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Short communication

Identification and characterization of a cathepsin D homologue from lampreys (*Lampetra japonica*)

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ABSTRACT

Cathepsin D (EC 3.4.23.5) is a lysosomal aspartic proteinase of the pepsin superfamily which participates in various digestive processes within the cell. In the present study, the full length cDNA of a novel cathepsin D homologue was cloned from the buccal glands of lampreys (*Lampetra japonica*) for the first time, including a 124-bp 5′ terminal untranslated region (5′-UTR), a 1194-bp open reading frame encoding 397 amino acids, and a 472-bp 3′-UTR. Lamprey cathepsin D is composed of a signal peptide (Met 1-Ala 20), a propeptide domain (Leu 21-Ala 48) and a mature domain (Glu 76-Val 397), and has a conserved bilobal structure. Cathepsin D was widely distributed in the buccal glands, immune bodies, hearts, intestines, kidneys, livers, and gills of lampreys. After challenging with *Escherichia coli* or *Staphylococcus aureus*, the expression level of lamprey cathepsin D in the buccal gland was 8.5-fold or 6.5-fold higher than that in the PBS group. In addition, lamprey cathepsin D stimulated with *Escherichia coli* was also up-regulated in the hearts, kidneys, and intestines. As for the *Staphylococcus aureus* challenged group, the expression level of lamprey cathepsin D was found increased in the intestines. The above results revealed that lamprey cathepsin D may play key roles in immune response to exogenous pathogen and could serve as a potential antibacterial agent in the near future. In addition, lamprey cathepsin D was subcloned into pcDNA 3.1 vector and expressed in the human embryonic kidney 293 cells. The recombinant lamprey cathepsin D could degrade hemoglobin, fibrinogen, and serum albumin which are the major components in the blood, suggested that lamprey cathepsin D may also act as a digestive enzyme during the adaptation to a blood-feeding lifestyle.

1. Introduction

Cathepsin D is an intracellular aspartic protease of the pepsin superfamily which is synthesized in rough endoplasmic reticulum with a signal peptide, a propeptide and a mature peptide (Zaidi et al., 2008). After the signal peptide was removed, cathepsin D with a propeptide and a mature peptide reaches its targeted intracellular vesicular structures (lysosomes, endosomes, and phagosomes), where it could be activated by endopeptidases (Laurent-Matha et al., 2006). Usually, cathepsin D is widely distributed in most eukaryotic cells, and plays key roles in the lysosomal digestive process (Fusek and Větvička, 2005; Luca et al., 2009). Recently, a great number of studies have demonstrated that cathepsin D participates in the regulation of apoptosis, activation of polypeptide hormones, growth factors, enzymatic precursors, etc. (Benes et al., 2008). In addition, the expression pattern of cathepsin D usually changed during many pathological processes, such as Alzheimer's disease, atherosclerosis and cancer (Benes et al., 2008). Thus, cathepsin D might also act as a biomarker for the prevention and detection of diseases in the near future (Vetvicka and Fusek, 2012). Lately, several studies have focused on cathepsin D from the blood feeding parasites which was proved to be related to the blood digestion process due to its ability to degrade hemoglobin (Brinkworth et al., 2001). In 2012, three cathepsin D (*Ixodes ricinus* cathepsin D, *Ir*CD) forms were identified from the gut tissue of ticks (*Ixodes ricinus*). Among the three paralogs, *Ir*CD1 with a shortened propeptide region and a unique post-translational modification was recombinant and also proved to act

Abbreviations: BCA, bicinchoninic acid; BLAST, basic local alignment search tool; DTT, dithiothreitol; *E. coli*, *Escherichia coli*; ESTs, expressed sequence tags; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEK 293 cell, human embryonic kidney 293 cell; *Ir*CD, *Ixodes ricinus* cathepsin D; *L. japonica*, *Lampetra japonica*; PBS, phosphate buffered saline; RACE, rapid amplification of cDNA ends; *S. aureus*, *Staphylococcus aureus*; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TiCaD, *Triatoma infestans* cathepsin D; UTR, terminal untranslated region.

