lobster *Homarus americanus. P. clarkii* and *H. americanus* are belongs to the same order as crab; Decapoda, but grouped

Among crab, *S. olivacea* tropomyosin has the highest similarity with tropomyosin of partial *S. olivacea* in the database (98.69%), followed by *Portunus sanguinolentus* under Family Cambaridae [28] and Nephropidae [29], respectively. Allergenic and cross-reactive tropomyosin has been previously reported in *P. clarkii* [30] and *H. americanus* [31], but their sequence similarities with crab species have not been reported.

Similarly, the tropomyosin of *S. olivacea* when compared with prawn species indicated high percentage similarities between 90.14% (*Penaeus latisulcatus*) to 95.42% (*Macrobrachium nipponense*), but relatively lower compared to percentage similarity between crab species. It was reported that tropomyosins from various species of crustaceans are highly cross-reacted with one another and also with numerous other invertebrates, such as mites and cockroaches [1, 10, 14, 25, 26].

Understanding of the amino acid sequence of tropomyosin from various species of crustaceans is the main determination to identify the allergenic cross-reactivity among them. However, further study on comparison of tropomyosin sequence of *S. olivacea* with other invertebrates such as molluscs, insects and nematodes are also necessary to explore the possible cross-reactivity among *S. olivacea* and other species of invertebrates.

Figure 1: Nucleotide Sequence of cDNA Encoding tropomyosin of *Scylla olivacea* and the Deduced Amino Acid Sequence. The deduced amino acid sequence is shown under the nucleotide sequence of the cDNA.

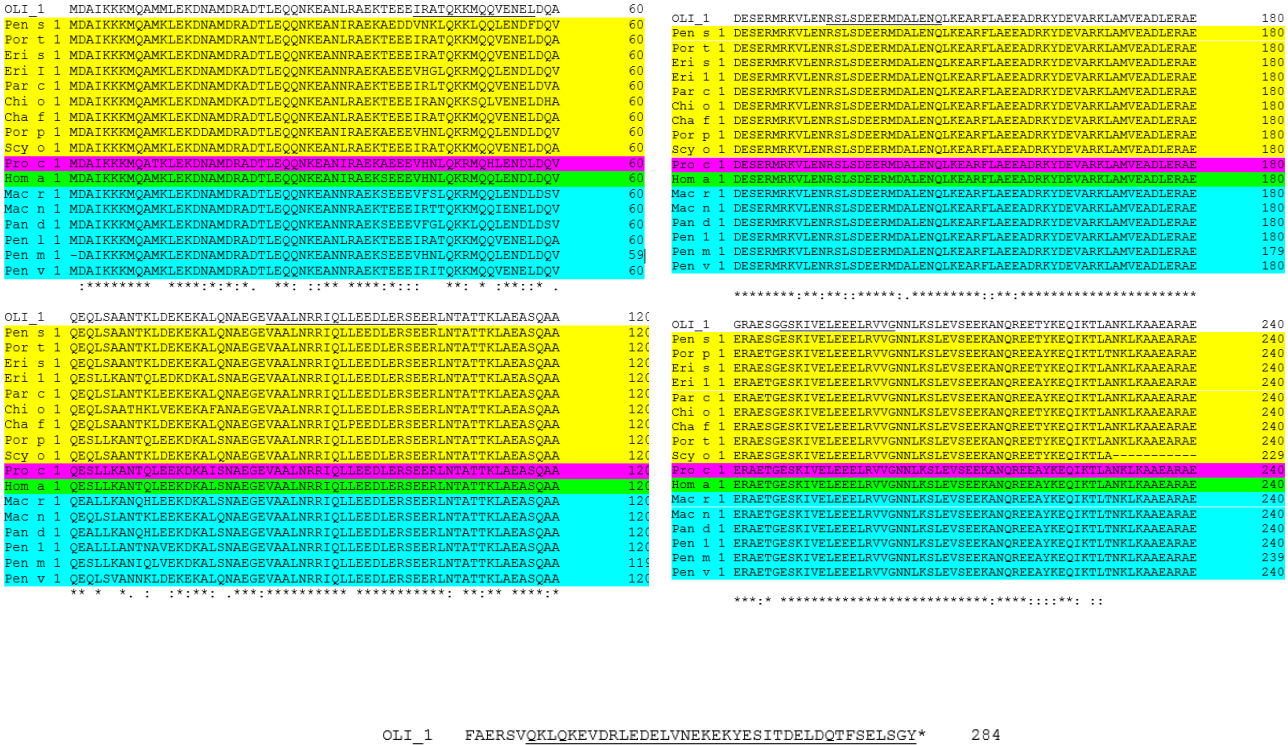

Figure 2: Amino acid sequence alignment of tropomyosins from *Scylla olivacea* (OLI_1) and tropomyosins from other crustaceans. Pen s 1: Three-spot swimming crab, *Portunus sanguinolentus* (ABL89183.1); Por t 1: Japanese blue crab, *Portunus trituberculatus* (ABS12234.1); Eri I 1: Horsehair crab, *Erimacrus isenbeckii* (BAF47265.1); Eri s 1: Chinese mitten crab *Eriocheir sinensis* ABO71783.1); Par c 1; red king crab, *Paralithodes camtschaticus* (BAF47266.1); Chi o 1; Snow crab, *Chionoecetes opilio* (A2V735.1); Cha f 1; Crucifix swimming crab, *Charybdis feriata* (Q9N2R3.1); Por p 1; flower crab, *Portunus pelagicus* (AGE44125.1); Scy o 1; Orange mud crab (partial), *Scylla olivacea* (AAX37289.1); Pro c 1; cambarid freshwater crayfish, *Procambarus clarkii* (ACN87223.1); Hom a 1; American lobster, *Homarus americanus* (AAC48288.1); Mac r 1*;* giant river prawn, *Macrobrachium rosenbergii* (AHA85706.1); Mac n 1; Oriental river prawn, *Macrobrachium nipponense* (AHJ10946.1) Pan d 1; caridean shrimp, *Pandalus borealis* (P86704.1); Pen m 1; giant tiger prawn, *Penaeus monodon* (ADM34184.1); Pen l 1; western king prawns, *Penaeus latisulcatus* (AGF86397.1); Pen v 1; Whiteleg shrimp, *Penaeus vannamei* (XP_027229140.1) The code (R1–R5) signifies the site of IgE-binding epitopes on shellfish tropomyosin as reported by Ayuso et al. [25].

4. Conclusion

This study demonstrated the sequence of amino acid in tropomyosin from *S. olivacea* is highly conserved between crab and other crustacean species, which might contributing to highly allergenic cross-reactivity between crab species. However, further clinical, immunological and characterization studies are needed to confirm the allergenic cross-reactivity among crustacean shellfish.

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