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Identification and activity of a paralog of cathepsin S from yellow catfish (*Pelteobagrus fulvidraco*) involved in immune response

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ABSTRACT

Cathepsin S, a papain-like cysteine peptidase, is an important regulator and signaling molecule with diverse biological actions in addition to immune presentation. However, our understanding of its structure and properties remains limited. Herein, a full-length cathepsin Sa from yellow catfish was cloned and named *PfCTSSa*. It contained 1366 bp, including a 981 bp ORF flanked by a 123 bp 5′-untranslated region (UTR) and a 262 bp 3′-UTR. This ORF encoded a 36.5 kD cysteine protease with the deduced amino acid sequence having a 76% sequence identity with *Ictalurus punctatus* ctssa. Additionally, PfCTSSa was found to be a paralog of cathepsin S since it generated a new cluster with cathepsin Sa in the phylogenic tree. Furthermore, PfCTSSa was found to contain more *N*-glycosylation sites than cathepsin S. The recombinant PfCTSSa was overexpressed in *E. coli* BL21 (DE3) and appeared to have the strongest activity at pH 8.5 and 35 °C in a concentration-dependent manner, with activity further affected by metal ions and detergents. Moreover, *PfCTSSa* mRNA was highly expressed in classic and mucosal immune tissues, although constitutively distributed in all of the examined tissues. Yellow catfish were then challenged with inactivated *Aeromonas hydrophila* and *PfCTSSa* was remarkably increased in the head kidney, liver and spleen when compared to the PBS control. Collectively, these results indicate that *PfCTSSa* is a paralog of cathepsin S and functions in the yellow catfish immune response.

1. Introduction

Cathepsins are proteases responsible for lysosome protein degradation and are widely distributed among prokaryotes and eukaryotes [1]. When synthesized as an inactive precursor, the typical domain architectures seen include a signal peptide, a propeptide, and a mature peptide. The signal peptide guides the ribosome-synthesized zymogen to the endoplasmic reticulum where it is hydrolyzed. The remaining procathepsin is modified in the Golgi complex, where a mannose-6-phosphate tag is added. This tag is then recognized by a lysosome receptor. After translocation to the lysosome, the acidic pH within facilitates the excision of the propeptide from the procathepsin by other proteases or by an autocatalytic mechanism, thus generating a mature peptide with proteolytic activity [2].

Cathepsins are classified into serine (A and G), asparagine (D and E), and cysteine proteases (B, C, F, H, L, K, O, S, V, W, and X) according to the active site amino acids [3]. Furthermore, 11 human cysteine cathepsins can be further divided into the L-like (L, V, K, S, W, F, and H) and B-like subfamily (B, C, O, and X) based on their propeptide structures [3,4]. Despite the mature peptides of these two subfamilies being highly conserved, the propeptides differ significantly in their lengths and motifs. The L-like family (~100 residues) propeptides are longer than the B-like family (~60 residues) and contain two conserved motifs, ERF(W)NIN and GNFD. The ERF(W)NIN is lacking in the B-like family [3].

In cysteine cathepsins, the inhibitory propeptide is removed from the active site by exposure to the acidic pH within the lysosome to enable activity [5]. The neutral pH outside of the lysosome will cause them to be rapidly irreversibly inactivated. Uniquely, cathepsin S is stable at a neutral or slightly alkaline pH [2], thus allowing it to function outside of lysosomes [6]. Moreover, abnormal cathepsin S expression has been implicated with several human pathologies, such as rheumatoid arthritis [7] and cancer [2]. In humans, cathepsin S is highly expressed in antigen-presenting cells (APCs), such as the dendritic cells (DCs) and B-cells [8] and plays a more specific role by participating in the endosome antigen process and presentation. In antigen presentation, cathepsin S cleaves the MHC class II-associated invariant chain so that

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exogenous antigenic peptides can bind MHC class II molecules and be presented to $CD4^+$ T cells for recognition [2,9–12].

