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The first CUB-domain containing serine protease from *Chlamys farreri* which might be involved in larval development and immune response

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ABSTRACT

Serine proteases (SPs) are one of the most well understood enzyme families, which play an important role in regulating many physiological events. In the present study, one CUB-domain containing serine protease was identified from *Chlamys farreri* (designated as CfCUBSP). The full-length cDNA of CfCUBSP was of 3181 bp with an open reading frame of 2688 bp encoding a polypeptide of 896 amino acids. CfCUBSP shared closer phylogenetic relationship with those multi-domain SPs which consisted of one SP domain, and different numbers of CUB domain and LDLa domain than other SPs. The mRNA transcripts of CfCUBSP were detected in all developmental stages with the highest expression level in fertilized eggs and the lowest in trochophore larvae. In adult scallop, the CfCUBSP mRNA could be detected in all examined tissues with the highest level in hepatopancreas, and CfCUBSP protein was dominantly located in the gills, hepatopancreas, gonad and kidney. The mRNA expression of CfCUBSP in hemocytes was significantly up-regulated after the stimulation of lipopolysaccharide (LPS), peptidoglycan (PGN) and β-glucan (GLU) ($P < 0.05$). All the results collectively indicated that CfCUBSP was a primitive member of the invertebrate SPs which might be involved in larval development and immune response against Gram-negative (G−) and Gram-positive (G+) bacteria and fungus in scallop.

1. Introduction

Serine proteases (SPs) are the largest classes of proteases widely found in viruses, bacteria and eukaryotes (Rawlings et al., 2006). By far, 36 SP families have been identified, and grouped into at least 12 clans (Rawlings et al., 2015). In invertebrates, large numbers of SP and SP homolog (SPH) genes have been identified in the genome of *Drosophila melanogaster* (Ross et al., 2003), *Apis mellifera* (Zou et al., 2006), *Crassostrea gigas* (Zhang et al., 2012) and *Nilaparvata lugens* (Bao et al., 2014), which are divided into single or multi-domain SPs according to their domain composition. Single domain SPs contain only one SP domain, while multi-domain SPs contain additional domains such as clip domain, Low Density lipoprotein receptor class A (LDLa) domain and C1r/C1s/Uegf/bone morphogenetic protein 1 (CUB) domain (Zou et al., 2006; Bao et al., 2014). The structural diversity of SPs suggests their multi-functions in maintaining the normal physiological activity.

Accumulating evidences indicated that SPs played important roles in regulating many physiological events, such as embryogenesis and development (Hong and Hashimoto, 1995; Konrad et al., 1998), food digestion (Neurath, 1984; Zou et al., 2006) and immune response (Ren et al., 2011). Trypsin was the most well-studied single domain SP, which was originally identified in digestive process (Zou et al., 2006), and subsequently was confirmed to be involved in development, fertilization, apoptosis and immunity (Page and Di Cera, 2008; Lee et al., 2004; Shi et al., 2009; Balasubramanian et al., 2010). The clip domain SPs (cSPs), a multi-domain SP firstly identified in arthropods, have been proven to mediate dorsoventral pattern formation, embryonic development and immune defense processes (Jiang and Kanost, 2000; Ligoxygakis et al., 2002). Mannose-binding lectin-associated serine proteases (MASPs), consisted of CUB domain, epidermal

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growth factor (EGF)-like domain, complement control protein (CCP) domain and SP domain, are involved in complement activation through the lectin pathway (Endo et al., 2003).

Compared with those in arthropods and vertebrates, the information about molluscan SPs is mainly from cSPs (Zhu et al., 2007; Zhang et al., 2009a,b), and there is still no report on CUB domain containing SP (CUBSP) in mollusk. As the cascades of protease are consisted of multiple steps of protease activation to mediate many well-known innate immune processes (Jiravanichpaisal et al., 2006; Iwanaga and Lee, 2005; Cerenius and Söderhäll, 2004), the investigation on the structure characteristics and immune activities of CUBSP in *C. farreri* (designated as CfCUBSP) will provide new insights into the role of CUBSPs in scallop larval development and innate immune responses. The main objectives of the present study were (1) to characterize the molecular structure of CfCUBSP, (2) to detect its expression in various tissues at mRNA and protein level, and (3) to investigate its temporal expression in different development stages and after the stimulations with different pathogen-associated molecular patterns (PAMPs).

2. Material and methods

2.1. Scallops, RNA isolation and cDNA synthesis

Zhikong scallop *C. farreri*, averaging about 55 mm in shell length and 50 g in weight, were collected from a scallop farm in Qingdao, Shandong Province, China.

Total RNA isolation and cDNA synthesis were performed as previously described (Yang et al., 2014).

2.2. Cloning and sequence analysis of the full-length cDNA of CfCUBSP

Based on the EST sequence (No. rscag0_003163, Wang et al., 2009) homologous to a CUB domain containing protein identified previously (EKC26355, Zhang et al., 2012), two specific primers were designed to clone the full-length cDNA of CfCUBSP by rapid amplification of cDNA ends (RACE) approach according to the Usage information of 5'/3' RACE system (Invitrogen). The information of all primers used in this assay was shown in Table S1. After gel-purification, the PCR products were cloned into pMD 18-T vector (TaKaRa) and sequenced in both directions with primers M13-47 and RV-M. The sequencing results were verified and subjected to sequence analysis.

