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Research Article

Characterization and expression analysis of chymotrypsin after bacterial challenge in the mud crab, *Scylla paramamosain*

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Abstract

Chymotrypsin is one of the serine proteases families that have various biological functions. A chymotrypsin gene was isolated from hepatopancreas of the mud crab, *Scylla paramamosain* (designated *SpCHY*) in this study. The full-length cDNA of *SpCHY* contained 942 nucleotides with a polyadenylation sequence and encoded a peptide of 270 amino acids with a signal peptide of 17 amino acids. The *SpCHY* gene contains seven exons, six introns, a TATA box and several transcription factor binding sites that were found in 5'-promoter region which is 1221 bp in length. Real-time quantitative PCR analysis indicated that the expression level of *SpCHY* mRNA in hepatopancreas was significantly higher than that in other tissues. Immunocytochemistry and *in situ* hybridization exhibited the *CHY*-like reactivity presented in resorptive cells of the hepatopancreas. After bacterial challenge with *Vibrio alginolyticus*, the expression level of *SpCHY* mRNA was extremely up-regulated at 3 h in hepatopancreas. Our results suggest that *SpCHY* might play an important role in the mud crab's immune response.

Keywords: chymotrypsin, *Scylla paramamosain*, immune response, immunocytochemistry, *in situ* hybridization.

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Introduction

Belonging to one of the largest gene family in the animal kingdom, serine proteases (SP) have a tryp-spc domain, which is conserved with the catalytic triad (His, Asp and Ser), part of an extensive hydrogen bonding network (Szabo and Bugge, 2008; Zhou *et al.*, 2012). In the human genome, approximate 500 protease-encoding genes have been identified, of which about 30% are SP or SP homologues (SPH) (Southan, 2001). In *Drosophila melanogaster*, around 200 SP- and SPH-encoding genes have been identified (Ross *et al.*, 2003). SPs participate in various biological processes, including protein digestion (Mazumdar and Broadway, 2001; Broehan *et al.*, 2008), immune response (Jiang *et al.*, 2003a, b), and molting (Samuel and Reynolds, 1993; He *et al.*, 2009).

As one of the SP, the chymotrypsin family includes chymotrypsin A and chymotrypsin B, two structurally related, but phylogenetically distinct subfamilies (Rawlings *et al.*, 2008). Chymotrypsin B plays an important role in intracellular protein turnover, while chymotrypsin A is prevalent in the extracellular space and performs different functions (Broehan *et al.*, 2010). The chymotrypsin A subfamily contains a variety of enzymes, such as chymotrypsin, trypsin, elastase, granzyme and different matrix peptidases, with different cleavage specificities. The substrate-binding pocket near the catalytic site determines these types of specificity (Perona and Craik, 1995). These proteins are all synthesized as inactive zymogens, which can be activated by specific proteolytic cleavage. The canonical catalytic triad residues (Ser, His and Asp) form the active site (Hedstrom, 2002).

In invertebrates, studies on chymotrypsin are mostly focused on the digestive system of some pest insects. In the lepidopteran, *Spodoptera exigua*, chymotrypsin was found likely to mediate the proteolytic remodeling in the gut during larval-pupal transition (Herrero *et al.*, 2005). The injection of dsRNA for chymotrypsin 5C/6C in the red flour beetle, *Tribolium castaneum*, resulted in severe molting defects, which indicate that chymotrypsin plays an important role in molting process (Broehan *et al.*, 2010). In addition, chymotrypsin was associated with immune defense reactions against bacteria in *D. melanogaster* (de Morais *et al.*, 2005). In crustaceans, only few studies report on chymotrypsin (Sellos and Wormhoudt, 1992; Shi *et al.*, 2008; Serrano, 2013), and only few chymotrypsin cDNA and genomic DNA sequences have been cloned and characterized. The polymorphism and evolution of this gene have been analyzed in the pacific white shrimp, *Litopenaeus vannamei* (Sellos and Wormhoudt, 1992, 1999). Chymotrypsin in Chinese shrimp, *Fenneropenaeus chinensis*, was observed to be involved in innate immune reactions after bacterial and viral challenges (Shi *et al.*, 2008).

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The mud crabs of the genus *Scylla* are important cultured crustaceans that live in intertidal and subtidal sheltered soft-sediment habitats (Keenan, 1999). In Southeast Asia, mud crabs are a valuable source of income for coastal communities (Le Vay, 2001; Ye *et al.*, 2011). The bacterium, *Vibrio alginolyticus*, can cause many diseases (such as exoskeleton ulcer disease, black gill disease) that seriously affect crustacean aquaculture and thus receive increasing attention in recent years (Zhu *et al.*, 2008).

