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Short communication

Molecular characterization, expression analysis, and bactericidal activity of the derivative peptides of TFPI-1 and TFPI-2 in half-smooth tongue sole, *Cynoglossus semilaevis*

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ABSTRACT

Tissue factor pathway inhibitors (TFPIs) are Kunitz-type serine protease inhibitors that reversibly regulate the blood coagulation induced by tissue factor. TFPI family contain two members, TFPI-1 and TFPI-2. Recent studies have shown TFPI-1 and TFPI-2 also play important roles in innate immunity, however, the potential function of teleost TFPI are very limited. In this study, we characterized two TFPI (CsTFPI-1 and CsTFPI-2) molecules from half-smooth tongue sole (*Cynoglossus semilaevis*), examined their tissue distributions and expression patterns under pathogens stimulation as well as investigated the antibacterial activity of the C-terminal peptides. Quantitative real time RT-PCR analysis showed that constitutive *CsTFPI-1* expression occurred, in increasing order, in head kidney, intestine, brain, spleen, liver, skin, gills, heart, and muscle; *CsTFPI-2* was expressed, in increasing order, in the gills, intestine, skin, head kidney, liver, brain, spleen, muscle, and heart. Under *Vibrio anguillarum*, *Streptococcus agalactiae* and fish megalocytivirus stimulation, both *CsTFPI-1* and *CsTFPI-2* expression increased significantly in a manner that depended on the pathogen, tissue type, and infection stage, which suggested CsTFPI-1 and CsTFPI-2 play important roles in anti-bacterial and anti-viral infection. Finally, C-terminal peptides of CsTFPI-1 and CsTFPI-2, were synthesized and proved to have antibacterial effect against *Micrococcus luteus* that were independent of host serum. Take together, these results indicate that CsTFPI-1 and CsTFPI-2 play important roles in antimicrobial immunity of this fish.

1. Introduction

Tissue factor pathway inhibitors (TFPIs) are matrix-associated Kunitz-type serine protease inhibitors [1]. In mammalians, there are two members in the TFPI family, TFPI-1 and its structural homologue TFPI-2 [2,3]. TFPIs are mainly known for their roles as regulators of blood coagulation. In the process of blood coagulation, tissue factor (TF), which is constitutively expressed on the surface of fibroblasts and smooth muscle cells surrounding blood vessels [4], binding to its enzymatic partner, factor VII (FVII), the interaction resulting in the formation of TF-VII complex. Then the complex activates factor X and factor IX, leading to the generation of thrombin [5–7]. TFPIs reversibly inhibit the TF-VII complex, thus effectively shutting off the TF activity [8]. Compared to TFPI-1, TFPI-2 is a weak inhibitor of coagulation induced by the TF-VII complex, while it targets a wide range of proteases such as trypsin, chymotrypsin, plasmin, MMPs, factor Xia and plasma kallikrein [9,10].

Structurally, both TFPI-1 and TFPI-2 consist of a highly negatively charged N terminus, three tandem Kunitz domains, and a highly positively charged C-terminus [11–13]. The roles of the three Kunitz domains are different, the first and second domain participated in binding and inhibition of the TF-VII complex and factor Xa [14], while the third domain could interact with heparin [15]. Previous reports have shown that the C-terminal region of TFPI-1 are required for interactions with plasma lipoproteins, thrombospondin-1, clearance receptors [16], and lipopolysaccharide [17]. The C-terminal region of both human TFPI-1 and TFPI-2 could be cleaved off by a number of proteinases such as thrombin, plasmin, and matrix metalloptoteinase-8 and ADAMTS1at different positions *in vivo* and *in vitro*, resulting in various TFPI and TFPI C-terminally truncated forms [18–25].

Recent studies have shown both TFPI-1 and TFPI-2 are participated in innate immunity and inflammation response except for functioning as inhibitors of blood coagulation. Expression of TFPI-1 and TFPI-2 are increased under stimulation with inflammatory

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mediators, endotoxin, or molecules involved in infection, inflammation, and growth [16,26,27]. Moreover, mammalian TFPIs play important roles in tumor cell growth and invasion. For example, human TFPI-1 and TFPI-2 located at the surface of breast cancer cells were able to inhibit TF activity and breast cancer cell growth locally [28]. In addition, C-terminal fragments of human TFPI-1 and TFPI-2, and several invertebrate TFPI-2 including Chimpanzee (*Pan troglodytes*), Gorilla (*Gorilla gorilla gorilla*), Mouse (*Mus musculus*), Turkey (*Meleagris gallopavo*), Chicken (*Gallus gallus*), Alligator (*Alligator Mississippiensis*), Turtle (*Alligator Mississippiensis*) and Frog (*Xenopus tropicalis*) were proved to possess antimicrobial activity, and have great potential in antimicrobial therapies [24,25,29,30].

Although many TFPI-1 and TFPI-2 molecules have been identified, the expression and potential function of these TFPIs are limited to only a few species. In red drum (*Sciaenops ocellatus*), Zhang found that recombinant TFPI-1 and TFPI-2 exhibits serine protease inhibitor activity and bactericidal activity in a manner that is independent of any host factors [31,32], further study indicated the C-terminal peptide of TFPI-1 possess antibacterial activity higher than recombinant TFPI-1 [31]. Recently, C-terminal peptides of Shark (*Callorhinchus milii*) and Zebrafish (*Danio rerio*) were proved to be antibacterial against *Escherichia coli* and *Pseudomonas aeruginosa* [30]. The function of teleost TFPIs in other fish species remain unknown.

