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Short communication

Identification, expression, and responses to bacterial challenge of the cathepsin C gene from the razor clam *Sinonovacula constricta*

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ABSTRACT

Cathepsin C (dipeptidyl-peptidaseI, DPPI) is a lysosomal cysteine proteinase that belongs to the papain superfamily, and it is involved in protein degradation and proenzyme activation. However, very little is known about the function of cathepsin C in bivalves. In the present study, we identified the cathepsin C gene in the razor clam *Sinonovacula constricta* (*Sc-CTSC*). The full-length *Sc-CTSC* cDNA contained a complete open reading frame (ORF) of 1371 nt encoding 456 amino acids, a 98 bp 5′ UTR, and a 1043 bp 3′ UTR. The ORF of *Sc-CTSC* consisted of a putative signal peptide of 22 aa, a propeptide of 229 aa, and a mature peptide of 205 aa containing the active site triad of Cys, His, and Asn. The *Sc-CTSC* transcript was expressed in a wide range of tissues but exhibited the greatest level of expression in the digestive gland. During the early developmental stages, the transcript was detected widely. Upon injection with *Vibrio anguillarum*, the *Sc-CTSC* transcript was significantly up-regulated in digestive gland, mantle, and gill tissues. The results provided important information for further exploring the roles of cathepsin C in the innate immune responses.

1. Introduction

Cathepsin is a family of lysosomal proteases that consists of three groups, which are based on the amino acid residues in their active sites: serine protease (cathepsin A and G), aspartic protease (cathepsin D and E), and cysteine protease (cathepsin B, C, F, H, K, L, O, S, W, and Z) (Chen et al., 2011). Most lysosomal cysteine cathepsins are members of the papain family of cysteine proteases and belong to the C1 peptidase family (Colbert et al., 2009). The cysteine cathepsins are synthesized as inactive proenzymes, and their activation requires the removal of an N-terminal propeptide region (Qiu et al., 2013).

Cathepsin C, also known as dipeptidyl peptidase I (DDPI) (EC 3.4.14.1), is a lysosomal cysteine protease that belongs to the papain superfamily (Kominami et al., 1992), and it exhibits both endopeptidase and dipeptidyl carboxy peptidase activities (McGrath, 1999). Most cysteine proteinases in the papain family are monomers containing R- and L-domains (Turk et al., 1996). However, cathepsin C has an unique structure consisting of four identical subunits, which are each composed of three polypeptide chains: the pro-region and the heavy and light chains (Cigić et al., 2000, 1998; Dolenc et al., 1995). The heavy and light chains form a papain-like structure that consists of two domains, with an active site at the interface. It has been postulated that the pro-region contains intramolecular disulfide bonds and that it is glycosylated (Cigić et al., 2000, 1998). The pro-region has no analogy in the papain family, leading to its unique amino dipeptidyl peptidase activity by extending and capping the active site cleft and preventing endopeptidase activity (Tran et al., 2002). The pro-region is required for the proper transport and expression of newly synthesized cathepsin C (Santilman et al., 2002). Moreover, it is important that pro-cathepsin C is cleaved by a certain proteinase, such as cathepsin L or S (Dahl et al., 2001), which allows cathepsin C to form an oligomeric structure with proteinase activity (Matsui et al., 2002; Qiu et al., 2008).

Cathepsin C is part of the lysosomal hydrolytic armaments, which degrade proteins and peptides conveyed to the lysosome through endocytosis or autophagy (Santilman et al., 2002). Cathepsin C is a central coordinator for the activation of many serine proteinases in immune and inflammatory cells. It activates many chymotrypsin-like serine proteases by removing one or several dipeptides from the N-termini of the zymogens (Tran et al., 2002). Additionally, cathepsin C deficiency is associated with known diseases. Papillon–Lefèvre syndrome (PLS) and Haim–Munk syndrome (HMS) are associated with premature periodontal

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destruction. Mutations of the cathepsin C gene have been identified as the underlying genetic defect in PLS. The mutations of exon 6 of cathepsin C were identified in PLS and HMS. Moreover, PLS and HMS were allelic variants of cathepsin C gene mutations (Hart et al., 2000, 1999). As part of the DPPI-dependent neutrophil recruitment and cytokine response, cathepsin C and neutrophils play a critical role in the Sendai virus-induced asthma phenotype (Akk et al., 2008). In invertebrates, cathepsin C perhaps is important for anti-bacterial immune responses. Cathepsin C expression is up-regulated in hemocytes, hepatopancreas, gills and intestine in response to *Vibrio anguillarum* and WSSV injections in *Fenneropenaeus chinensis* (Wang et al., 2012), lipopolysaccharide (LPS) stimulation in *Penaeus monodon* (Qiu et al., 2008), and *V. anguillarum* stimulation in *Eriocheir sinensis* (Li et al., 2010a).

Cathepsin C is active in a variety of mammalian tissues, particularly lung, liver and spleen (Kominami et al., 1992; McGuire et al., 1997). Cathepsin C is also present in fish, crab and shrimp, including *Danio rerio* (NM-214722), *Takifugu rubripes* (XP_003968362), *E. sinensis* (ADO65981), *Litopenaeus vannamei* (ACK57788), *P. monodon* (ABW74905), *F. chinensis* (ACG60902), and *M. japonicus* (AB104735). In bivalves, only two incomplete cathepsin C sequences in *Crassostrea gigas* are known, and there are no reports on the function of the gene.