In this study, we first cloned the cDNA, 5-promoter region and genomic DNA of a chymotrypsin gene from the mud crab, *Scylla paramamosain* (designated *SpCHY*), and investigated its expression in various tissues by real-time quantitative PCR. The localization of chymotrypsin protein and mRNA in hepatopancreas was detected by immunocytochemistry and *in situ* hybridization. The temporal responses of *SpCHY* to the bacterium *V. alginolyticus* were investigated to study the role of *SpCHY* in the immune response.

Materials and Methods

Sample collection

Vigorous female crabs (~250 g), with both claws intact and antennae in movement, were purchased from a local fish market in Xiamen city, China. Brain, thoracic ganglion, heart, gill, hepatopancreas, stomach, muscle, and ovary tissues were dissected and immediately preserved in liquid nitrogen. Total RNA was extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol and potential genomic contamination was removed by DNase I treatment. RNA quality was determined by agarose gel electrophoresis and quantification was done with an ND-1000 NanoDrop UV spectrophotometer (NanoDrop Technologies, USA). RNA aliquots of 1 μg were reversely transcribed using a reversed first strand cDNA synthesis kit (Fermentas, USA) and stored at -20 °C.

Cloning of full-length *SpCHY* cDNA

The degenerate primers *CHY*f1 and *CHY*r1 (Table 1), directed to highly conserved sequences of various chymotrypsin orthologs, were used to amplify a partial chymotrypsin-like sequence of *S. paramamosain*. The *SpCHY* sequence was completed by 3' and 5' rapid amplification of cDNA ends (RACE) by means of a 3', 5' full race kit (Takara, Dalian, China). The specific primers *CHY*3' and *CHY*5' are listed in Table 1.

Polymerase chain reactions (PCR) were carried out in a total volume of 25 μL that contained 1 μL of cDNA template, 2.5 μL of 10xPCR buffer (containing Mg^{2+}), 1 μL of each primer (10 μM), 2.5 μL of dNTP (2.5 mM), 0.2 μL (2.5 U) of LA *Taq* polymerase (Takara, Dalian, China) and 16.8 μL of PCR-grade water. PCR conditions were as follows: 94 °C for 3 min; 32 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min; followed by a final extension at 72 °C for 10 min. After agarose gel electrophoresis, the DNA fragment of expected size was ligated into pMD19-T vectors (Takara, Dalian, China) and then used to transform competent cells of *Escherichia coli.* Positive recombinant clones were sequenced using the specific primers RV-M

Table 1 - Summary of primers used in this study.

Primer name	Primer Sequence (5'- 3')	Purpose	Amplified fragment length
*CHY*f1	GGYGTYGTYTGCATYGACGGHRC	fragment amplication	203 bp
*CHY*r1	GCTCAGGGWKTGACRCCRGTCTT	fragment amplication	
*CHY*3'	CTCGCTCTGCTCCTTGTCTG	3' amplication	904 bp
*CHY*5'	GAAAGATGTGATGCCGTAGGTC	5' amplication	728 bp
*CHY*f2	ATGATTGCCAAGCTCGCTCTG	genomic DNA amplify	1994 bp
*CHY*r2	TCAGGGGGTGACACCGGTC	genomic DNA amplify	
*CHY*f3	ACGAGCAGGGACTTCTTCACC	real-time RT-PCR for *SpCHY*	286 bp
*CHY*r3	AGACGACGCCACTTCCAACA	real-time RT-PCR for *SpCHY*	
*CHY*5-1	CAGCAACGCAGACAAGGAGCA	promoter region clone	1261 bp
*CHY*5-2	TGGGGAAAGAAGGAAAGTGGC	promoter region clone	1155 bp
*CHY*5-3	GCAAAACATCTACGACCACAGCA	promoter region clone	974 bp
T*CHY*f1	GCCAGAACGAGCCCTCTCAG	riboprobe amplication clone	314 bp
T*CHY*r1	GACGACGCCACTTCCAACAAT	riboprobe amplication clone	
T7	TAATACGACTCACTATAGGG	riboprobe amplication clone	
M13-47	CGCCAGGGTTTTCCCAGTCACG	colony PCR	
RV-M	GAGCGGATAACAATTTCACACA	colony PCR	
β-actin F	GAGCGAGAAATCGTTCGTGAC	internal control	183 bp
β-actin R	GGAAGGAAGGCTGGAAGAGAG	internal control	

and M13-47 (Table 1) at Sangon Biotech Co, Ltd (China). Finally, the full-length of *SpCHY* cDNA was assembled from 3' end and 5' end sequences.

