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Full Length Research Paper

Identification of a trypsin gene from *Scylla paramamosain* and its expression profiling during larval development

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The mud crabs, *Scylla* spp., are economically valuable marine species, which are distributed in many coastal countries. Here, an anionic trypsin (EC 3.4.21.4) gene was isolated from *Scylla paramamosain*, the most common mud crab in southeast China. The full-length cDNA (GenBank No. FJ917598, denoted as *SpTryp*) was 881 bp, with a 780-bp open reading frame encoding a polypeptide of 259 amino acid residues. Comparison with genomic DNA revealed that it contained two introns. The deduced monomer of trypsin had a molecular weight of 28.15 KD and the isoelectric point was 4.49. Alignment analysis and structure prediction showed that *SpTryp* shared high homologies with other trypsin genes. The phylogenetic tree of trypsins indicated that *S. paramamosain* clustered with crustacean group. Real-time quantitative PCR analysis demonstrated that trypsin mRNA accumulated most abundantly in zoeal I stage, the expression level declined with little fluctuation in the latter stages of larval ontogeny, and abruptly increased again in crab I stage. The transcript of trypsin gene was proposed to correlate with the dietary condition. The characterization of trypsin gene in *S. paramamosain* would contribute to the understanding of trypsin gene in crustacean.

Key words: Larval development, mud crab, real-time quantitative PCR, *Scylla paramamosain*, trypsin.

INTRODUCTION

The mud crab (*Scylla* spp.) is a ubiquitous marine creature and artificially cultivated in brackish coast along Southwest Pacific Ocean and North Indian Ocean (Wang et al., 2005). The total yield is more than 100, 000 ton each year, while the supply of seeds mainly depends on catching from wild population, which cannot guarantee the amount and seed size as demanded. For a long time, though biological and ecological studies have been widely carried out, large-scale seedling rearing of mud crab is less stable than other economic crabs such as Chinese mitten crab (Eriocheir sinensis) and swimming crab (Portunus trituberculatus). In order to meet the demand, more investigations on nutritional and environmental details in seedling rearing of *Scylla* spp. are impellingly required to improve the stability and production of seedling rearing. Especially, the intake, digestion, and dissimilation of dietary feed occupy a central role in the ontogeny of crustacean larvae.

Digestive enzymes play fundamental roles in assimilating substance and energy from the food. Digestive enzymes reported in larval decapods include protease, trypsin, carboxy-peptidase, amino-peptidase, pepsin, chymotrypsin, elastase, collagenase, carbohydrase, lipase, amylase, and laminarinase (Jones et al., 1997). Like in other primarily carnivorous animals, trypsin serves

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Abbreviation: RT-qPCR, Real-time quantitative PCR.

as a central role in digesting dietary protein in mud crab.

Trypsin (EC 3.4.21.4) is a polymorphic molecule in crustacean (Sainz-Hernández and Cordova-Murueta, 2009) and recognized as the most abundant protease present in crustaceans (shrimps, crabs, crayfish and lobsters) (Johnson et al., 2002), which belongs to the serine proteases family. It hydrolyzes peptide bonds at the carboxylic side of positive charged amino acid residues within a protein, and triggers activation of zymogens, acting on Lysine and Arginine residues (Harris et al., 2000). However, most studies are performed on shrimps; there are still little data for crabs. Given that artificial larval cultivation of commercially important crabs has drawn much attentions, and is nearing a breakthrough (Qiao et al., 2010), detailed investigations of physiological and genetic process are in really worthy requirement. In order to investigate the role of trypsin gene in larval development of mud crab, here, a novel trypsin gene was isolated from the most common mud crab in China, *Scylla paramamosain* (Ma et al., 2006), and its expressing profile during larval development was examined as well.

MATERIALS AND METHODS

Crab, RNA isolation and cDNA library construction

S. paramamosain was captured from Qinglan Harbor, Hainan Island, China (19º33′N, 110 º49′E). Total RNA was isolated from hepatopancreas and further purified using Unizol Reagents (Biostar, Shanghai, China) following the protocol of the manufacturer. Quality and concentration were checked by agarose gel electrophoresis (Universal Hood II, Bio-Rad Laboratories Inc., Hercules, CA, USA) and spectrophotometer (DU 800 Nucleic acid/Protein Analyzer, Beckman Coulter Inc., Brea, CA, USA) and the RNA was stored at -80°C until use.

SMART^{TM} cDNA Library Construction Kit (Clontech, Palo Alto, CA, USA) was employed to construct a cDNA library following the description. The cDNA was inserted into the modified vector pBluescript II SK(-) followed by transforming, screening, and sequencing.

Obtaining of *trypsin* gene, 5'-RACE confirming for cDNA completeness

Random sequencing was performed after cDNA library construction. All the EST sequences were subjected to BLAST analysis. According to the screening of obtained sequences, a sequence with Poly A which shared high homologies with *trypsin* genes deposited in NCBI was selected for further analysis.

