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

Jie Gong¹, Yinjie Xie¹, Kun Yu¹, Ya’nan Yang¹, Huiyang Huang¹ and Haihui Ye¹,²

¹College of Ocean and Earth Sciences, Xiamen University, Xiamen, Fujian, China. ²Center for Marine Biotechnology, Xiamen University, Xiamen, Fujian, China.

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.

Introduction

Belonging to one of the largest gene family in the animal kingdom, serine proteases (SP) have a tryp-sp 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 (Mazurek 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 inactivezymogens, 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).
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 *CHYf1* and *CHYr1* (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 *CHY3’* and *CHY5’* 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 Mg²⁺), 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 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

| Primer name | Primer Sequence (5'-3') | Purpose | Amplified fragment length |
|-------------|------------------------|---------|--------------------------|
| *CHYf1*     | GGYGTYGTYTGCATYGACGGHRC | fragment amplification | 203 bp |
| *CHYr1*     | GCTCAGGGWKTGACRCCRGTCTT | fragment amplification | 286 bp |
| *CHY’*      | CTGCCTCTCTCCTTCTGCTG   | 3’ amplification 904 bp |
| *CHY5’*     | GAAAGATGTGATGCCTAGGGTC | 5’ amplification 728 bp |
| *CHY2*      | ATGATTGCCAAGCTGCTCTG   | genomic DNA amplification 1994 bp |
| *CHY2*      | TCAGGGGTTGACACCGGTC   | genomic DNA amplification 1994 bp |
| *CHY3*      | ACGAGCAGGGACTTCTCTTACC | real-time RT-PCR for *SpCHY* 286 bp |
| *CHY3’*     | AGACGACGCGACTTCTCCACA | real-time RT-PCR for *SpCHY* 314 bp |
| *CHY5-1*    | CAGCAACGCAAGACAGGACA | promoter region clone 1261 bp |
| *CHY5-2*    | TGGGGGAAGAAGGAAGTGGGC | promoter region clone 1155 bp |
| *CHY5-3*    | GCAAAACATCTACGACCACAGCA | promoter region clone 974 bp |
| *TCHf1*     | GCCGAAACGAGCCTCCCTCAG | riboprobe amplification clone 314 bp |
| *TCHy1*     | GACGGAGCACTCCCTCAAAT | riboprobe amplification clone 314 bp |
| *T7*        | TAATACGACTACATGTAGGG | colony PCR 183 bp |
| *M13-47*    | CGCCAGGGTTTCTCCAGTCAG | colony PCR 183 bp |
| *RV-M*      | GACGGATACCAATTTTCACACA | colony PCR 183 bp |
| *β-actin F* | GACGGAGGAATCGTTGTGAC | internal control 183 bp |
| *β-actin R* | GGAGGAGGCTGGAAGAGAG | internal control 183 bp |
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 CHYr2 and CHYr1 (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 CHYS-1, CHYS-2, CHYS-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 CHYr3 and CHYr3; 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 18S fragment of S. paramamosain was amplified as the internal control. All samples were run in triplicate and relative expression was calculated as 2^ΔΔCt (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 diethylyrocarbonate (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 TCHY1, TCHYr1 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 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...
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
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 al., 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 (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.

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Internet Resources

ORF Finder, http://www.ncbi.nlm.nih.gov/orf (July 3, 2013).
NCBI, http://www.ncbi.nlm.nih.gov (July 3, 2013).
Expasy, http://www.expasy.org/ (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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