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CsPTX1, a pentraxin of *Cynoglossus semilaevis*, is an innate immunity factor with antibacterial effects

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ABSTRACT

Pentraxin 1 (PTX1) is a member of the pentraxin protein family, which plays important roles in the innate immunity of vertebrates. In fish, the biological function of PTX1 is essentially unknown. In this study, we examined the expression and function of a PTX homologue (CsPTX1) from the tongue sole, *Cynoglossus semilaevis*. CsPTX1 contains 223 amino acids and shares 49.3%–38.8% overall sequence identity with other known fish pentraxins. CsPTX1 is expressed in multiple tissues and is upregulated by bacterial and viral infection. CsPTX1 contains a pentraxin domain, which is known to bind extracellular antigens, and recombinant CsPTX1 (rCsPTX1) bound a wide range of Gram-positive and Gram-negative bacteria. rCsPTX1 also agglutinated all the bacteria tested in a Ca^{2+}-dependent manner and the agglutinating capacity of rCsPTX1 was abolished in the absence of calcium. As well as its ability to agglutinate bacterial cells, rCsPTX1 displayed apparent bacteriostatic activity against *Pseudomonas fluorescens in vitro* by influencing the permeability of the microbial envelope. When introduced *in vivo*, rCsPTX1 enhanced the host's resistance to bacterial infection. These results indicate that CsPTX1 is a classic pattern recognition molecule that defends *C. semilaevis* against bacterial infection.

1. Introduction

Innate immunity is the evolutionarily oldest mechanism of defense against microbes in both invertebrates and vertebrates, and plays a key role in the activation and orientation of the adaptive immune response and immune homeostasis [1,2]. Like adaptive immunity, the innate immune system consists of cellular and humoral arms [3]. Pathogen recognition by the innate immune system is based on a set of germline-encoded receptors, known as pattern recognition molecules (PRMs), that recognize conserved microbial moieties, collectively designated pathogen-associated molecular patterns [2]. Based on their cellular localization and functions, PRMs are classified into two major groups: (i) cell-associated receptors, localized in different cellular compartments (plasma membrane, endosomes, cytoplasm) and belonging to different molecular classes, such as toll-like receptors, NOD- and RIG-like receptors, and scavenger receptors; and (ii) humoral PRMs, which are the functional ancestors of antibodies and belong to different molecular families, including collectin, ficolin, and pentraxin [4,5]. Humoral PRMs are essential effectors and modulators of the humoral innate immune system in humans and other animals. They constitute an integrated system of diverse molecules, with complementary specificities, tissue distributions, and modes of production [1,3]. Despite their molecular diversity, humoral PRMs share basic, evolutionarily conserved effector mechanisms, including the activation and regulation of the complement cascade, agglutination and neutralization, and the regulation of inflammation. A bidirectional interaction between the humoral and cellular arms of the innate immune system also sustains and regulates the innate responses [1,6].

The pentraxins (PTX) are a family of highly conserved, multimeric pattern-recognition proteins that are also host-defense-related prototypic components of the humoral PRMs [7,8]. They are characterized by the presence of a 200-amino-acid pentraxin domain at the C terminus, which contains the eight-amino-acid pentraxin signature (HxCxS/TWxS) [3,5]. The pentraxins are divided into two groups based on the primary structure of the subunit: short pentraxins and long pentraxins. The classic short

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pentraxins are represented by C-reactive protein (CRP), also known as pentraxin-1 (PTX-1), and pentraxin-2 (PTX-2), commonly referred to as serum amyloid P (SAP), whereas pentraxin 3 (PTX3) and other subsequently identified proteins constitute the long pentraxin family [5,9–12].

CRP (PTX-1) was first identified as an acute-phase-response protein because it binds to the polysaccharide of *Streptococcus pneumonia* [11,13]. SAP (PTX-2) was the second member of the short PTX family to be identified, based on its sequence homology to CRP [1,12]. The sequence identity between CRP and SAP is approximately 51%, reflecting their highly conserved and unique molecular structures [10,11,14]. CRP and SAP are produced primarily in the liver, and their levels in the blood rise rapidly in response to inflammation and tissue injury [1]. The capacity to recognize a number of diverse ligands, usually in a calcium (Ca^{2+})-dependent manner, is a common feature of the pentraxin family members [3,7]. CRP binds modified low-density lipoproteins, apoptotic cells [15], and various microorganisms through phosphorylcholine and carbohydrate structures [16]. SAP recognizes carbohydrates located on pathogen surfaces, such as microbial lipopolysaccharide, phosphoethanolamine, polysaccharides, cyclic 4,6-pyruvate acetal galactose, 6-phosphorylated mannose, and the 3-sulfated saccharides galactose, glycosaminoglycans, shiga toxin 2, and influenza virus hemagglutinin [7,17–20]. CRP and SAP have also been shown to bind nuclear material, such as histones, chromatin, and small nuclear ribonucleoprotein particles [21,22]. By aggregating or attaching to most of their ligands, CRP and SAP interact with the globular head modules of C1q or all three classes of Fcγ receptors and activate the classical complement cascade, mediate the phagocytosis of apoptotic cells and microorganisms, and mediate the protective immune responses [3,7]. However, the functional properties of PTX so far identified have been limited to mammalian studies.