Genomic DNA and promoter cloning of *SpCHY*

Genomic DNA was extracted from muscle tissue of the mud crab by means of a DNA extraction kit (Takara, Dalian, China) PCR amplified by two specific primers *CHY*f2 and *CHY*r2 (Table 1) PCR and cloned as described above. The promoter region was cloned by genome walking using the Universal Genome Walker kit (Takara, Dalian, China). Nested PCR was performed with primers *CHY*5-1, *CHY*5-2, *CHY*5-3 (Table 1) according to the manufacturer's protocol. The PCR product was purified and sequenced as before.

Phylogenetic and sequence analysis of *SpCHY*

A homology analysis of *SpCHY* with *CHY* genes of other species was performed using the Blastp algorithm. Characteristics of the protein were predicted using algorithms.of the Expasy site. The putative signal peptide was identified with SignalP software (Nielsen *et al.*, 1997), and the ClustalW program was used to perform multiple sequence alignments. The neighbor-joining method implemented in MEGA3.1 software was used to construct the phylogenetic tree based on protein sequences (Kumar *et al.*, 2004), with a bootstrapping replication of 1000. SSRHunter software was used to search for microsatellite sequences.

Tissue expression of *SpCHY*

mRNA transcripts of *SpCHY* in different tissues were examined by real-time quantitative PCR (Applied Biosystems 2770 Thermal Cycle, New York, USA). The reactions were performed in a 20 μL reaction volume containing 10 μL of SYBR premix, 2 μL of cDNA template (1/10x dilution of cDNA), 0.8 μL of each primer (10 μM *CHY*f3 and *CHY*r3; Table 1) which amplify a product of 286 bp, and 6.4 μL of PCR-grade water. PCR conditions were as follows: 94 °C for 10 min; 40 cycles of 94 °C for 20 s, 56 °C for 30 s and 72 °C for 40 s; final extension at 72 °C for 10 min. A 183 bp β-actin (GU99242) fragment of *S. paramamosain* was amplified as the internal control. Standard curves were run for each primer and the cDNA templates were tested in a graded dilution series (1, 1/10, 1/100, 1/1000). Based on these analyses, PCR efficiency was calculated to be > 96% (according to the PCR amplification formula $E = 10^{(-1/slope)}-1$; where E is the PCR efficiency). The negative control was performed with PCR-grade water replacing the cDNA template. All samples were run in triplicate and relative expression was calculated as $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

Immunocytochemistry

Hepatopancreas tissue removed from adult female crabs was fixed in Bouin's fixative overnight, dehydrated, embedded in paraffin, and then sectioned at 7 μm thickness. The sections were immunocytochemically stained by the streptavidin-peroxidase method with a primary antiserum generated in mouse against CHY (1:100 dilution, Abcam, UK) following an immunocytochemical protocol of the supplier (Transgen, China). The presence of CHY-like immunoreactivity in the tissues was visualized by a DAB enhanced liquid substrate system (Sigma-Aldrich, USA). Thereafter, the sections were dehydrated and observed on an Olympus multifunction microscope BX51 (Olympus, Japan). Control sections were prepared simultaneously by substituting PBS buffer solution in place of the primary antibody.

In situ hybridization

Digoxigenin-labeled cRNA riboprobes were synthesized with a DIG-RNA labeling Kit (Roche, Switzerland) using a 314 bp template of *SpCHY* that was ligated into the pGEM-T easy vector (Promega, USA). Hepatopancreas tissue was dissected and immediately fixed overnight in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) made in diethypyrocarbonate (DEPC) water. Tissue sections of 7 μm thickness were hybridized with the digoxigenin-labeled riboprobes at 57 °C overnight followed by incubation in an anti-DIG alkaline phosphatase-conjugated antibody (Roche, Switzerland). Hybridization signals were visualized with the colorimetric substrates nitroblue tetrazolium/4-bromo-4-chloro-30-indolylphosphate (NBT/BCIP). The riboprobe templates for *SpCHY* were generated by RT-PCR from hepatopancreas cDNA using the specific primers T*CHY*f1, T*CHY*r1 containing *T7* adapters. Photographs were taken on an Olympus multifunction microscope BX51 (Olympus, Japan).