5'-RACE including RT, dC tailing, and PCR amplification was carried out following the protocol provided by the manufacturer (SMART^{TM} RACE cDNA Amplification Kit, Clontech, Palo Alto, CA, USA) to confirm the completeness of cDNA sequence. Two gene-specific primers, SpTryp-5-1 (5'-CCA GCC ACA GCC TGC ACA ATT CCT GG AT-3', as 5'-RACE first amplification primer) and SpTryp-5-2 (5'-CCA CCG CAG AAG TGC CAT GGA TTG CTG T-3', as 5'-RACE nested amplification primer) were designed according the obtained sequence and used for 5'-RACE.

Cloning of genomic sequence of *trypsin* gene

In order to unveil if introns were present in the genome region of *trypsin* gene, PCR amplification was carried out by using 1.5 μg of total genomic DNA, isolated from muscle of *S. paramamosain* using the method described by Klinbunga et al. (2000). The used primers were SpTrypFull5 (5'-GAC TCG TAT CCA TCC TGG GCA GTC-3') and SpTrypfull3 (5'-CGA CCA CCA TAT TGT ATT TAA TTT GAT G-3'). The PCR condition were as the follow: 3 min at 94°C, 30 cycles (45 s at 94°C, 45 s at 55°C, 3 min at 72°C), and 8 min at 72°C . The PCR product was purified and cloned into pMD19-T vector (TaKaRa, Shiga, Japan), followed by sequencing. Three independent clones were sequenced to avoid mistakes cause by PCR or sequencing.

Bioinformatics analyses

The molecular information about the full-length cDNA of *SpTryp*, characterizations, and alignments were carried out with Vector NTI Suite 8.0. Secondary structure and three-dimensional structure predictions were performed at http://cn.expasy.org. The alignment of trypsin protein deposited in NCBI was done with ClustalX, and the bootstrap test of phylogeny based on UPGMA method was carried out with MEGA3.

Expression pattern analysis during larval development

S. paramamosain larvae were cultured in standard cement pools with rotifers at zoea I and II stages, with *Artemia* since zoea III stage, and with smashed shellfish as the main feed since crab I stage (Davis et al., 2005a; Davis et al., 2005b). The water temperature was 26-30°C, and salinity was 22-30 ppt. Real-time quantitative PCR (RT-qPCR) was carried out to investigate the expression profile of *trypsin* gene during larval development of *S. paramamosain*. The larvae of zoea I, II, III, IV, and V stages, Megalopa stage, and Crab I stage were collected for RNA isolation with Unizol method. All RNA samples were digested with DNase I (RNase-free) prior to use. 2 μl of total RNA were employed in the RT-qPCR reaction using random hexamer primers for the synthesis of first strand cDNA. The amplification reactions were performed on StepOne Plus^{TM} Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with gene-specific primers SpTryp-Real-F (5'-GGA ATT GTG CAG GCT GTG G-3') and SpTryp-Real-R (5'-ACG AAG AAT CAA AGT AGG GAT GGA-3'), *16S rRNA* primers Sp16S-Real-F (5'-GGG ACG ATA AGA CCC TAT AAA GCT T-3') and Sp16S-Real-R (5'-TTA TAC CAT TGT CGC CCC AAC-3'), and the SYBR ExScript RT-PCR kit (TaKaRa, Shiga, Japan) protocol to confirm changes in gene expression. For each reaction, 2 μl of diluted cDNA (10^{-3} of original cDNA solution for *SpTryp*, 10^{-3} of original cDNA solution for *16S rRNA*), 10 μl of 2×SYBR *Premix Ex* Taq^{TM}, 0.2 μM of forward primer, 0.2 μM of reverse primer, and nuclease free water were added to a final volume of 20 μl. The thermal cycle conditions used were 10 s at 95°C followed by 40 cycles of amplification (5 s at 95°C and 35 s at 60°C). Melting curve analysis (15 s at 95°C, 60 s at 60°C, and 15 s at 95°C) and agarose gel electrophoresis following each RT-qPCR were performed to assess product specificity.

The expression levels of *16S rRNA* and *SpTryp* in investigated samples were quantified for three repeats by measuring the cycle threshold values (Ct) and extrapolation to their calibration graphs constructed with serial dilution cDNA templates in zoea II stage of known concentrations (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} of the original cDNA solution). Then the ratio of expression levels of *SpTryp*/*16S rRNA* in zoea II stage was initiated as "1.0", so the relative ratios of expression levels of *SpTryp*/ *16S rRNA* in other stages were

determined by comparing with zoea II stage.

RESULTS AND DISCUSSION

Cloning of the full-length cDNA and genomic DNA of *SpTryp*

The full-length cDNA of *SpTryp* was 881 bp with 5' and 3' un-translated region, poly-A tail and contained a 780-bp open reading fragment (GenBank accession No. FJ917598) encoding a 259-amino-acid protein (Figure 1). These characteristics were consistent with previous reports (Klein et al., 1996; Magalhaes et al., 2007; Rudenskaya et al., 2004) for other trypsins. Speaking with strict definition, the sequence cloned in this study was a trypsinogen gene.

