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Short communication

Molecular cloning, characterization and expression analysis of trypsin-like serine protease from triangle-shell pearl mussel (*Hyriopsis cumingii*)

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ABSTRACT

Trypsin-like serine protease (TLS) is ubiquitous in animals and plays a number of diverse roles, including dietary protein digestion, hemolymph coagulation, antimicrobial activity and immune responses, among others. This study reports the isolation of a 1048 bp full-length cDNA sequence of TLS from triangle-shell pearl mussel (*Hyriopsis cumingii*), including a 12 bp 5' UTR (untranslated region), a 172 bp 3' UTR, and an open reading frame (ORF) of 864 bp by rapid amplification of cDNA ends (RACE). Bioinformatic analysis shows that the gene belongs to the trypsin-like serine protease superfamily, and contains a 15 residues N-terminal signal peptide and a conserved C-terminal domain. In comparison to other serine proteases, the catalytic triad were identified as His-98, Asp-149, and Ser-240. Quantitative real-time PCR (qPCR) showed a broad expression of the TLS gene in ten tested tissues. Time-course expression analysis demonstrated that the expression level of the TLS mRNA was significantly up-regulated in eight tested tissues (liver, intestine, gill, heart, axe foot, adductor muscle, kidney and gonad), but down-regulated in mantle and stomach after *Aeromonas hydrophila* injection. This is one of the results indicate that TLS may be involved in innate defense reactions against *A. hydrophila* in triangle-shell pearl mussel.

1. Introduction

The triangle-shell pearl mussel (*Hyriopsis cumingii*), commonly known as the freshwater bivalve mollusk, belongs to the Unionidae family. It is a unique resource in China, prized for its pearl-making capabilities [1]. Like other invertebrates, the triangle-shell pearl mussel utilizes a series of innate immune responses which are believed to be its sole defense against microbial threats and invasion [2]. Deterioration of local aquaculture environments has led to a greater susceptibility of disease outbreaks among triangle-shell pearl mussel, causing a significant decline of its production. Previous study showed that *A. hydrophila* caused great damage to the aquatic animals [3] and it was the main pathogen which caused triangle-shell pearl mussel to be dead [4].

Serine proteases are involved in several functions including digestion, activation of the complement system, cell differentiation and hemostasis, and this has been demonstrated in several microorganisms, plants, and animals [5]. TLS was a member of the chymotrypsin/trypsin family of serine proteases and was almost totally confined to animals [6]. TLS shared a common mechanism of catalysis that rely upon the coordinate action of three catalytic residues: His (H), Asp (D), Ser (S), which selectively hydrolyze peptide bonds C-terminal to basic amino acid residue [7,8]. TLS mostly function extracellularly in roles that include dietary protein digestion, hemolymph coagulation, antimicrobial peptide synthesis, and the activation of a rapidly immune pathway in response to pathogen detection [9–13]. TLS was found to be one of the most important proteases, contributing approximately 6% of soluble protein in the digestive gland [14]. Studies showed that prophenoloxidase (proPO) can be activated in vitro by exogenous trypsin in shrimp [15] and trypsin was involved in the regulation of innate immunity in the small intestine by acting an antimicrobial peptide prodefensin convertase [16].

Several TLS genes had been previously identified. Four trypsin-like serine protease genes were obtained from the hepatopancreas of the Chinese shrimp, with all showing upregulation during

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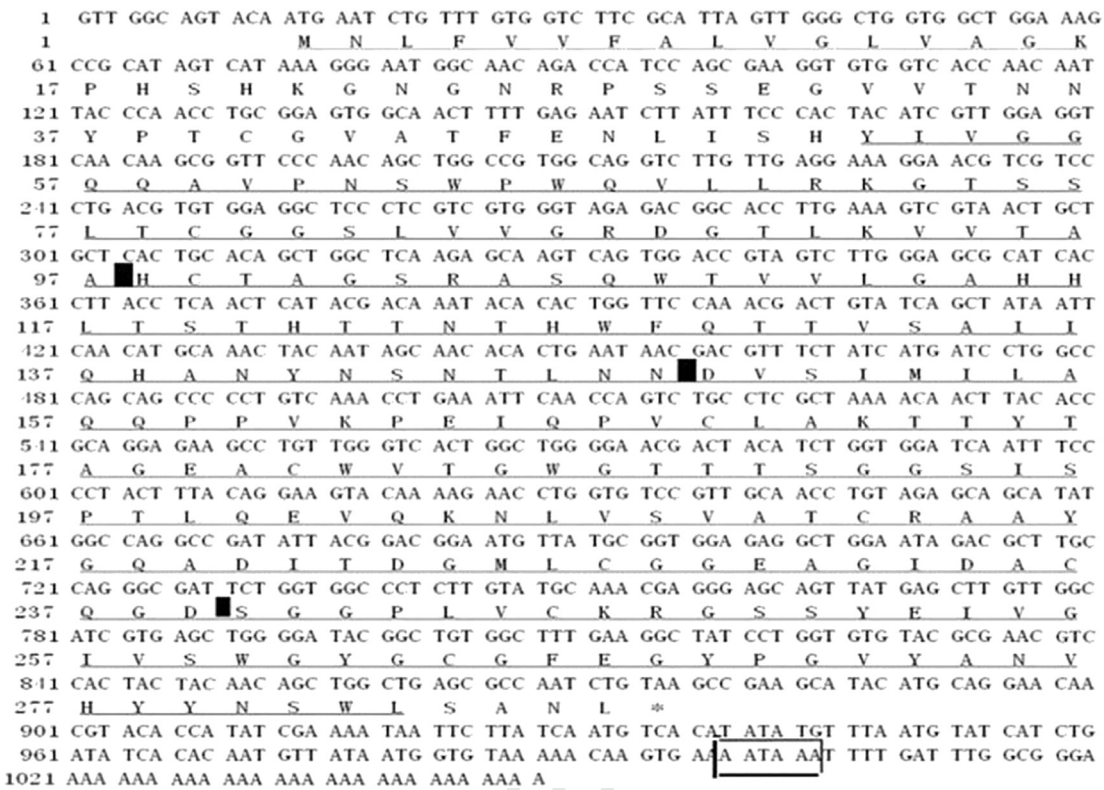