PTXs have been identified in several species of fish, including the CRP- and SAP-like proteins of the dogfish (*Mustelus canis*) [23], rainbow trout (*Oncorhynchus mykiss*) [24], channel catfish (*Ictalurus punctatus*) [25], common carp (*Cyprinus carpio*) [26], cod (*Gadus morhua* L.) [27], European plaice (*Pleuronectes platessa*) [28], Atlantic salmon (*Salmo salar*) [29], northern pike (*Esox lucius*) [30], rock bream (*Oplegnathus fasciatus*) [31], and half-smooth tongue sole (*Cynoglossus semilaevis*) [32,33]. However, research into the functions of fish PTX1, a short pentraxin homologue, has been limited, particularly in terms of its role in immunology. *Cynoglossus semilaevis* is an important economic species farmed extensively in north China. In this study, we analyzed the expression profile of *C. semilaevis* PTX1 (CsPTX1) under different conditions. Furthermore, we found that recombinant CsPTX1 binds and aggregates both Gram-negative and Gram-positive bacterial pathogens and plays a role in innate immune defense against bacterial infection.

2. Materials and methods

2.1. Ethics statement

Live animal study was performed in accordance with the "Regulations for the Administration of Affairs Concerning Experimental Animals" promulgated by Shandong Province. The methods and protocols of the animal study were approved by the Ethics Committee of Institute of Oceanology, Chinese Academy of Sciences.

2.2. Fish

Clinically healthy tongue sole (average 14.8 g) were purchased from a commercial fish farm in Shandong Province, China. Fish were acclimatized in the laboratory for two weeks and maintained at 20 °C in aerated seawater before experimental manipulation as reported previously [34]. Briefly, fish were kept at in 40 L tanks containing aerated seawater under natural daylight (~12 h/day). The density of the fish was maintained at less than 30 fish/tank. Fish were fed daily with commercial dry pellets {content (%): protein, ≧45; fat, ≧10; fiber, ≦4; calcium, ≧1.5; phosphate, ≧1.2; lysine, ≧2.2; ashes, ≦17} (Shandong Sheng-suo Fish Feed Research Center, Shandong, China) at ~0.3 g/day. Before experiment, fish were randomly sampled and verified to be absent of bacterial pathogens in liver, kidney, and spleen. For tissue collection, fish were euthanized with tricaine methanesulfonate (Sigma, St. Louis, USA).

2.3. Sequence analysis

The cDNA sequence of CsPTX1 were obtained from GenBank (accession no. XM_008323050.1). Sequence analysis was performed using the BLAST program at the National Center for Biotechnology Information (NCBI). Domain search was performed with the simple modular architecture research tool (SMART) version 4.0 and the conserved domain search program of NCBI. The theoretical molecular mass and theoretical isoelectric point (PI) were predicted by using EditSeq in the DNASTAR (Madison, WI) software package. Multiple sequence alignment was created with DNAMAN and signal peptide search was performed with SignalP3.0.

2.4. Quantitative real-time polymerase chain reaction (qRT-PCR)

qRT-PCR was determined as reported previously [35,36]. Briefly, kidney, spleen, gill, blood, liver, intestine, heart, muscle, and brain were taken aseptically from five tongue sole and used for total RNA extraction with EZNA Total RNA Kit (Omega Bio-tek, Doraville, GA, USA). qRT-PCR was carried out in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the SYBR ExScript qRT-PCR Kit (Takara, Dalian, China), and the expression level of CsPTX1 was analyzed using $2^{-\Delta\Delta CT}$ with beta-actin (ACTB) as an internal reference [36]. For bacterial infection, *Pseudomonas fluorescens* [37] was cultured in Luria-Bertani broth (LB) medium at 28 °C to an OD_{600} of 0.8; the cells were washed with PBS and resuspended in PBS to 1×10^7 CFU/ml. Tongue sole were injected intraperitoneally with 50 μl *P. fluorescen* or PBS. At 6 h, 12 h, 24 h, and 48 h post-infection, kidney, spleen, and liver were taken (five fish/time point), and CsPTX1 expression was determined by qRT-PCR as described above. For viral infection, megalocytivirus RBIV-C1 [38] was suspended in PBS to 5×10^5 copies/ml; tongue sole were injected intraperitoneally with 50 μl megalocytivirus or PBS. At 1 d, 3 d, 5 d and 7 d post-infection, kidney, spleen, and liver were taken (five fish/time point), and CsPTX1 expression was determined by qRT-PCR as described above. All experiments were performed three times.

2.5. Plasmid construction

To construct pEtCsPTX1, which expresses His-tagged SmLMWPTP was constructed as follows. The coding sequence of CsPTX1 was amplified by PCR with primers F1 (5′ −GATATCATGGTTCCTCAAGACTTGTCTGG− 3′, underlined sequence, EcoRV site) and R1 (5′ −GATATCATCATCTGGTGCATCATCAA− 3′, underlined sequence, EcoRV site); the PCR products were ligated with the T−A cloning vector pEASY-T1 Simple (TransGen Biotech, Beijing, China), and the recombinant plasmid was digested with EcoRV to retrieve the CsPTX1 -containing fragment, which was inserted into pET259 [39] at the SwaI site, resulting in pEtCsPTX1.