The comparison of the full-length cDNA and the genomic DNA of *SpTryp* revealed that there were two introns. The first one was 89 bp, which consisted of GT-AG rule, located between the signal and the activation peptides. The insertion site appeared two nucleotides before the sequence encoding the last residue of the signal peptide, which is in line with the previous data and case (Breathnach and Chambon, 1981; Klein et al., 1998). The other was 91 bp, which showed TA-GG at the both ends, locating in the region of mature peptide. It was rare for TA-GG to exist in introns sequence, indicated that there might be SNP site or other causes. As expected, the two location were quasi-conserved with those of trypsin from *Litopenaeus vannamei* (Klein et al., 1998), one was before the sequence encoding the signal peptide and the other was in the region of mature peptide. Nevertheless, the length and sequence of the introns was not so conservative.

Characterization of *SpTryp*

BLAST analysis on http://www.ncbi.nlm.nih.gov revealed that *Sptryp* gene shared high homologies with trypsin or trypsin-like genes from other crustacean organisms, such as *Portunus pelagicus* (86% maximum identities with EF120993), *L. vannamei* (78% maximum identities with Y15041; 77% maximum identities with X86369; 77% maximum identities with Y15040; 77% maximum identities with Y15039), *Fenneropenaeus chinensis* (77% maximum identities with FJ904936; 77% maximum identities with FJ904937), and *Eriocheir sinensis* (93% maximum identities with EF530707; 93% maximum identities with EF530705; 92% maximum identities with EF530706).

Protein-Protein BLAST analysis (www.ncbi.nlm.nih.gov) also showed that SpTryp had high homologies to other crustacean trypsins or trypsin-like serine proteinases, and belonged to Tryp_SPc superfamily. SpTryp showed the highest homologies with trypsin from *P. pelagicus* (88% identities, 94% Positives with ABM65758).

The deduced SpTryp had a calculated molecular weight of 28.15 KD and a *p*I value of 4.49. With the exception of the N-terminal 29-amino-acid peptide, the deduced mature enzyme had 230 amino acids, showing a theoretical molecular mass of 25.02 KD and a *p*I of 4.15.

SpTryp protein also contained an active-site triad (His-Asp-Ser), which is specific for the trypsin activity, an Asp which determines the substrate specificity of trypsin, a Gly in the region that binds the substrate. There were 9 Cys residues in the mature peptide region of SpTryp, they might form 4 disulfide bonds, with the information of prediction of three-dimensional structure also combined. Judging from the alignment of all the trypsins from crabs, these proteins were quite conservative especially on the active sites (Figure 2).

Secondary and three-dimensional structure of SpTryp

The analysis results suggested that the gene product was initially synthesized as precursor proteins, and subsequently processed to a mature enzyme. SignalP analysis (www.cbs.dtu.dk/services) illustrated that SpTryp contained an N-terminal signal peptide with the most likely cleavage site being between A_{15} and A_{16} (Dyrløv Bendtsen et al., 2004). However, according to the description of trypsin from *P. pelagicus*, another cleavage site for activation peptide should be between R_{29} and I_{30}. This was also supported by the existence of IVGGE motif at the N-terminus (Figure 1), which is proposed to be after the cleavage site for activation as in other cases(Johnson et al., 2002). The presence of two cleavage sites was an interesting subject, coinciding with what in *L. vannamei* (Klein et al., 1996). In the other way, the alignment of trypsins as well showed that PpTrypsin, SpTrypsin, and Pa-cTrypsin all had the N-terminal peptide composed of 48 amino acids and an additional C-terminal peptide composed of approximated 30 amino acids, compared with other trypsins from crabs (Figure 2). The N-terminal signal peptide was believed to help form a proenzyme before yielding the active enzyme with proteolytic cleavage(Sainz-Hernández and Cordova-Murueta, 2009). It is proposed that activation of trypsin zymogen and cleavage of signal peptides are two of various post-translational controls that digestive proteinases undergo (Muhlia-Almazán et al., 2008). All these results suggested that the modification and regulation of trypsin were not very identical in different species, only the trypsins from several species had the additional peptides. This dissimilarity still demanded further investigation.

It also appeared that the signal peptide and activation peptide were highly similar in amino acid sequence and length among the three investigated crabs (Figure 2). When referring to shrimp *L. vannamei* (Klein et al., 1998),

