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# CsSAP, a teleost serum amyloid P component, interacts with bacteria, promotes phagocytosis, and enhances host resistance against bacterial and viral infection

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ABSTRACT

Serum amyloid P component (SAP) is a member of the pentraxins family that plays important roles in innate immunity in vertebrates. In fish, the biological function of SAP is essentially unknown. In this study, we examined the expression and function of a SAP homologue (CsSAP) from tongue sole *Cynoglossus semilaevis*. CsSAP shares 46%–58% overall sequence identities with known fish SAP and was upregulated in expression by bacterial and viral infection. Recombinant CsSAP (rCsSAP) exhibited differential binding capacities to a wide range of Gram-positive and Gram-negative bacteria and promoted uptake of the bound bacteria by host phagocytes. When introduced *in vivo*, rCsSAP enhanced host resistance not only to bacterial infection but also to viral infection. Consistently, antibody blockage of CsSAP significantly weakened the ability of tongue sole to clear invading bacteria. These results provide the first evidence that fish SAP contributes significantly to both antibacterial and antiviral immunities.


## 1. Introduction

Innate immunity is the first line of defense against pathogens and plays an important role in pathogen clearance in vertebrates. It also affects the activation and orientation of adaptive immunity and the maintenance of tissue integrity and repair (Bayne and Gerwick, 2001; Iwasaki and Medzhitov, 2010). The innate immune system comprises a cellular and a humoral arm, components of the latter including soluble pattern recognition molecules (PRMs) that recognize pathogen associated molecular patterns (PAMPs) and initiate the immune response in coordination with the cellular arm (Bottazzi et al., 2010; Garlanda et al., 2005). Soluble PRMs share basic, evolutionarily conserved functions such as opsonization, complement activation, agglutination, neutralization, and regulation of inflammation (Bottazzi et al., 2010; Inforzato et al., 2012). PRMs comprise a heterogeneous group of molecules, including collectins, ficolins, and pentraxins (Garlanda et al., 2005; Holmskov et al., 2003; Inforzato et al., 2012).

As an essential component of soluble PRMs, pentraxins (PTX) are conserved in evolution from arachnids to humans. They have five identical subunits non-covalently associated as a pentamer and possess a 200-amino acid pentraxin domain in the C-terminus, which contains an 8-amino acid pentraxin signature (HxCxS/TWxS) (Bottazzi et al., 2010; Gewurz et al., 1995). Based on the primary structure of the protomers, pentraxins are divided into two groups, short and long pentraxins: C-reactive protein (CRP) and serum amyloid P component (SAP) together constitute the prototypic short pentraxin, while pentraxin 3 (PTX3) and other subsequently identified proteins represent the long pentraxins (Inforzato et al., 2012; Mantovani et al., 2008).

SAP was identified as a relative of CRP on the basis of amino acid sequence identity (51%) (Rubio et al., 1993). It is ~25 kDa with a structural organization that comprises five or ten identical subunits arranged in a pentameric radial symmetry (Lund and Olafsen, 1998). The physiological functions attributed to SAP involve recognition and binding to different ligands, mostly in a $Ca^{2+}$-dependent manner (Agrawal et al., 2009; Emsley et al., 1994). The ligands of SAP include agarose (Bayne and Gerwick, 2001; Jensen et al., 1995), Type IV collagen (Zahedi, 1996), heparin (Li et al., 1994), extracellular matrix proteins like fibronectin (Tseng and Mortensen, 1986), soluble immune complexes like aggregated IgG

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(Brown and Anderson, 1993), and carbohydrate structures on pathogen surface such as microbial polysaccharides, influenza virus hemagglutinin, shiga toxin 2, cyclic 4,6-pyruvate acetalgalactose, 6-phosphorylated mannose, 3-sulfated saccharides galactose, and glycosaminoglycans (Agrawal et al., 2009; Andersen et al., 1997; Loveless et al., 1992). In addition, SAP has also been shown to bind DNA, histones, chromatin, and small nuclear ribonucleoprotein particles (Breathnach et al., 1989; Butler et al., 1990; Pepys and Butler, 1987; Pepys et al., 1994); thus, SAP is thought to be involved in the recognition of damaged cells and their constituents and in the clearance of necrotic cells and apoptotic cells (Bottazzi et al., 2010; Hicks et al., 1992). However, it has to be said that the SAP functional properties so far discovered are limited to mammalian studies.

In fish, SAP proteins and sequences have been identified in several species, including European plaice (*Pleuronectes platessa*) (Pepys et al., 1982), dog-fish (*Mustelus canis*) (Robey et al., 1983), rainbow trout (*Oncorhynchus mykiss*) (Jensen et al., 1995; Murata et al., 1994), Atlantic salmon (*Salmo salar*) (Leong et al., 2010; Lund and Olafsen, 1999), northern pike (*Esox lucius*) (Leong et al., 2010), and rock bream (*Oplegnathus fasciatus*) (Choi et al., 2015). However, research on the immunological function of fish SAP is very limited. Half-smooth tongue sole (*Cynoglossus semilaevis*) is a farmed marine fish with high economic values. In this study, we analyzed the expression of the tongue sole SAP (CsSAP) and examined its potential in antibacterial and antiviral immunity.