The razor clam *Sinonovacula constricta*, belonging to Mollusca (Bivalvia: Veneroida: Solecurtidae), lives in lower-to-mid intertidal zones along coasts of the Western Pacific Ocean. The species has a high market value because of its short culture cycle, rapid growth rate, and delicious taste. Indeed, it is one of four major aquacultured clams along with *Crassostrea gigas, Ruditapes philippinarum, and Tegillarca granosa* in China (Xie, 2003). The clam has been cultured for 100 years in Zhejiang and Fujian Provinces in China. However, in recent years, aquaculture of this species is facing the high mortality mainly due to bacterial diseases (Qiu et al., 2010; Wang et al., 2006; Yang et al., 2003). A number of *Vibrio* species have been described as the main causal agents of diseases affecting all life stages of bivalve molluscs: larval, juveniles and adults (Castro et al., 1996) and have provoked severe mortality outbreaks in shellfish (Beaz-Hidalgo et al., 2010). *V. anguillarum* as potential pathogen was remarkable in *Argopecten purpuratus* (Riquelme et al., 1996). The diseases caused by a wide range of microorganisms are associated with large economic losses in *Ruditapes decussatus* and *Ruditapes philippinarum*, The majority of bacterial diseases are caused by members of the Vibrio genus. *Vibrio alginolyticus* was associated with high mortality rates (up to 73%) of *R. decussatus* larvae (Moreira et al., 2012). Both *Vibrio splendidus* and *V. anguillarum* significantly induced lysosomal membrane destabilisation and increases in the activities of antioxidant enzymes in *Mytilus galloprovincialis* (Canesi et al., 2010). In the present study, we analyzed the cathepsin C sequence, its expression patterns and its responses after bacterial injection in the razor clam *S. constricta.*

2. Materials and methods

2.1. Identification of cathepsin C and sequence analysis

We searched the cDNA library of *S. constricta* (Feng et al., 2010) using zebrafish cathepsin C as a query. The unique sequences obtained were analyzed using BlastX to confirm that the ESTs were related to cathepsin C genes. The chz_0021_B12 clone in the libary contained the full 3′ terminal and partial 5′ terminal cDNA of cathepsin C in the library. To confirm the transcript identity and sequence accuracy, re-sequencing was performed using the Sanger technology with the universal M13 primer (Supplementary Table 1) on the ABI3730 platform (Applied Biosystems, Forster City, CA, USA). To complete the cDNA sequence, the 5′ terminal sequence was obtained using the 5′-Full RACE kit (Takara, Otsu, Shiga, Japan) with a 5′ RACE primer (Supplementary Table 1), according to the manufacturers' instructions. In addition, cathepsin C gene ORF were identified using the Open Reading Frame Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), and the signal peptide was predicted by the SignalP4.1 Server (http://www.cbs.dtu.dk/services/SignalP/).

2.2. Alignment and phylogenetic analysis

The deduced amino acid sequences of cathepsin C from various vertebrates and invertebrates were compared using the ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/). Phylogenetic trees were constructed using the neighbor-joining (NJ) and UPGMA methods with 10,000 bootstrapping replications within the Molecular Evolutionary Genetics Analysis (MEGA 4.0) package. The data were analyzed using a Poisson correction, and gaps were removed by complete deletion.

2.3. Biological material

All experimental clams *S. constricta* were obtained from the Yuejingyang Farm, Ninghai City, Zhejiang Province, China. Embryos and larvae were reared in hatching tanks at 25–27 °C. The samples from different developmental stages included the post-fertilization embryo period, trochophore, veliger larvae, umbo larvae, creeping larvae, and juvenile clams. The embryos and larvae were collected using different mesh silk screen. The juveniles were picked by hands. These samples were stored into 1.5 ml microtubes with RNA storage liquid. Each tube contained dozens of individuals.

2.4. Bacterial challenge and collection of tissue samples

Clams used for the bacterial challenge had an average body weight of 9.2 g and an average body length of 5.5 cm, and they were kept in water at 25–27 °C and 30% salinity. The clams were acclimated for a week before processing. There were two experimental groups, a control group and a group for *V. anguillarum* challenge. *V. anguillarum* (byk0637) was from Aquatic Pathogen Collection Centre of Ministry of Agriculture, China. The bacteria were cultured at 28 °C with 2216E culture medium for 24 h and adjusted at 1×10^8/ml with phosphate-buffered saline (PBS, phosphate-buffered saline, pH 7.2–7.4). Clams in challenged group were injected from the feet with 50 μL of a *V. anguillarum* suspension each clam, whereas clams injected with 50 μL sterile PBS were used as the control group.

To determine the cathepsin C expression in healthy tissues, samples of six tissues including gill, mantle, gonad, foot, siphon, and digestive gland (four pools of six individuals each) were isolated and flash-frozen in liquid nitrogen. Tissues were homogenized in liquid nitrogen using a mortar and pestle and stored at −80 °C until RNA extraction. Similarly, the tissues (four pools of six clams each) were isolated for RNA extraction at 4, 8, 12, 24, 48, and 72 h after *V. anguillarum* treatment. Control samples were taken also at each time interval.

2.5. RNA isolation and first-strand cDNA synthesis

Total RNA was extracted using the RNeasy Plus kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The RNeasy kit included a genomic DNA elimination column. RNA was assayed for its A 260/280 ratio on a Nanodrop 2000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The RNA integrity was checked by electrophoresis on 1% agarose gels. First-strand cDNA synthesis was performed in a volume of 20 μL with 1 μg total RNA a PrimeScript™RT reagent kit (Takara,