In *Ictalurus punctatus*, two cathepsin S genes, *ctss* and *ctssa*, have been identified. These genes differ in sequence characteristics and expression profiles and play different roles in immune response [13]. The *ctss* has been extensively studied in mammals and fishes [8,10,13–20] and is involved in the immune response to bacterial infections [13,15–17,20] and in antigen presentation [8,10,12]. However, the enzymatic activity and function of *ctssa* is still unknown. Herein, a full-length cDNA of cathepsin Sa (designated as *PfCTSSa*) was cloned from yellow catfish, a commercially important species in southern China that often suffers *Aeromonas hydrophila* infection due to the intensive aquaculture [21]. The mRNA expression profile of this gene was then examined in healthy and challenged specimen, with its proteolytic activity and its role in immune response examined.

2. Materials and methods

2.1. Full-length cDNA cloning of PfCTSSa and sequence analysis

A yellow catfish cDNA library was generated to obtain the sequence of *PfCTSSa* minus its 3′-end [22]. The 3′-end was amplified by 3′-RACE with the following primers: ctssa-3′: 5′-CTCTGAGTGTTCAGAACCTGGTGGA-3′, ctssa-3′-1: 5′-CAGTTCTGAGGAGGGAAACCGT-3′. The characteristics of the deduced PfCTSSa amino acid sequence was analyzed by PredictProtein (https://www.predictprotein.org/). Its *N*-glycosylation sites were predicted by examining the sequence context of Asn-Xaa-Ser/Thr sequences using NetNglyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc). Homologous sequences were located using BLASTp (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and aligned using ClustalX 2.1 (http://www.clustal.org/download/current/). Based on the alignment, a phylogenic tree was constructed using the neighbor-joining algorithm with a bootstrap of 1000 in MEGA 6.0.

2.2. Expression profile of PfCTSSa RNA in different conditions

The quantitative PCR (qPCR) was performed to analyze the relative expression of *PfCTSSa* in healthy and challenged yellow catfish. The yellow catfish (~100 g) were obtained from the Pingxi Lake in Pingdingshan City, China. They were acclimated in a 500-L tank with aeration and filtration systems for 2 weeks. The tank was maintained at about 25 °C, with the water partially replaced daily, and the fish were fed with commercial pellets twice a day at a 2% feeding rate. Eleven kinds of tissues (trunk kidney, intestine, blood, spleen, muscle, skin, liver, head kidney, fin, gill and heart) were collected from the healthy specimen ($n = 3$) to detect *PfCTSSa* mRNA distributions. To analyze its temporal expression post-challenge, 200 μl PBS or inactivated *A. hydrophila* (1×10^7 cells/ml) [21] was injected into the abdominal cavity of a yellow catfish. The head kidney, liver and spleen were then collected at 0, 6, 12, 24, 72, 120, or 168 h for both groups ($n = 3$). Total RNA was extracted from each sample and transcribed into cDNA as previously described [21]. The obtained cDNA was then used to quantify *PfCTSSa* mRNA expression via qPCR using specific primers (qCTSSa-F: 5′-ACAGGAGAACAATCTGGGAGCA-3′, qCTSSa-R: 5′- GCACCCTCAGACCATTCAGTTTAG-3′) and with 18S rRNA used as a reference gene (q18S-F: 5′-GGACACGGAAAGGATTGACAGA-3′, q18S-R: 5′-GTTCGCTATCGGAATTAACCAGA-3′). The relative fold change was calculated using the $2^{-\Delta\Delta CT}$ method and significance between samples was determined by performing an ANOVA using SPSS 19.0, with significance defined as $^*P < 0.05$ or $^{**}P < 0.01$.