Temporal expression of *SpCHY* in hepatopancreas after immune challenge

In an attempt to determine whether *SpCHY* was involved in innate immune reactions, the expression profiles of *SpCHY* after bacterial challenge were measured and compared to the unchallenged (control group). The bacterium *V. alginolyticus* was prepared and washed for animal challenge. A dose of $1x10^7$ CFU mixed with 20 μL crab saline was injected at the base of the last pereiopods into each of 30 vigorous female crabs (~250 g) of the experimental group (Cheng *et al.*, 2004). Another 30 vigorous female crabs (~250 g) composing the control group were injected with 20 μL saline. These two groups were reared separately in culture tanks under the same conditions with seawater at a temperature between 26-28 °C, salinity at 26 ppt, and with continuous aeration. For real-time quantitative PCR assays, three crabs each were sampled at 0, 3, 6, 12, 24, 48 and 72 h post-injection and their hepatopancreas tissues were dissected and preserved in RNAsafer Stabilizer Reagent (Takara, Dalian, China). Total RNA extraction, first-strand

cDNA synthesis and real-time quantitative assays were performed according to the procedures described above.

Statistical analysis

One-way analysis of variance (ANOVA) and Student's t-test done with SPSS 11.5 software were used to determine the statistical significance of *SpCHY* expression in different tissues and challenge experiment respectively (SPSS, Chicago, IL, USA). Before the comparisons, Kolmogorov-Smirnov and Cochran tests were run to test for normality and homogeneity of variances. P values of < 0.05 were considered statistically significant.

Results

Cloning of the *SpCHY* gene

A 942 bp cDNA sequence of *SpCHY* (GenBank accession number: JF831535.1) was obtained in this study. It comprises an 813 bp open reading frame (ORF) encoding 270 amino acids with a signal peptide of 17 amino acids, an 115 bp 3'-untranslated region (UTR) with a polyA tail, and a 14 bp 5'UTR (Figure 1). The deduced molecular weight of mature SpCHY protein was 28.5 kDa and its isoelectric point 6.11. Conserved domain analysis done online in NCBI showed that SpCHY contained a trypsin-like SP domain including one cleavage site I-45, three active site (H-85, D-131, S-222), three substrate binding sites (S-216, S-237, G-239), and six cysteine residues, which were similar to other chymotrypsin members.

Similar to other chymotrypsin genes, *SpCHY* is composed of seven exons interrupted by six introns. In addition, all the intron-exon boundaries conformed to the GT-AG rule, which belonged to a 0-type intron/exon junction. Moreover, a 36 CA repeat microsatellite sequence was found by screening with SSRHunter software (Figure 2).

In order to study the regulation of *SpCHY* expression in the mud crab, we used a cloned 1221 bp fragment of the 5' flanking region of the *SpCHY* gene. Using the program Promoter 2.0, we found a putative TATA box that was located at 45 bp upstream of the translation start site. In addition, several putative transcriptional factor binding sites or *cis*-regulatory elements including HSF, Hb, Dfd, SP1, Bcd, CF1 and Ubx were also identified.

Phylogenetic analysis of SpCHY

Blastp data showed that the deduced amino acid sequence shared high similarity with chymotrypsins of *L. vannamei* CHYA (GenBank accession no. CAA71672, 82%), *F. chinensis* (ACC68669.1, 80%), *M. japonicus* (BAI49929.1, 79%), *L.* vannamei CHYB (CAA71673.1, 79%). The phylogenetic analysis suggested that three different groups were formed, representing CHYs from invertebrates, vertebrates and urochordates respectively. The vertebrate CHY group could be further separated into three distinct and well-supported clades: CHYA, CHYB, and CHYC (caldecrin). The invertebrate group contained 2 subgroups. As showed in Figure 3, crustacean CHY was well separated from insect CHY and formed a separate cluster.

Tissue distribution of *SpCHY* mRNA

Real-time quantitative PCR showed that *SpCHY* mRNA is expressed in a wide variety of tissues, including