Fig. 1. The full-length cDNA sequence and deduced amino acid sequence of Trypsin-like Serine Protease. The signal peptide is labeled by the dashed underline. The serine protease Tryp_SPc domain is labeled by the single underline. The mRNA polyadenylation signal is labeled by the solid line box. The catalytic triad amino acids are labeled with rectangles on the left side.

host infection with the white spot syndrome virus (WSSV). An additional protease, Fctry3, showed increased expression levels after a bacterial challenge [17]. From a 1216 bp full-length cDNA sequence of TLS that was cloned from *Apostichopus japonicus* using the RACE technique, TLS was shown to be regulated during different stages of regeneration, suggesting that TLS was important in the regeneration process of *A. japonicus* [18].

In this study, a trypsin-like serine protease gene from *H. cumingii* was cloned. The tissue-specific expression and temporal expression profiles of the gene after stimulation with *A. hydrophila* were analyzed by qPCR. The results of this study determine the involvement of TLS in the innate immunity of triangle-shell mussel.

2. Materials and methods

2.1. Animal and immune challenge

Triangle-shell mussels were obtained from a commercial farm in Changde, Hunan Province, China. The average weight was 280.73 ± 55.18 g and the average length was 13.57 ± 1.28 cm. The mussels were acclimatized for one week before the experiment at 24–28 °C. *A. hydrophila* was received as a gift from the Institute of Hydrobiology at the Chinese Academy of Sciences.

Healthy mussels were challenged by injection of 0.5 mL 10^9 cfu/ml *A. hydrophila*. Unchallenged mussels were used as control. Tissue samples including liver, stomach, intestine, gill, heart, mantle, axe foot, adductor muscle, kidney, and gonad from three control mussels and three experimental mussels were collected at 0 h, 3 h, 6 h, 12 h, 24 h, and 48 h after injection, frozen in liquid nitrogen immediately for use.

2.2. RNA extraction and cDNA synthesis

Total RNA contents from a variety of tissues (liver, stomach, intestine, gill, heart, mantle, axe foot, adductor muscle, kidney, gonad) were extracted according to the manufacturer's protocol of the RNAprep pure Tissue Kit (Tiangen biotech, China). The mRNAs of different tissues were reversely transcribed to cDNAs using the SMARTer™ RACE (Rapid amplification of cDNA ends) cDNA Amplification Kit (Clontech, USA).

2.3. Cloning and sequencing of trypsin-like serine proteases cDNA

Primers for TLS gene were designed according to the expressed sequence tag (EST, GenBank accession number: EX828678) which was obtained by constructing a subtractive hybridization cDNA library [19]. 5′-RACE-Ready cDNA was synthesized using the SMART II™ A Oligonucleotide(AAGCAGTGG TATCAACGCAGAGTACGCGGG) and 5′ -RACE CDS Primer A (AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$V N), then 5′-RACE was performed using the gene specific primer pairs UPM/TLS-GSPR-out (TCGCCCTGGCAAGCGTCTA.TTCC) and UPM/TLS-GSPR-in (AGCCTTCAAAGCCACAGCCGTAT) in nested PCRs. 3′ -RACE-Ready cDNA was synthesized using the 3′ -RACE CDS Primer A (AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$V N), then 3′-RACE was performed using the gene specific primer pairs UPM/TLS-GSPF-out (ACGGACGGAATGTTATGCGGTGGA) and UPM/TLS-GSPF-in (ACGGCTGTGGCTTTGAAGGCTAT) in nested PCRs. PCR for 5′ end of TLS was performed with the following program: 94 °C for 5 min, followed by 5 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 45 s, then 30 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 45 s. After the final cycle, samples were incubated at 72 °C for 10 min. PCR enrichment of 3′ end of TLS was performed

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