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as a hemoglobinase which facilitates the blood digestion process of ticks (Sojka et al., 2012). At the same time, two native cathepsin D proteins (*Triatoma infestans* cathepsin D, TiCaD and TiCaD2) purified from the lumen of the small intestine of the hematophagous bug (*Triatoma infestans*) were characterized as proteases targeting the ingested blood (Balczun et al., 2012). Moreover, cathepsin D might also act as an immune defender in fishes and parasites. During the infection process with pathogenic microorganisms, the expression level of cathepsin D was found increased in the various tissues of grass carp (*Ctenopharyngodon idella*), channel catfish (*Ictalurus punctatus*), half-smooth tongue sole (*Cynoglossus semilaevis*), hard tick (*Haemaphysalis longicornis* and *Ixodes ricinus*), and triatomine (*Rhodnius prolixus*) (Boldbaatar et al., 2006; Borges et al., 2006; Chen et al., 2011; Dong et al., 2012; Feng et al., 2011; Franta et al., 2010; Horn et al., 2009; Jeffers and Roe, 2008; Mu et al., 2013). In addition, cathepsin D is also involved in egg development and tissue invasion process (Fialho et al., 2005; Follo et al., 2013; Williamson et al., 2003). So far, several cathepsin D homologues have been identified and characterized in a variety of tissues from bugs, fishes, hookworms, mites, nematodes, schistosomes, and ticks (Balczun et al., 2012; Bartley et al., 2012; Chen et al., 2011; Dong et al., 2012; Feng et al., 2011; Fragoso et al., 2009; Mu et al., 2013; Sojka et al., 2012; Verity et al., 1999; Williamson et al., 2002, 2003).

The jawless lampreys (*Lampetra japonica, L. japonica*) are one of the most primitive vertebrates still alive, which are considered as ideal animal models to study vertebrate evolution, embryo development and the origin of the adaptive immune system due to their unique position during the long-term evolution process (Chang et al., 2014; Forey and Janvier, 1993; Nikitina et al., 2009). In addition, lampreys are also well known for their blood sucking habit which may bring harmful effects to marine fishes (Lennon, 1954). In 2007, Xiao et al. have reported the fibrinogenolytic properties of buccal gland secretion which may help lampreys counteract the blood coagulation for the first time (Xiao et al., 2007). In recent years, a variety of bioactive proteins and peptides have been identified in the buccal glands of lampreys, which were proved to be involved in counteracting the hemostasis, nociceptive, and immune responses of host fishes (Chi et al., 2009; Gao et al., 2005; Ito et al., 2007; Liu et al., 2009; Sun et al., 2008, 2010a, 2010b; Wang et al., 2010; Wong et al., 2012; Xiao et al., 2007, 2012; Xue et al., 2011).

In contrast to the extensive studies of cathepsin D from the other vertebrates and invertebrates, little work has been done on cathepsin D from lampreys. In the present study, a cathepsin D homologue was found in the buccal glands of lampreys for the first time. And the connection of this enzyme with immune defense in response to bacterial stimulation and digestion process of lampreys was also investigated.

2. Materials and methods

2.1. Animals

Adult lampreys (*L. japonica*) at spawning migration stage were obtained in December 2011 in Tong River, a branch of Songhua River in Heilongjiang province of China. The handling of lampreys was approved by the Animal Welfare and Research Ethics Committee of the Institute of Dalian Medical University (Permit Number: SYXK2004—0029).