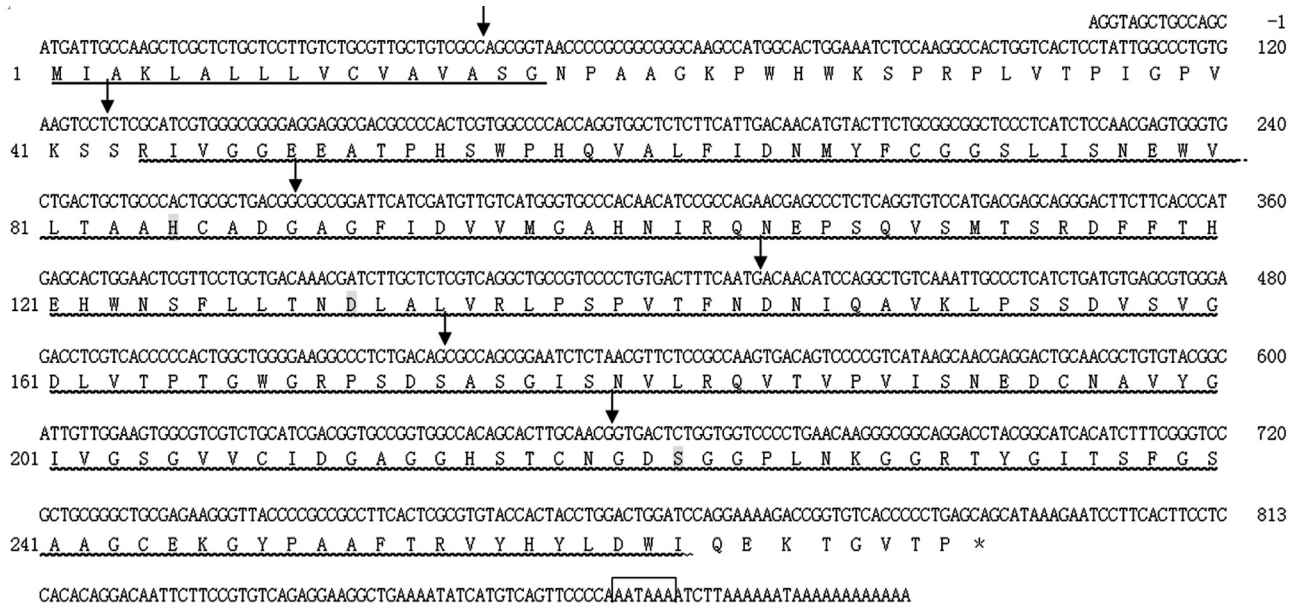

Figure 1 - Nucleotide and deduced amino acid sequences of the *SpCHY* gene (GenBank accession no. JF831535.1). The nucleotides are numbered on the right, and the amino acids on the left. The putative signal peptide is underlined. The trypsin-like SP domain is wave underlined. The catalytic triad (H, D, and S) is gray shadowed. The boxed letters are the polyadenylation signal. The asterisk (*) indicates the stop codon and arrows indicate the location of introns.

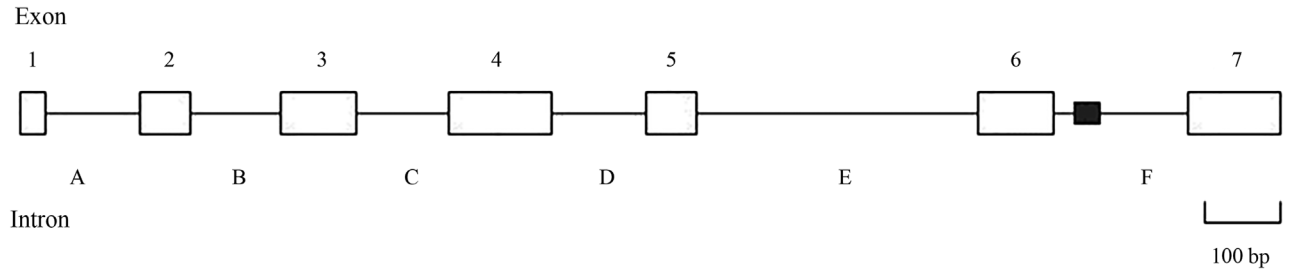

Figure 2 - Organization of the *SpCHY* gene. The positions of the exons (open boxes 1-7), introns (A-F), and CA repeat sequence (filled box) are denoted.