The cDNA sequence analysis, deduced amino acid sequence analysis, the protein domains predication and the multiple sequence alignmentwere performed as previously described (Yang et al., 2014). An unrooted phylogenetic tree was constructed based on the sequence alignment by the neighbor-joining (NJ) algorithm using the MEGA4.1 beta software (www.megasoftware.net). The reliability of the branching was tested by bootstrap re-sampling (1000 pseudo-replicates).

2.3. Examination of the CfCUBSP mRNA in different developmental stages

All embryos and larvae were sampled from the Yixiang farm in Rongcheng, Shandong Province, China in April and May, 2011 (Yue et al., 2013). Briefly, the embryo or larva at different stages was identified microscopically according to Cragg's description (Newsholme et al., 1978) and collected as oocytes, fertilized eggs, 4-cell embryos, morula (6 h post-fertilization, hpf), blastula (11 hpf), gastrula (18 hpf), trochophore (22 hpf), D-hinged larvae (2 day post-fertilization, dpf), mid-veliger larvae (7 dpf) and eyebot larvae. For each developmental stage, six samples were collected, re-suspended in 1 mL TRIzol reagent (Invitrogen, Carlsbad, CA), and stored in liquid nitrogen immediately. RNA isolation and cDNA synthesis were carried out as described above.

The mRNA transcripts of CfCUBSP in different developmental stages were analyzed by SYBR Green fluorescent quantitative real-time PCR (qRT-PCR). All reactions were performed as previously described (Yue et al., 2013). The information of all primers used in this assay was shown in Table S1. The expressions of CfCUBSP were normalized to that of β-actin gene for each sample. The $2^{-\triangle\triangle CT}$ method was used to analyze the expression level of CfCUBSP (Kenneth and Thomas, 2001). All data were given in terms of relative mRNA expressed as mean ± SE ($N \geq 4$). The data was subjected to one-way analysis of variance (one-way ANOVA) followed by a multiple comparison (S-NK). Differences were considered significant at $P < 0.05$.

2.4. Temporal expression patterns of CfCUBSP mRNA in different tissues and in hemocytes after PAMPs stimulation

Hemolymph from the scallops was collected from the adductor muscle and immediately centrifuged at 500×*g*, 4 °C for 10 min to harvest the hemocytes. The total RNA from hemocytes, hepatopancreas, gonad, mantle, gill, adductor muscle and kidney was extracted from six adult scallops as parallel samples using TRIzol reagent (Invitrogen). RNA isolation, cDNA synthesis, RT-PCR and statistical analysis were carried out as described above.

The PAMPs, including LPS, PGN and GLU stimulation experiment were performed using 150 scallops as previously described (Yang et al., 2014), and the final concentration of LPS, PGN and GLU stimuli used per scallop was 0.5 μg g^{-1}, 0.8 μg g^{-1} and 1 μg g^{-1}, respectively. The scallops received PBS stimulation and the untreated scallops were employed as control and blank group. After stimulation, the scallops were returned to water tanks and 6 individuals were randomly sampled at 3, 6, 12 and 24 h post-injection. The hemocytes collection, RNA isolation, cDNA synthesis, RT-PCR and statistical analysis were carried out as described above.

2.5. Preparation of recombinant protein and polyclonal antibody of CfCUBSP

The cDNA fragment encoding a polypeptide from T484 to P895 consisting of one CUB domain, one LDLa domain and one Trypsin-like serine protease (Tryp_SPc) domain was amplified with specific primers CfCUBSP-ReF and CfCUBSP-ReR (Table S1). The PCR products were gel-purified and cloned into pEASY-E1 expression vector with a His tag (Transgen, China). The recombinant plasmid (pEASY-E1-CfCUBSP) was transformed into *Trans*-T1 phage resistant chemically competent cell (Transgen), and the forward positive clones were screened by PCR (Table S1). The valid recombinant plasmid was extracted and transformed into *E. coli* BL21 (DE3)-Transetta (Transgen). The culture of positive transformants, the induction and purification of recombinant CfCUBSP proteins (rCfCUBSP), and the refolding of the purified rCfCUBSP were performed as previously described (Yang et al., 2014). The resultant proteins were separated by reducing 15% SDS-PAGE, and visualized with Coomassie Bright Blue R250. The concentration of purified rCfCUBSP was quantified by BCA method (Smith et al., 1985).

For preparation of antibody, the renatured rCfCUBSP was continued to dialyse against ddH_2O before protein were freeze concentrated. Then 6 weeks old rats were immunized by rCfCUBSP to acquire polyclonal antibody as previously described (Zhou et al., 2013). The titers of anti-rCfCUBSP antibodies were detected by Western blotting analysis (Zhou et al., 2013). Rats' preimmune serum was used as negative control.