a

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   1                                    gactcgtatccatcctgggcagtc
  25 atgaagaccctcgtgctgtgcctgcttgttgccggggccttagcc
   1 M  K  T  L  V  L  C  L  L  V  A  G  A  L  A
  70 gccccatcccgcaagcccaccttccgtcggggactgaacaggatt
  16 A  P  S  R  K  P  T  F  R  R  G  L  N  R  I
 115 gttggcggtgaggacacagttcacggcgaatttcagtaccaactt
  31 V  G  G  E  D  T  V  H  G  E  F  Q  Y  Q  L
 160 agccttcaagatacgtcgtacagcaatccatggcacttctgcggt
  46 S  L  Q  D  T  S  Y  S  N  P  W  H  F  C  G
 205 ggtaccctgtacaatgagcactggggcatcactgcttgtcactgt
  61 G  T  L  Y  N  E  H  W  G  I  T  A  C  H  C
 250 ctgcagtatgatgtggataatccaggaattgtgcaggctgtggct
  76 L  Q  Y  D  V  D  N  P  G  I  V  Q  A  V  A
 295 ggtgaatacactcttgaggcaaatgacggttccgagcaggcagca
  91 G  E  Y  T  L  E  A  N  D  G  S  E  Q  A  A
 340 agactggatgagatcatcctccatccctactttgattcttcgtta
 106 R  L  D  E  I  I  L  H  P  Y  F  D  S  S  L
 385 ctcgttaacgacgtcgccctcattcactttccgcaaacaatggtt
 121 L  V  N  D  V  A  L  I  H  F  P  Q  T  M  V
 430 tatgacgattacgttaatcccatcggtcttcaagaagaaaaagag
 136 Y  D  D  Y  V  N  P  I  G  L  Q  E  E  K  E
 475 cttgtaggcgtcgagtgcaccgtcacgggatggggagctttgtca
 151 L  V  G  V  E  C  T  V  T  G  W  G  A  L  S
 520 gagggagggagtgccgccacagtcttgcagaaggttcatgtccct
 166 E  G  G  S  A  A  T  V  L  Q  K  V  H  V  P
 565 actgtgtccgatgaggaatgtagaatatcttactacggtattgaa
 181 T  V  S  D  E  E  C  R  I  S  Y  Y  G  I  E
 610 gattccatgatctgtgctggatatcccgagggaggaaaggacgcc
 196 D  S  M  I  C  A  G  Y  P  E  G  G  K  D  A
 655 tgccagggtgactctggtggacctatggtgtgcaagggatacctt
 211 C  Q  G  D  S  G  G  P  M  V  C  K  G  Y  L
 700 accggcatcgtgtcctggggctacggctgtgctcgccccaactac
 226 T  G  I  V  S  W  G  Y  G  C  A  R  P  N  Y
 745 ccgggggtctacacggaggtggcctactttgtggattggattatt
 241 P  G  V  Y  T  E  V  A  Y  F  V  D  W  I  I
 790 gccaatgctgtataaattgtaccattgatatgatcaaaacatggc
 256 A  N  A  V  *
 835 acatcaaattaaatacaatatggtggtcgaaaaaaaaaaaaaaaa
 880 aa
```

b

Intron I:

5'-GTCAGTGCCTCCATGCAGCAATGCACTATTCAACTGTTTTTTTGTTTGACAAACAATATT
TTTAACATTGACTTTCTTTCTACCCACAG-3'

Intron II:

5'-TATTTGCAATAGCCAAATGCTCACATGAAATGCACAAAACCACTTCCATAGTTAGTCAA
GATATATTAACTGAGATGATTCTCTTTGCAGG-3'

Figure 1. The full-length cDNA sequence, deduced amino acid sequence, and introns sequence of trypsin from *Scylla paramamosain*. The start codon (atg) and the stop codon (taa) are underlined. (a) The cDNA sequence and deduced amino acid sequence. The three amino acids which composing the active site are boxed with squares. The circle denotes the amino acid of the substrate-binding site. The conservative motif of catalyzing-site is indicated by the shadow. The solid arrow indicates the theoretical possible cleavage site of the deduced signal peptide. The hollow arrow indicates the cleavage site of the activation peptide. The triangles show the positions where the introns exist. The N-terminal IVGGE motif is underlined. The polyadenylation signal sequence in the 3' non-coding region is boxed. (b) The sequence of introns.