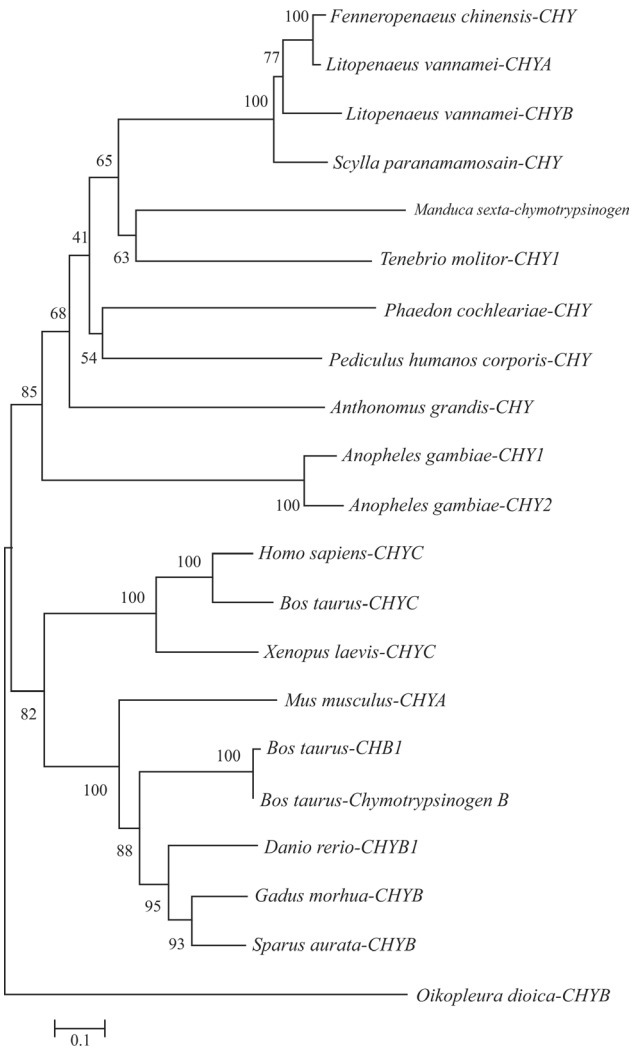

Figure 3 - Phylogenetic analysis of SpCHY with other chymotrypsins. A NJ tree was produced with Mega3.1 software. One thousand bootstraps were carried out to check the repeatability of the result. *L. vannamei*-CHYA (CAA71672), *L. vannamei*-CHYB (CAA71673), *F. chinensis*-CHY (ACC68669), *P. humanus corporis*-CHY (AAV68346), *P. cochleariae*-CHY (CAA76928), *T. molitor*-CHY (DQ356031.1), *M. sexta*-CHY (2120321A), *A. grandis*-CHY (AAT09847.1), *A. gambiae*-CHY1 (CAA79325), *A. gambiae*-CHY2 (CAA79326), *B. Taurus* - CHYB (P00767), *G. morhua*-CHYB (P80646), *S. aurata*-CHYB (AAT45258), *M. musculus*-CHY (AAL11034), *H. sapiens*-CHY (CAA74031.1), *B. taurus*-CHYC(AAI51507.1), *X. laevis*-CHYC(NP_001085458), *B. taurus*-CHYB (NP_001098800.1), *D. rerio*-CHYB1 (NP_997783.1), and *O. dioica*-CHYB (AAT47850).

brain, thoracic ganglion, heart, gill, hepatopancreas, stomach, muscle, and ovary. The mRNA expression level in hepatopancreas was considerably higher than that of other tissues, with the expression level in muscle being the lowest (Figure 4).

Immunocytochemistry and *in situ* hybridization

The histological results showed that hepatopancreas of *S. paramamosain* consists of many blind ending tubules (hepatopancreatic tubules). The hepatopancreas cells could be classified into four types: embryonic cells, fibrillar cells, resorptive cells, and blister cells (Figure 5A).

Using immunocytochemistry, SpCHY protein was detected in resorptive cells of the hepatopancreas, and the positive signals were mottled (Figure 5B). *SpCHY* gene expression was determined by *in situ* hybridization. Positive hybridization signals with the antisense *SpCHY* riboprobe were also mainly localized in resorptive cells (Figure 5C). However, specific signals were also detected in some small cells around the blind ending tubules. No positive signal was detected with the sense *SpCHY* riboprobe in hepatopancreas (Figure 5D).

Expression of *SpCHY* in hepatopancreas following bacterial challenge

In order to determine whether *SpCHY* may be involved in innate immune reactions, the expression profiles of *SpCHY* after bacterial challenge were evaluated. Total

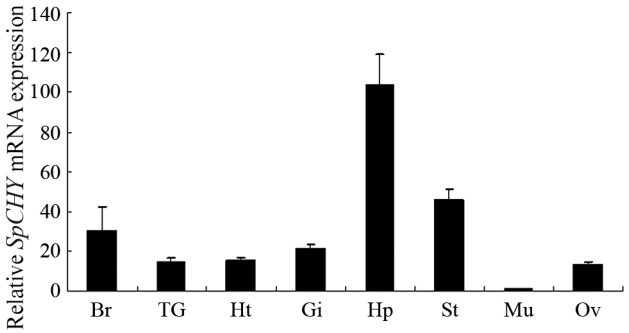

Figure 4 - The results of quantitative real-time PCR analysis of *SpCHY* expression in various tissues. Expression of a β-actin gene was used as control. Values were shown as means ± S.E. (N = 3). Abbreviations: Br, brain; TG, thoracic ganglion; Ht, heart; Gi, gill; Hp, hepatopancreas; St, stomach; Mu, muscle; Ov, ovary.

