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Kunitz-type serine protease inhibitor is a novel participator in anti-bacterial and anti-inflammatory responses in Japanese flounder (*Paralichthys olivaceus*)

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ABSTRACT

Kunitz-type serine protease inhibitor (KSPI) interacts with serine protease (SP) to regulate cascade reactions *in vivo* and plays essential roles in innate immunity. Theoretical considerations support various functions of *kspi*, but further studies are required for full characterization of these functions. In this study, a KSPI molecule was identified from Japanese flounder (*Paralichthys olivaceus*), and was named *Pokspi*. The full-length cDNA sequence of *Pokspi* was 2810 nt, containing an open reading frame of 1527 nt, which encoded a polypeptide of 509 amino acid residues. PoKspi protein contained five conversed domains, namely, MANEC, PKD, LDLa and two Kunitz domains. Homology analysis revealed that *Pokspi* shared the highest similarity (83%) with its homolog in *Cynoglossus semilaevis*. Phylogenetic analysis indicated that *Pokspi* clustered with the homologs in other fishes. The mRNA transcripts of *Pokspi* were detected in all tested tissues, with the highest expression level in gill, followed by kidney and intestine. Its elevated expression in response to the application of *Edwardsiella tarda* (*in vivo*) and pathogen-associated molecular pattern (*in vitro*) suggested the involvement of *Pokspi* in the essential immune defense against various pathogens. Recombinant PoKspi (rPoKspi) purified from *Escherichia coli* exhibited not only serine protease inhibitor activities but also a broad spectrum of anti-microbial effect in a manner that was independent of any host factors. In addition, the recombinant PoKspi protein could cause the down-regulation of pro-inflammatory factors TNF-α and IL-1β. In conclusion, *Pokspi* is a biologically active serine protease inhibitor endowed with anti-bacterial and anti-inflammatory property. This study provides strong evidences for understanding the innate immune defense in Japanese flounder.

1. Introduction

Protease inhibitor (PI) inhibits proteases by regulating proteolytic activities inside the cells. They form stable complex with target proteases, resulting in either blockage or alteration of active structures of the proteases [1,2]. Serine proteinase inhibitor (SPI) is widely distributed in organisms and are intensively studied in recent years. They play various and crucial roles in many biological processes, such as blood coagulation, fibrinolysis, development, inflammation, tissue injury, immune defense, prophenoloxidase activation, and the complement system in vertebrates and invertebrates [3–9]. SPI can be categorized into several families based on their primary sequences, tertiary structures and mechanisms of binding motifs [10]. Some families of SPI, known as Kazal, Kunitz, Serpin and α-macroglobulin, have been well-characterized in various organisms [11,12].

Kunitz-type SPI (KSPI) exists in a variety of organisms, including plants, insects, ectoparasites, animals and microbes [10,13–16]. Generally, KSPI is characterized by one, two, three or even more Kunitz domains that are composed of approximately 60 amino acid residues and stabilized by three conserved disulfide bonds [17]. It selectively exhibits inhibitory activities against different serine proteases, such as trypsin, chymotrypsin and thrombin [18]. Several families of SPI, including Kunitz-type SPI, are believed to be involved in immune signal pathways by modulating the proteolytic activities of SP, owing to their prominent inhibitory abilities [4].

Increasing studies, particularly in some marine animals, have demonstrated the immune-defensive ability of KSPI. For instance, *kspi* may play vital roles in the immune response of shrimp, scallop, solen and rock bream, since its mRNA are significantly up-regulated by viral or bacterial infections [19–22]. The study of *Oplegnathus fasciatus* is the

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first to describe the multi-domain *kspi* from a fish species, revealing the potential immune-defensive role against invading bacterial and viral pathogens, and the potential association with the wound-healing mechanism of rock bream *kspi* [22]. More than one KSPI homolog has anti-inflammatory effects, protects animals from tissue damages and promotes tissue repair [9,23,24]. However, the expression and potential function of teleost *kspi* remain limited.

Japanese flounder (*Paralichthys olivaceus*) is an important maricultural fish in China. With the enlargement of breeding scale, the harm of diseases has attracted attention. Many previous reports have referred to the mass deaths of Japanese flounder caused by *Edwardsiella tarda* [25,26]. In this study, we identified and characterized a *kspi* homolog from Japanese flounder (*Pokspi*), described its tissue distribution and temporal expression after *E. tarda* and pathogen-associated molecular pattern (PAMP) stimulation. In addition, the inhibitory activity of the recombinant protein (rPoKspi) toward trypsin was examined. This study explored the anti-bacterial activity and anti-inflammatory function of Japanese flounder *kspi* for the first time, which will help to understand the role of *kspi* in the immune system.

2. Materials and methods

2.1. Identification of Pokspi cDNA

A *kspi* sequence based on the homologs of other teleosts, was selected from the Japanese flounder database [27] by means of homology screening using Basic Local Alignment Search Tool (BLAST) of NCBI. A pair of primers *kspi*-Fw1/Rv1 (Table S1) specific to this sequence was designed using Primer Premier 5.0 program. The cDNA sequence was identified by PCR, gel purification, sub-cloning and sequencing.