## 2. Materials and methods

### 2.1. Fish

Clinically healthy tongue sole (average 13.8 g) were purchased from a commercial fish farm in Shandong Province, China and maintained at 20 °C in aerated seawater. Before experiment, fish were acclimatized in the laboratory for two weeks and verified to be absent of bacterial and viral pathogens in liver, kidney, and spleen by plate count (Zhang et al., 2015). For tissue collection, fish were euthanized with tricaine methanesulfonate (Sigma, St. Louis, USA) at the dose of 0.1 g/l.

### 2.2. Sequence analysis

The cDNA sequence of CsSAP has been deposited in GenBank database (accession no. KT025856). The cDNA and amino acid sequences of CsSAP were analyzed using the BLAST program at the National Center for Biotechnology Information (NCBI). Domain search was performed with the conserved domain search program of NCBI. The theoretical molecular mass and theoretical isoelectric point were predicted by using EditSeq in the DNASTAR (Madison, WI) software package. Multiple sequence alignment was created with DNAMAN.

### 2.3. Quantitative real time reverse transcription-PCR (qRT-PCR)

To determine constitutive *CsSAP* expression by qRT-PCR, spleen, blood, liver, kidney, intestine, heart, muscle, gill, 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) (Sun and Hu, 2015). The expression level of *CsSAP* was analyzed using comparative threshold cycle method ($2^{-\Delta\Delta CT}$) with beta-actin (ACTB) as an internal reference (Long et al., 2014). The entire experiment (i.e. extraction of RNA, cDNA synthesis, and qRT-PCR) was performed three times, each time with five fish.

*CsSAP* expression during bacterial and viral infection was determined as reported previously by Hu and Zhang (2015). *Vibrio harveyi* (Yu et al., 2013) 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 $1 \times 10^6$ CFU/ml. Tongue sole (as above) were divided randomly into two groups (20/group) and injected intraperitoneally (i.p.) with 50 μl *V. harveyi* or PBS. At 6 h, 12 h, 24 h, and 48 h post-infection, five fish were taken for tissue collection, and *CsSAP* expression was determined by qRT-PCR as above. For viral infection, megalocytivirus RBIV-C1 (Zhang and Li, 2015) was suspended in PBS to $5 \times 10^5$ copies/ml; tongue sole were divided randomly into two groups (20/group) and injected i.p. with 50 μl megalocytivirus or PBS. At 1 d, 3 d, 5 d and 7 d post-infection, five fish were taken for tissue collection, and *CsSAP* expression was determined by qRT-PCR as above. All experiments were performed three times.

### 2.4. Construction of plasmids

To construct pEtCsSAP, which expresses His-tagged recombinant CsSAP (rCsSAP), the coding sequence of CsSAP was amplified by PCR with primers SAPF1 (5′–GATATCATGGTAACACAAGATC TCTCAGGAAA– 3′, underlined sequence, EcoRV site) and SAPR1 (5′–GATATCCATATCATCTGGTGCATCATC– 3′, underlined sequence, EcoRV site); the PCR products were ligated with the T–A cloning vector T-Simple (TransGen Biotech, Beijing, China), and the recombinant plasmid was digested with EcoRV to retrieve the *CsSAP*-containing fragment, which was inserted into pET259 (Zhou et al., 2015) at the SwaI site. To construct pEtCsIL-6, which expresses His-tagged interleukin 6 of tongue sole (CsIL-6), the coding sequence of CsIL-6 was amplified by PCR with primers IL-6F1 (5′–GATATCAT GGAGGTGACCGACATTCTGG– 3′) and IL-6R1 (5′–GATATCATT-CTTCAGTGCTGTAAAAAAGTG– 3′), which were designed based on the IL-6 sequence of tongue sole (GenBank accession number XP_008330269.1). The PCR products were ligated with T-Simple as above, and the recombinant plasmid was digested with EcoRV to retrieve the CsIL-6-containing fragment, which was inserted into pET259 as above.

### 2.5. Purification of recombinant proteins

Protein purification was performed as described previously (Zhang et al., 2014b). *Escherichia coli* BL21(DE3) (TransGen Biotech Beijing, China) was transformed separately with pEtCsSAP and pET32a (Novagen, San Diego, USA), the latter expressing Trx; the transformants were cultured in LB medium at 37 °C to mid-logarithmic phase, and isopropyl-β-D-thiogalactopyranoside was added to the culture to a final concentration of 0.4 mM. After growing at 28 °C for an additional 8 h, the cells were harvested by centrifugation (4200 g), and His-tagged proteins were purified using Ni-NTA Agarose (QIAGEN, Valencia, USA) as recommended by the manufacturer. The purified proteins were reconstituted and removed of endotoxin as reported previously (Chen et al., 2013b). The proteins were then concentrated with PEG20000 (Solarbio, Beijing, China). The concentrated proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized after staining with Coomassie brilliant blue R-250. For purification of rCsIL-6, BL21(DE3) was transformed with pEtCsIL-6; growth of the transformant, induction of protein expression, and purification of the recombinant protein were performed exactly as above.