2.2. Cloning of a cathepsin D homologue from lampreys

In the previous studies, the cDNA libraries from the buccal gland, liver, and leukocyte cell of lampreys (*L. japonica*) have been constructed, respectively (Gao et al., 2005; Zhang et al., 2010; Zhu et al., 2008). Based on the analysis of the expressed sequence tags (ESTs) of the buccal gland cDNA library from the lampreys, two cDNA sequences which are homologous to cathepsin D were identified by using the Basic Local Alignment Search Tool X (BLASTX) in the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/). All PCR primers were designed based on the sequences in the lamprey buccal gland cDNA library which are homologous to cathepsin D (Supplementary Table S1). Total RNA was isolated from the buccal gland of lampreys (*L. japonica*) according to the manufacturer's protocol (TaKaRa, Dalian, China) and treated with the RNase-free DNase I (TaKaRa, Dalian, China) to remove genomic DNA contamination. First-strand cDNA was synthesized from the total RNA with PrimeScript™ RT-PCR Kit (TaKaRa, Dalian, China) and stored at –80 °C. To obtain the full length cDNA of lamprey cathepsin D, rapid amplification of 5′ cDNA ends (5′-RACE) and 3′-RACE was performed with the 5′ Full RACE Core Set Kit (TaKaRa, Dalian, China) and 3′ Full RACE Core Set Kit (TaKaRa, Dalian, China), respectively. All the PCR amplifications were carried out by using LA Taq DNA polymerase (TaKaRa, Dalian, China) and cloned into a pMD19-T Simple Vector for sequence confirmation.

2.3. Sequence analysis

The full length cDNA sequence of lamprey cathepsin D was spliced by Sequencher 4.2 software and the deduced amino acids were analyzed with DNAMAN V6 and DNASTAR 5.0 software, respectively. The primary structure was analyzed by ProtParam (http://www.expasy.org/). A three-dimensional structure model of lamprey cathepsin D was constructed using the Phyre software (version 0.2) with the X-ray structure of pepsin-like acid proteases (d3psga_) as a template, and visualized using the UCSF Chimera program package (Kelley and Sternberg, 2009; Pettersen et al., 2004).

2.4. Sequence alignment and phylogenetic tree construction

Additional 29 cathepsin D sequences from the other species were obtained from ExPASy (http://www.expasy.ch/tools/blast). The multiple sequence alignments of cathepsin D were performed by ClustalX 1.83 software using default settings (Thompson et al., 1997). A neighbor-joining tree was constructed by MEGA 4.1 software based on the pair-wise deletion of gaps/missing data and a p-distance matrix of an amino acid model with 1000 bootstrapped replicates (Tamura et al., 2007).

2.5. Real-time quantitative PCR analysis

The lampreys with an average body weight of 200 g were kept in aquaria at 16 ± 2 °C for two weeks before challenging. The *Escherichia coli* (*E. coli*) or *Staphylococcus aureus* (*S. aureus*) was cultured at 37 °C to logarithmic growth in LB liquid medium. The cells were then inactivated with formalin (He et al., 2005). The inactivated bacteria cells were resuspended after centrifugation to approximately 2×10^8 CFU/ml in phosphate buffered saline (PBS, pH 7.2). The lampreys were randomly divided into three groups (ten lampreys in each group) and intraperitoneally injected with PBS (0.1 ml), inactivated *E. coli* (0.1 ml) and *S. aureus* (0.1 ml) at 8-day intervals, respectively. After the fourth injection, the buccal glands, gills, hearts, livers, intestines, kidneys, and immune bodies of the lampreys were respectively dissected for RNA extraction as described above. Three tissues were pooled together as one sample (three lampreys per pool, three pools per tissue). The expression level of lamprey cathepsin D was assayed by using TaKaRa SYBR® PrimeScript™ RT-PCR Kit with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control (Kawano-Yamashita et al., 2007; Pfaffl, 2001). Each reaction contained 1 × SYBR Premix Ex Taq, forward primer (10 μM), reverse primer (10 μM) and cDNA (100 ng/μl) with a final volume of 25 μl. The amplification was carried out on a TaKaRa PCR Thermal