2.3. Recombinant expression of a mature PfCTSSa peptide

To overexpress a mature PfCTSSa peptide, specific primers were designed (32a-ctssa-F: 5′-GATGGATCCCTCCCTCCCAGTGTGA-3′, 32a-ctssa-R: 5′- GAACTCGAGTTAGACAATGGGGACGA-3′) with restriction enzyme sites *Bam*HI and *Xho*I (underlined). The PCR products were purified using a DNA Gel Extraction Kit (Promega) and excised using the *Bam*HI and *Xho*I restriction sites. The obtained amplicon was then cloned into the pET-32a expression vector, thus generating a recombinant pET-32a/PfCTSSa plasmid. The constructed plasmid was then transformed into *E. coli* BL21 (DE3) and recombinant rPfCTSSa protein expression was induced using 1 mM IPTG at 37 °C. After sonication, the rPfCTSSa inclusion bodies were first washed in buffer A (20 mM Tris-Cl, 500 mM NaCl, and 10 mM imidazole) and then buffer B (20 mM Tris-Cl, 500 mM NaCl, and 2M urea), denatured in buffer C (20 mM Tris-Cl, 500 mM NaCl, 5 mM DTT, and 8 M urea), and purified using a Ni-NTA column (GE, Histrap FF 1 mL) in AKTA purifier (GE Healcare). The purified rPfCTSSa was then diluted in refolding buffer D (100 mM Tris-Cl, 5 mM EDTA, 5 mM β-mercaptoethanol, 5% glycerol, 2 mM GSH, 1 mM GSSG, and 300 mM arginine) and dialyzed in buffer D. A soluble Trx tag was used as a negative control and was purified from *E. coli* BL21 (DE3): pET-32a. First, the *E. coli* BL21 (DE3): pET-32a was induced with 1 mM IPTG at 37 °C and sonicated in buffer A with an additional 30 mM imidazole and 2 mM DTT. Second, the supernatant was loaded on a Ni-NTA column and the Trx was eluted using buffer A supplemented with 60, 90, and 120 mM imidazole and 5 mM DTT. The purified Trx was then dialyzed in PBS for 24 h. The obtained rPfCTSSa and Trx were then quantified via Bradford Protein assay (CWBIO, China) and then used for an enzymatic activity assay.

2.4. Enzymatic activity of rPfCTSSa

To analyze rPfCTSSa enzymatic activity, a fluorogenic substrate, *Z*-Gly-Gly-Arg-7-amido-4-methylcoumarin hydrochloride (*Z*-GGR-AMC; Sigma Aldrich) was utilized. First, 10 μl of rPfCTSSa (0.2 μmol/L) or Trx (0.2 μmol/L) were added into 85 μl of 100 mM Tris-Cl buffer (pH 3–10) containing 1 mM DTT and incubated at 37 °C for 2 h. Next, 5 μl of fluorogenic substrate (100 μM) was added and allowed to incubate at 37 °C for 10 min. Finally, the enzymatic activity was measured using a Microplate Fluorometer (PerkinElmer, USA) at an excitation wavelength of 380 nm and an emission wavelength of 460 nm.

To determine the optimal temperature for rPfCTSSa, the enzymatic activity was assessed at different temperatures using *Z*-GGR-AMC as a substrate while at the optimal pH. To analyze rPfCTSSa activity at various concentrations, the rPfCTSSa was double diluted and assessed using *Z*-GGR-AMC as the substrate under the optimal pH and temperature. To study the effects of different metal ions and detergents on rPfCTSSa activity, $ZnSO_4$, $CuSO_4$, $CoCl_2$, KCl, $MgSO_4$, $CaCl_2$, $HgCl_2$, SDS, Tween 20, and Triton X-100 were added at both 1 and 5 mM concentrations.

3. Results

3.1. Identification and characterization of PfCTSSa

The full-length *PfCTSSa* cDNA (Fig. 1) was 1366 bp and included a 123 bp 5′-UTR, a 981 bp ORF, and a 262 bp 3′-UTR with a polyadenylation signal-AATAAA located 159-bp upstream of the 20-bp poly (A) tail (GenBank accession number: KX495164). The ORF encoded an inactive preprocathepsin (326 aa) with a theoretical pI/MW of 6.00/36.5 kDa, which contains a signal peptide (1–17 aa), a propeptide inhibitor domain (18–113 aa), and a mature peptide