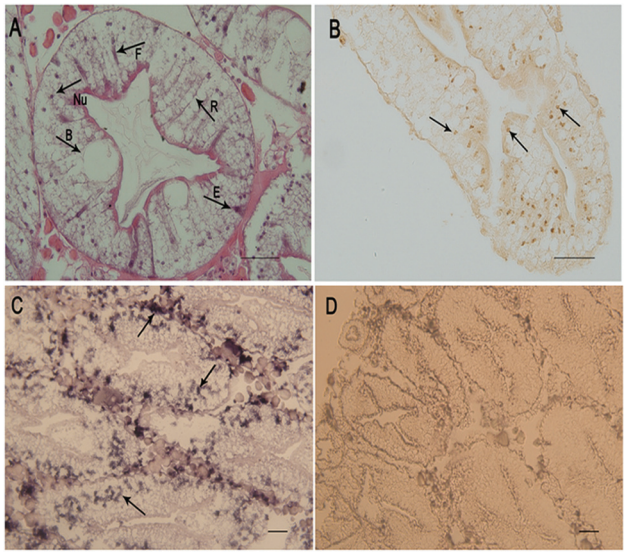

Figure 5 - Location of *SpCHY* by immunocytochemistry and *in situ* hybridization in hepatopancreas of *S. paramamosain*. (A) histological observation; R resorptive cells, B blister cells, E embryonic cells, F fibrillar cells, Nu nucleus. (B) immunocytochemistry results; the arrows point to immunocytochemical positive signals. (C) *in situ* hybridization results; arrows indicate the specific *SpCHY* mRNA hybridization signal with the antisense riboprobe. (D) The negative control with the sense riboprobe showed no specific signal. Scale bars: 50 μm.

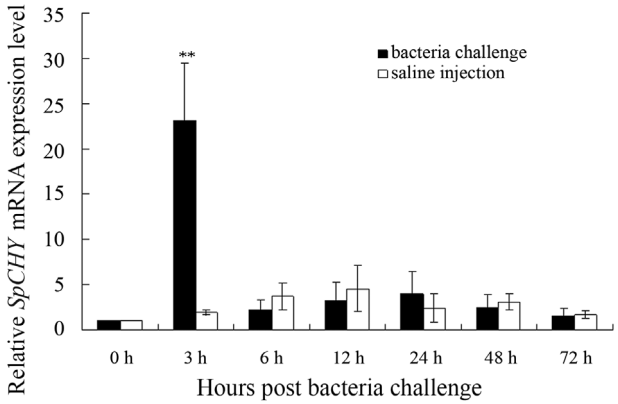

Figure 6 - Transcript profiles of *SpCHY* in hepatopancreas of *S. paramamosain* following challenge with *V. alginolyticus*,. The relative *SpCHY* transcript levels in crabs challenged with *V. alginolyticus* were compared to those of saline injected animals. The expression of a β-actin gene was used as endogenous control. Significant differences of *SpCHY* expression between the challenged and the control group are indicated with asterisks. **$p < 0.01$.

hepatopancreas RNA was extracted from control and bacterial challenged crab at 0, 3, 6, 12, 24, 48 and 72 h. Compared to the control group, in crabs injected with the bacterium *V. alginolyticus* the *SpCHY* mRNA expression level increased distinctly about 20-fold at 3 h ($p < 0.01$) and then decreased to normal level (Figure 6). During this 72 h time interval *SpCHY* expression levels in the control group fluctuated slightly but not significantly.

Discussion

In present study, a new chymotrypsin gene was identified from the mud crab, *S. paramamosain*, and was designated as *SpCHY*. The full-length cDNA contained an 813 bp open reading frame which encoded a putative chymotrypsin of 270 amino acids. The putative amino acids sequence has high identity with the other known crustacean chymotrypsins such as *L. vannamei* and *F. chinensis*. ClustalX alignment of the CHY sequence revealed that the tryp-spc domain was conserved among arthropod chymotrypsins. In addition, the catalytic triad (H, D, S) characteristic of chymotrypsins was observed in the deduced amino sequence. Furthermore, three disulfide bonds formed by six cysteines were found at the same location as in other chymotrypsins. This indicates the importance of secondary structure conservation for the enzymatic activity of this family. Another free cysteine residue found in the signal peptide was also identical to chymotrypsins from other invertebrates. The high similarity, together with the conservation of tryp-spc domain and catalytic triad, indicated that *SpCHY* is a true member of the chymotrypsin family.