2.2. Bioinformatic analysis

Homology searches in the GenBank database were carried out by BLASTP network server (https://blast.ncbi.nlm.nih.gov/Blast.cgi) of NCBI. Multiple alignment analysis of the protein sequences was performed using MegAlign of the LASERGENE software suite (DNASTAR) by ClustalW method. To determine the molecular distance among *kspi* homologs, a phylogenetic tree was constructed by MEGA7.0 using p-distance based on the neighbor-joining method. Several tools from the Expert Protein Analysis System (ExPASy) were used, such as SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) for canonical signal peptides, NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/) for possible N-glycosylation sites, and SMART program (http://smart.embl-heidelberg.de/) for domain architecture. The three-dimensional (3D) structure prediction was performed by SWISS-MODEL online software at Expert Protein Analysis System (http://www.expasy.org/). All the sequences used are from GenBank (Table 1). The reliability of each node was estimated by bootstrapping with 1000 replications.

Table 1
The GenBank accession numbers of *kspi* used in the present study.

Species	Accession number
Homo_sapiens	NM_181642.2
Mus_musculus	NM_016907.3
Manacus_vitellinus	XM_008919799.2
Alligator_mississippiensis	XM_006270353.3
Xenopus_tropicalis	XM_002939545.4
Callorhinchus_milii	XM_007893199.1
Danio_rerio	NM_213152.1
Oryzias_latipes	XM_004082359.3
Takifugu_rubripes	XM_011616372.1
Oplegnathus_fasciatus	KP793739.1
Hippocampus_comes	XM_019870186.1
Paralichthys_olivaceus	XM_020085203.1
Cynoglossus_semilaevis	XM_017035580.1
Crassostrea_gigas	XM_011440482.2

2.3. Specimen and tissue collection

Japanese flounder specimens used in this study were obtained from a fish farm in Yantai, Shandong Province, China, and maintained at 17 °C. For the gene expression experiments, tissues and organs were collected from 1-year-old individuals (three females and three males). The 6 fishes were anesthetized by MS-222 (30 mg/mL) and then killed by severing the spinal cord. Specimens of brain, heart, intestine, head kidney, liver, spleen, gill and muscle were collected from each fish, immediately frozen in liquid nitrogen and stored at −80 °C until further use.

2.4. Immune challenge assay

In total, 140 flounders with good growth state were randomly divided into the control group ($n = 60$) and the experimental group ($n = 80$). The control group was intraperitoneally injected with 1 mL of phosphate buffered saline (PBS), and the experimental group was intraperitoneally injected with 1 mL of *E. tarda* with a concentration of 10^7 CFU/mL (the concentration was determined based on pre-experiments). The two groups were then reared separately. Four individuals were sampled at every time point (1, 5, 8, 12, 24, 48, 72 and 96 h post-injection). After anesthesia administration, the fishes were anatomized, and gills and head kidneys were sampled. The tissues were immediately frozen in liquid nitrogen and stored at −80 °C until further use.

Japanese flounder gill cell line FG9307 from the Cell Engineering Technology Laboratory of Ocean University of China was cultured in DMEM (Invitrogen, Carlsbad, CA, USA) with 10% (*V/V*) fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 1% (*V/V*) NEAA (Invitrogen, Carlsbad, CA, USA), and 100 unit/mL penicillin (Invitrogen, Carlsbad, CA, USA) at 24 °C. Monolayer-cultured gill cells with good growth state were transferred to 12-well plates and cultured for 24 h. When the rate of cell coverage reached 90%–95%, LPS (lipopolysaccharide), PGN (peptidoglycan) and poly I:C (polyinosinic-polycytidylic acid) were added to each well of the experimental group to a final concentration of 50 μg/mL (the concentration was determined based on pre-experiments). The control group was added with the same concentration of PBS. Four wells of each group were sampled at every time point (0, 1, 2, 4, 8 and 12 h after stimulation). After washed 3 times in PBS, cells were sampled and stored in TRIzol at −80 °C until further use.

2.5. Construction of expression vector

The cDNA region except signal peptide and transmembrane region was amplified by PCR with the specific primers *kspi*-Fw2/Rv2 (Table S1). The reaction was performed under the following conditions: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing for 30 s at 60 °C and extension at 72 °C for 1 min, and an additional extension at 72 °C for 7 min. The PCR product was digested with *Eco*RV (TaKaRa, Dalian, China) and *Sac*I (TaKaRa, Dalian, China), and sub-cloned into the expression vector pET-32a (Novagen, USA) that had been cut with the same restriction enzymes. The recombinant plasmid was verified by sequencing and named pET32a/*Pokspi*.

2.6. Expression and purification of rPoKspi

The constructed plasmid pET32a/*Pokspi* was transformed into *E. coli* BL21 (DE3) and cultured overnight in Luria-Bertani (LB) broth containing kanamycin (100 mg/mL). The culture was diluted 1:100 with LB and incubated at 37 °C for 4 h. The expression of rPoKspi was induced by adding isopropyl β-*D*-thiogalactoside (IPTG) to the culture at a final concentration of 0.01 mM. After further incubation at 19 °C overnight,