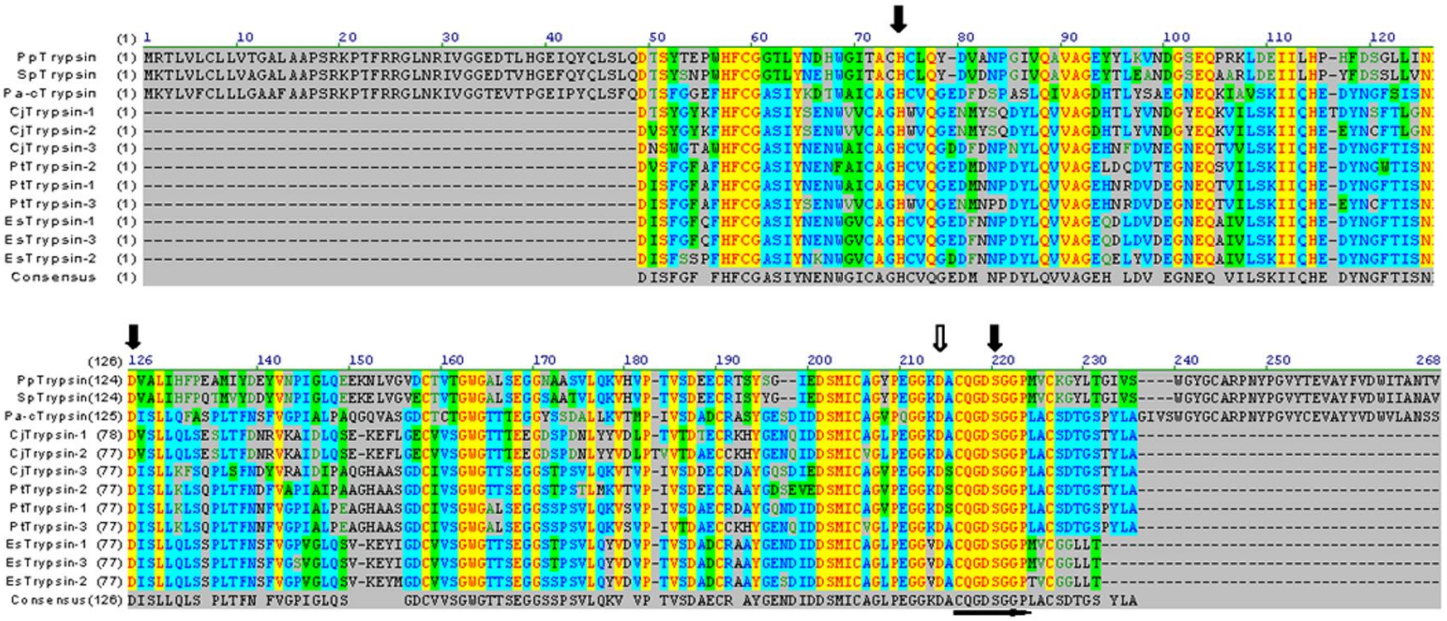

Figure 2. The alignment of trypsins from crabs. The sequences used are from *Scylla paramamosain*, *Portunus trituberculatus* (PtTrypsin-1: ABQ02537; PtTrypsin-2: ABQ02538; PtTrypsin-3: ABQ02539), *Charybdis japonica* (CjTrypsin-1: ABQ02512; CjTrypsin-2: ABQ02513; CjTrypsin-3: ABQ02514), *Portunus pelagicus* (PpTrypsin: ABM65758), *Paralithodes camtschaticus* (Pa-cTrypsin: AAL67442), and *Eriocheir sinensis* (EsTrypsin-1: ABQ02519; EsTrypsin-2: ABQ02520; EsTrypsin-3: ABQ02521). Dashes represent gasps introduced to maximize similarities. Residues of non-similar are shown by black letters on white background, conservative shown by dark blue on light blue, block of similar shown by black on green, identical shown by red on yellow, and weakly similar shown by green on white. The active-site triad (His-Asp-Ser) are denoted with vertical solid arrows respectively. The Asp which determines the substrate specificity of trypsin is denoted with a vertical hollow arrow. The conservative motif of catalyzing-site is denoted with a horizontal arrow.

they were still considerably conservative. While further referring to vertebrates (Roach et al., 1997), they became less similar as expected.

Based on the Hierarchical Neural Network analysis (Combet et al., 2000), SpTryp was composed of 16.99% alpha helix, 27.03% extended strand, and 55.98% random coil. The secondary structure of trypsins from *P. pelagicus* (GenBank No. AMB65758), *P. trituberculatus* (GenBank No. ABQ02537), and *L. vannamei* (GenBank No. CAA75309) could also be compared to display the similarities and dissimilarities.

The three-dimensional structures of trypsin from *S. paramamosain*, *P. pelagicus*, *P. trituberculatus* and *L. vannamei* were established by Swiss Model, and displayed in Figure 3, resulting in comparison of computational spatial architectures. It was quite apparent that the four structures were very similar, which suggested high conservative level of trypsins in construction and function. Combining the analysis of sequence alignments and three-dimensional structure, it supported that almost all conserved residues can be associated with one of the basic functional features of the protein: zymogen activation, catalysis and substrate specificity (Rypniewski et al., 1994).

However, the structural differences between psychrophilic and mesophilic trypsins from vertebrate were previously revealed and discussed, as well as the molecular mechanisms of cold-adaptation (Schrøder et al., 2000). Further attentions should also be directed to the examination of counterpart in invertebrate, such as trypsins from crustacean.