Half-smooth tongue sole (*Cynoglossus semilaevis*) is a flat fish culturing widely in China as an economic species. However, tongue sole industry is long-suffering from serious diseases, and the immune mechanism of tongue sole responses to pathogens infection is still limited. In this study, the sequence signatures of tongue sole TFPI-1 and TFPI-2 (CsTFPI-1 and CsTFPI-2) were analyzed, and their expression patterns under normal or pathogens stimulation conditions were investigated. Moreover, the antibacterial activity of the C-terminal peptides of CsTFPI-1 and CsTFPI-2 were examined.

2. Materials and methods

2.1. Fish

Half-smooth tongue sole (average 14.2 ± 1.5 g) were purchased from a commercial fish farm in Shandong Province, China and maintained at 20 °C in aerated seawater. Fish were acclimatized in the laboratory for two weeks before experimental manipulation. Fish were kept at 22 °C in containing aerated seawater that was changed twice daily. Before experimental manipulation, fish were randomly sampled and verified to be absent of pathogens in tissues as reported previously [33,34]. Before tissue collection, fish were euthanized with an overdose of tricaine methanesulfonate (Sigma, St. Louis, MO, USA) as reported previously [35].

2.2. Bacterial and viral strains

The fish pathogens *Pseudomonas putida* C1, *Streptococcus agalactiae* G1, *Vibrio anguillarum* CJ, *Vibrio parahaemolyticus* PL2 and *Vibrio scophthalmi* ZS1 have been preserved in laboratory previously. *Escherichia coli* DH5α was purchased from Tiangen (Beijing, China); *Micrococcus luteus* 1D00051 were purchased from China General Microbiological Culture Collection Center (Beijing, China). Fish megalocytivirus, infectious spleen and kidney necrosis virus (ISKNV) was kindly provided by Doctor Li of Pearl River Fishery Research Institute, Chinese Academy of Fishery Sciences. Except for *Streptococcus agalactiae* G1, which was cultured in Brain Heart Infusion (BHI) broth, all other strains were cultured in Luria-Bertani (LB) medium. All strains were cultured at 37 °C (for *Escherichia coli* and *Micrococcus luteus*) or 28 °C (for all others). ISKNV was propagated in a continuous cell line (named as CPB) established previously from brain of *Siniperca chuatsi* [36].

2.3. Sequence analysis and phylogenetic analysis

The cDNA sequences of *CsTFPI-1* and *CsTFPI-2* are available from GenBank database (GenBank accession numbers XP_008327257 and XP_008329541). The cDNA and amino acid sequences of *CsTFPI-1* and *CsTFPI-2* were analyzed using the BLAST program at the National Center for Biotechnology Information (NCBI). The signal peptide search and domain search were performed with the simple modular architecture research tool (SMART) version 4.0. The molecular mass and theoretical isoelectric point (pI) were predicted using DNAman software package (Lynnon Biosoft, Quebec, Canada). Phylogenic neighbor-joining trees were constructed based on the deduced amino acid sequences with the MEGA 6.0 software package. Data were analyzed using Poisson correction, and gaps were removed by pairwise deletion. The reliability of the tree was assessed by 1000 bootstrap repetitions.

2.4. Quantitative real time polymerase chain reaction (qRT-PCR) analysis of CsTFPI-1 and CsTFPI-2 expression in fish tissues under normal physiological conditions

Brain, heart, gills, head kidney, spleen, liver, muscle, skin and intestine were taken aseptically from five fish and used for total RNA extraction with the RNAprep Tissue Kit (Tiangen, Beijing, China). One microgram of total RNA was used for cDNA synthesis with the Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). qRT-PCR was carried out in a LightCycler 96 system (Roche Applied Science, North Carolina, USA) by using the SYBR ExScript qRT-PCR Kit (Takara, Dalian, China) as described previously [33]. The tongue sole β-actin gene was used as an internal control, which was previously proved as an appropriate internal control for qRT-PCR normalization [37,38]. The primers used to amplify *CsActin*, *CsTFPI-1* and *CsTFPI-2* were listed in Table 1. Melting curve analysis was carried out at the end of each PCR to confirm the specificity of PCR products. The expression level of *CsTFPI-1* and *CsTFPI-2* were analyzed using comparative threshold cycle method ($2^{-\Delta\Delta CT}$). All data are given in terms of relative mRNA levels to that of tissue in which *CsTFPI-1* or *CsTFPI-2* expression was the lowest.

2.5. qRT-PCR analysis of CsTFPI-1 and CsTFPI-2 expression upon bacterial and viral infection

Pathogens infection were performed as reported previously [39]. Briefly, *V. anguillarum* and *S. agalactiae* were cultured as above to an OD_{600} of 0.8. The cells were washed and resuspended in PBS to 10^6 CFU (colony forming unit)/ml. ISKNV was prepared as reported previously [36] and resuspended in PBS to 1×10^7 copies/ml. Tongue sole were divided randomly into four groups (30 individuals/group) and injected intraperitoneally (i.p.) with 100 μl *V. anguillarum*, *S. agalactiae*, ISKNV or PBS per fish, and maintained at 20 °C. Five fishes were euthanized at 0 h, 4 h, 8 h,

Table 1
PCR primers used in this study.

Primers	Sequences (5′-3′)	Target genes
CsActinRTF	GAACCCCAAAGCCAACAGG	β-actin
CsActinRTR	CCAAGTCAAGACGCAGGATG	
CsTFPI-1RTF	GACTCTGGAAGAATGTGAGGAAAC	TFPI-1
CsTFPI-1RTR	AGCCGCCGTAGAAGAAGGT	
CsTFPI-2RTF	TTTGTCTCCAAGCGAAACTGC	TFPI-2
CsTFPI-2RTR	GTCTCAAGCGGCGAATCAT-3′	