The genomic sequence of *SpCHY*, here first reported in crabs, is composed of seven exons and six introns, with the first intron inserted near the end of the putative signal peptide. The locations of introns were almost the same as in the white shrimp, *L. vannamei*, chymotrypsin gene (Sellos and Wormhoudt, 1992). The active site residues (His85, Asp131 and Ser222) involved in catalysis, as well as the residues (Ser216, Ser237 and Gly239) forming the binding pocket to interact with the hydrophobic side chains of the substrate, were encoded by separate exons. These functionally important amino acids and binding regions in separate exons are typical for the *SP* genes that have been described (Swift *et al.*, 1984; Craik *et a*l., 1984). Hence, the joining of different exons, encoding intrinsically catalytically inactive protein segments, resulted in the substrate specificity and catalytic activity of the enzyme. Moreover, the similarity between *SpCHY* and other *SP* genes in the number and location of intron/exon junctions revealed an evolutionary conservation of chymotrypsin gene.

In our study, *SpCHY* expression was detected in various tissues and strongly so in hepatopancreas. The high expression level of *SpCHY* in hepatopancreas was consistent with the role of the hepatopancreas as the main site for synthesizing digestive enzymes in crustaceans (Shi *et al.*, 2008). Furthermore, crustacean hepatopancreas plays important roles in initiating humoral immunity and mediating cellular immune responses performed by certain specialized cells and phagocytes (Gross *et al.*, 2001), which is supported by the discovery of several immunity-related genes in crustacean hepatopancreas post bacterial infection (Pan *et al.*, 2005; Zhao *et al.*, 2007).

The results obtained by immunocytochemistry and *in situ* hybridization indicated that the hepatopancreas is the

site of expression and translation of *SpCHY*. CHY-immunoreactivity was found in resorptive cells, supplying morphological evidence for the secretory function of resorptive cells. The localization of *SpCHY* mRNA in resorptive cells by *in situ* hybridization further strengthens this conclusion. All these findings indicated that *SpCHY* is synthesized in resorptive cells and might be secreted to implement the digestive and immune roles.

Lacking an acquired specific immune system, the innate immune system in crustaceans is considered as the major microbial infection defense mechanism (Chaikeeratisak *et al.*, 2012; Kiruthiga *et al.*, 2012). In recent years, non-specific immune system has been found to be of equal importance as a specific immune system, especially for the production of anti-bacterial and anti-viral proteins (Liu *et al.*, 2010). Pathogen molecules can trigger these immune responses by pattern recognition proteins (PRPs) (Medzhitov and Janeway, 1997). These PRPs bind to microbes and then activate the prophenoloxidase system (proPO-system), stimulate the release of antimicrobial peptides (AMPs), or initiate other biological defense processes. Recently, the clip domain SP was demonstrated to be cofactor for the activation of the proPO cascade in invertebrates (Cerenius and Söderhäll, 2004; Gai *et al.*, 2009). For example, in Sydney rock oysters, *Saccostrea glomerata*, the increase in chymotrypsin could activate ProPO to PO (Aladaileh *et al.*, 2007).

The immune function of chymotrypsin has been reported in *F. chinensis* (Shi *et al.*, 2008). However, little research has focused on the function of innate immunity in crabs. In this study, *SpCHY* was strongly up-regulated in *S. paramamosain* at 3 h after infection with the bacterium *V. alginolyticus*. In appropriate hosts, this kind of bacteria could proliferate unceasingly. The infection caused by unceasing reproduction of bacteria could induce the formation of reactive oxygen species (ROS) and severely destroy the functionality of crab cells (Li *et al.*, 2011). Similar results showing that *SpCHY* expression is significantly shortly after bacterial infection were also obtained in other crustaceans (Amparyup *et al.*, 2007; Qin *et al.*, 2009; Cui *et al.*, 2010). Hence we hypothesize that increasing the expression of *SpCHY* could activate PO production triggering an immune response and killing the bacteria.

In conclusion, our data suggest clearly for the first time that *SpCHY* is involved in the immune reaction against invading bacteria in the mud crab, *S. paramamosain.* The result should be helpful to understand the antibacterial defense mechanisms of crabs and provide biological information for mitigating crab diseases. Notwithstanding, the exact role of *SpCHY* in the activation of the immune response cascade needs further investigation.

Acknowledgments

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Internet Resources

ORF Finder, http://www.ncbi.nlm.nih.gov/gorf (July 3, 2013).

NCBI, http://www.ncbi.nlm.nih.gov (July 3, 2013).

Expasy, http://www.expasy.ch/ (July 3, 2013).

SignalP 4.0 software, http://www.cbs.dtu.dk/services/SignalP (July 3, 2013).

ClustalW, http://www.ebi.ac.uk/Tools/msa/clustalw2/.

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