Phylogenetic tree

Trypsin appears early in evolution and occurs in all phyla (Muhlia-Almazán et al., 2008). The evolutionary position of SpTryp was explored with the phylogenetic tree (Figure 4) constructed from a range of Tryp_SPc superfamily members with the ClustalX program based on UPGMA method. The trypsins from all the available crabs and white shrimp (*L. vannamei*), fresh prawn (*F. chinensis*), fruit fly (*Drosophila melanogaster*), and mouse (*Mus musulus*) were included in the phylogenetic tree. According to the tree, the evolution of trypsin was consistent with the category. However, no significant separation was found between the trypsins from crabs and shrimps, which might indicate that crustacean were a more big family than several morphological groups, such as crabs and shrimps. Based on previous analysis (Rypniewski et al., 1994), the phylogenetic tree of trypsins showed a continuous evolutionary divergence

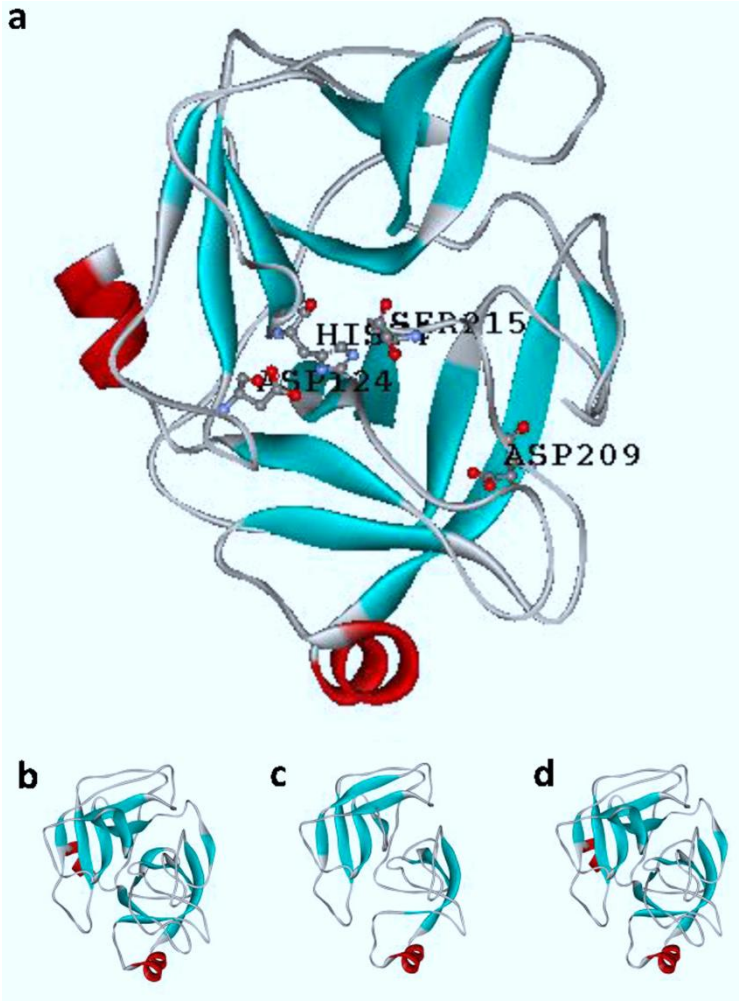

Figure 3. The three-dimensional structure established based on Swiss Model at http://cn.expasy.org. The amino acids from (a) *Scylla paramamosain* which determinate the active-site triad and substrate specificity are showed with atoms. Trypsins from (b) *Portunus pelagicus* (GenBank No. AMB65758), (c) *Portunus trituberculatus* (GenBank No. ABQ02537), and (d) *Litopenaeus vannamei* (GenBank No. CAA75309) are also displayed for comparison.

from a common ancestor of both prokaryotes and eukaryotes. The present tree also reflected a short evolutionary distance between trypsins of crustacean. They were much more related to each other than those from insect (fruit fly) and mammal (mouse).

In most crustacean species, trypsin is a polymorphic gene. In case of *L. vannamei*, there are six isoforms or at least three families, which may benefit adaptable characteristics for acclimatizing to the environment (Klein et al., 1996; Klein et al., 1998; Sainz-Hernández et al., 2004b). Based on the high homologies of the trypsin gene copies in one species, it coincided with the suggestion that the isoforms of trypsin gene may originate from gene duplication (Wang et al., 1995). This duplication might be useful to achieve adaptive advantages (Muhlia-Almazán et al., 2008).

Expression profile during larval development

As protease such as trypsin is prominently important in crustacean larval culture (Jones et al., 1997). It is demonstrated that digestive enzymes are expressed exclusively during some ontogenetic stages. Trypsin and chymotrypsin isoenzymes in *Penaeus monodon* varies during ontogenesis (Fang and Lee, 1992). Transcription has been suggested as a major regulatory mechanism of trypsin activity (Muhlia-Almazán et al., 2008). Storage and activation of trypsinogens also take part in activity regulation of trypsin (Sainz-Hernández et al., 2004a). It calls for more detailed researches in biochemistry, physiology, and nutriology to explore what is the regulatory mechanism like in *S. paramamosain*.

The result of expression profiling during larval ontogeny

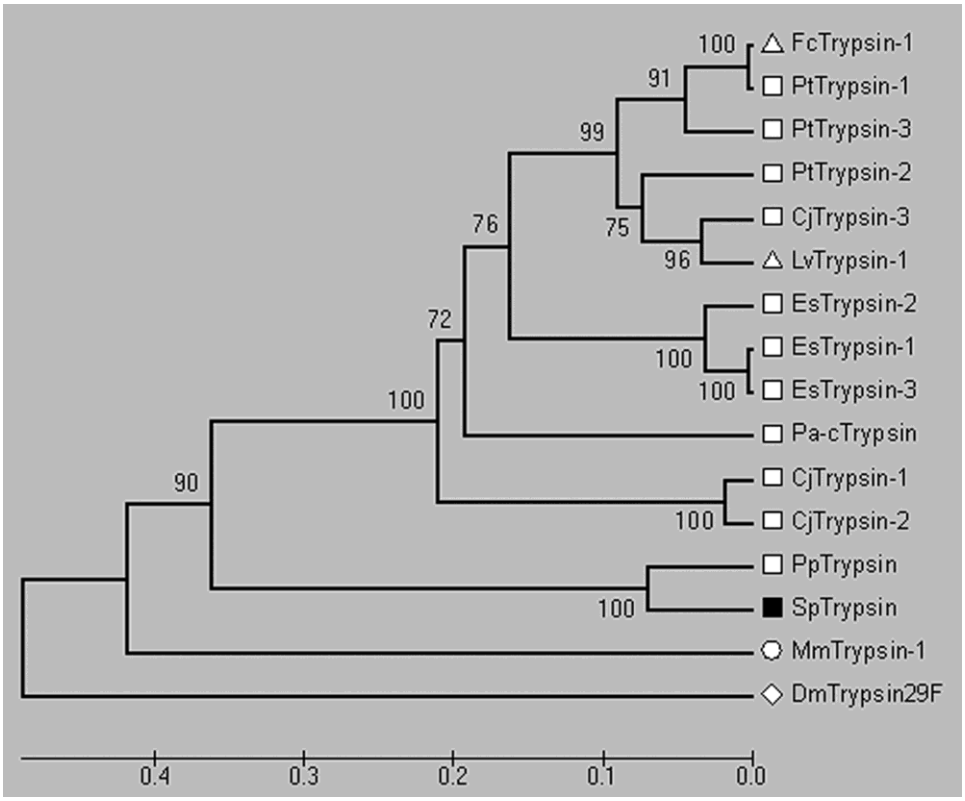

Figure 4. The phylogenetic tree of trypsins from crustaceans and fruit fly, mouse. ClustalX was used to do the complete alignment; bootstrap test was performed with MEGA3 using UPGMA method. The numbers on the branches represent bootstrap support for 1000 replicates. The other sequences used in the phylogenetic tree were from *Portunus trituberculatus* (PtTrypsin-1: ABQ02537; PtTrypsin-2: ABQ02538; PtTrypsin-3: ABQ02539), *Charybdis japonica* (CjTrypsin-1: ABQ02512; CjTrypsin-2: ABQ02513; CjTrypsin-3: ABQ02514), *Portunus pelagicus* (PpTrypsin: ABM65758), *Paralithodes camtschaticus* (Pa-cTrypsin: AAL67442), *Eriocheir sinensis* (EsTrypsin-1: ABQ02519; EsTrypsin-2: ABQ02520; EsTrypsin-3: ABQ02521), *Litopenaeus vannamei* (LvTrypsin-1: CAA75309), *Fenneropenaeus chinensis* (FcTrypsin-1: ABQ02525), *Drosophila melanogaster* (DmTrypsin29F: AAF52738), and *Mus musculus* (MmTrypsin-1: AAH94921). □ indicates crab. ■ indicates *Scylla paramamosain*. △ indicates shrimp. ◇ indicates fruit fly. ○ indicates mouse.

in this study showed that *SpTryp* expressed constitutively in all the phases during the development (Figure 5). Among the zoeal stages, the highest expression level emerged in zoea I stage. Lower expression levels appeared in zoea III and V stages. During the whole larval ontogeny, slight fluctuations were observed. After molting to crab phase, trypsin transcription was significantly enhanced. The decline of trypsin mRNA transcription after zoea I stage might be due to the full feed supply in the artificial condition. This result implies that digestive enzyme levels in cultured crustacean larvae are reduced as energy requirements are met without need for highly efficient digestion (Harms et al., 1991; Le Vay et al., 2001). Thus the prominent increase of expression level in crab I stage was likely to be an adaption prepared for larger amount of feed and more complicated diet composition. As fresh smashed shellfish was employed as the main feed since the crab I stage, the higher expression level of trypsin gene might indicate the response in crab I stage for digesting more complex food, and for activation of more zymogens.

Trypsin activity in *L. vannamei* is modulated by several factors (Sainz-Hernández and Cordova-Murueta, 2009) including genetic factors, frequency of feeding, dietary protein, molting, and stress. Nevertheless, differences between taxa might also influence the regulation mechanism of trypsin activity as in *Sparus aurata;* the mechanisms controlling adaptation of the activity of the trypsin to the amount of dietary protein were activated quite slowly (Naz and Türkmen, 2009). In transcription level, *trypsin* gene was proposed to quantitatively regulated by molting, dietary protein, starvation, and time (Le Moullac et al., 1997; Muhia-Almazán and García-Carreño, 2002; Sainz-Hernández et al., 2005; Sánchez-Paz et al., 2003). In *P. pelagicus*, *trypsin* genes were up-regulated in the intermolt stage and pre-molt stage

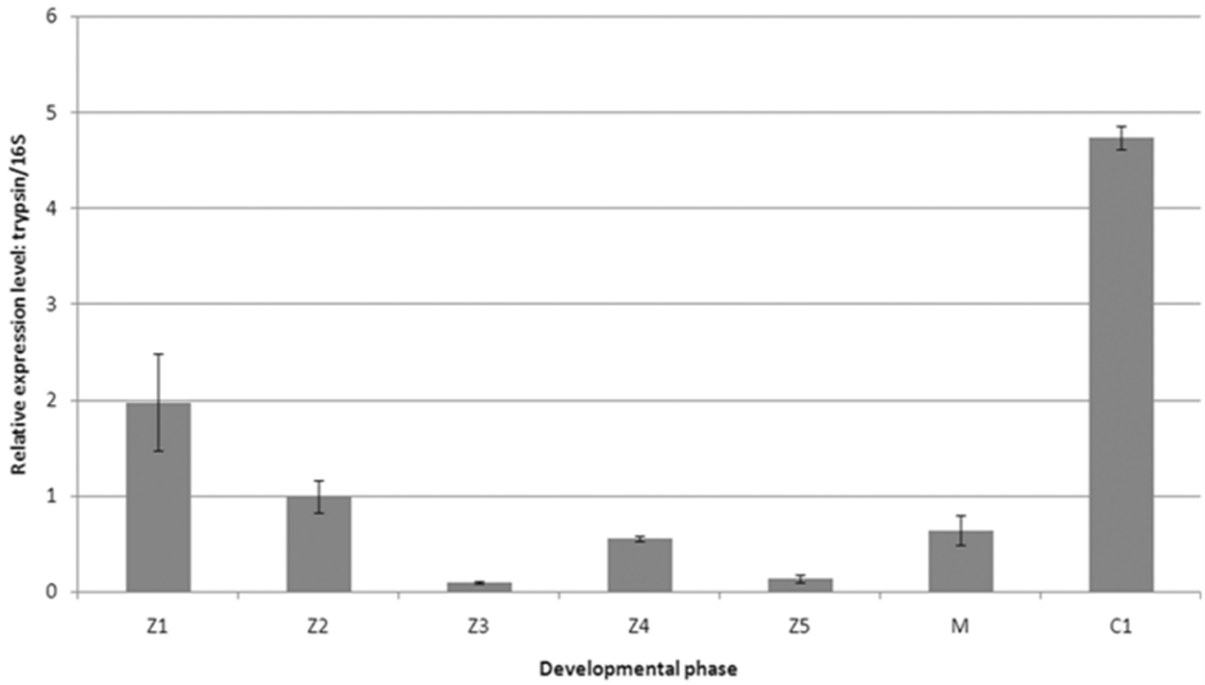

Figure 5. Expression profile of SpTrypsin during larval development. Total RNA was isolated respectively from larvae in various phases, including zoea I stage (Z1), zoea II stage (Z2), zoea III stage (Z3), zoea IV stage (Z4), zoea V stage (Z5), megalopa stage (M), and crab I stage (C1) followed by real-time quantitative PCR analysis with 16S rRNA as an internal control. The ratio of transcripts of SpTrypsin against which of 16S rRNA in zoea II stage was initiated as "1.0". The "y" axis represents the relative ratio of expression levels of SpTrypsin/16S rRNA in various stages. The data represent the means ± SD (standard deviation) of three repeated samples.

(Kuballa and Elizur, 2008). This indicated that trypsin acted its role in digestion and assimilation for the preparation of substance and energy, which is required during ecdysis. The expression pattern of trypsin gene should correlate with cyclic molting in this way.

Artificial feeds with algal extracts have been demonstrated to enhance tryptic activity in *Penaeus indicus* to promote increased growth and survival (Metin and Jones, 1995). Given that the enzyme activities including trypsin reflected each specific dietary and trophic resource utilization in some crabs (Johnston and Freeman, 2005), so the expression level of trypsin gene was related with the dietary. When novel diets in larval culture of mud crab were tested, the expression level of trypsin gene could be investigated as one of the factors to evaluate the tested diet.

Conclusion

Anyway, the identification and expression profiling of SpTryp gene provided the first elucidation of a zymogen sequence from the commercially important crab, and would benefit the exploitation of trypsin activities, evolution, and regulation of larval rearing. Whether the isoforms of trypsin existed in *S. paramamosain*, it remained unclear for further examination. In order to clarify the whole mechanism of trypsin activity, more attentions should be paid towards the cooperation with other digestion enzyme genes and the alteration of dietary. The understanding of trypsin gene would also shed new light on feeding ecology, feeding strategy and ecdysis control in larval cultivation of *Scylla* spp., and make contributions to the optimization of mud crab aquaculture.

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