Fish & Shellfish Immunology 42 (2015) 430–438

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

A potential Kazal-type serine protease inhibitor involves in kinetics of protease inhibition and bacteriostatic activity

Venkatesh Kumaresan [a], Ramaswamy Harikrishnan [b], Jesu Arockiaraj [a, *]

[a] Division of Fisheries Biotechnology & Molecular Biology, Department of Biotechnology, Faculty of Science and Humanities, SRM University, Kattankulathur, 603 203 Chennai, Tamil Nadu, India
[b] Department of Zoology, Pachaiyappa's College for Men, Kanchipuram 631 501, Tamil Nadu, India

ARTICLE INFO

Article history:
Received 17 September 2014
Received in revised form
31 October 2014
Accepted 21 November 2014
Available online 26 November 2014

Keywords:
Murrel
Protease inhibitor
Antimicrobial protein
Gene expression
Kinetic activity

ABSTRACT

Kazal-type serine protease inhibitor (KSPI) is a pancreatic secretary trypsin inhibitor which involves in various cellular component regulations including development and defense process. In this study, we have characterized a KSPI cDNA sequence of freshwater striped murrel fish *Channa striatus* (*Cs*) at molecular level. Cellular location analysis predicted that the *Cs*KSPI was an extracellular protein. The domain analysis showed that the *Cs*KSPI contains a Kazal domain at 47–103 along with its family signature between 61 and 83. Phylogenetically, *Cs*KSPI is closely related to KSPI from *Maylandia zebra* and formed a sister group with mammals. The 2D structure of *Cs*KSPI showed three α-helical regions which are connected with random coils, one helix at signal sequence and two at the Kazal domain region. The relative gene expression showed that the *Cs*KSPI was highly expressed in gills and its expression was induced upon fungus (*Aphanomyces invadans*), bacteria (*Aeromonas hydrophila*) and poly I:C (a viral analogue) challenge. The *Cs*KSPI recombinant protein was produced to characterize and study the *Cs*KSPI gene specific functions. The recombinant *Cs*KSPI strongly inhibited trypsin compared to other tested proteases. The results of the kinetic activity of *Cs*KSPI against trypsin was V_{max}s = 1.62 nmol/min, K_Ms = 0.21 mM and K_is = 15.37 nM. Moreover, the recombinant *Cs*KSPI inhibited the growth of Gram-negative bacteria *A. hydrophila* at 20 μM and Gram-positive bacteria *Bacillus subtilis* at the MIC_{50} of 15 μM. Overall, the study indicated that the *Cs*KSPI was a potential trypsin inhibitor which involves in antimicrobial activity.

1. Introduction

Proteases are inevitable component in all organisms ranging from microbes to mammals including fishes that act as a catalyst in cleavage of peptide bond [1,2]. The action of proteases is tightly regulated with endogenous inhibitors. The interaction between proteases and their inhibitors was a target of intensive study in the last two decades. Protease inhibitors (PIs) are ubiquitous in all living organisms and it is divided into four classes based on their protease active site such as serine, cysteine, aspartic and metalloprotease. Until now, more than 16,000 PIs have been reported from various organisms; these PIs are categorized into 67 families (http://merops.sanger.ac.uk/) [3].

Serine protease inhibitors (SPIs) play vital roles in many physiological functions including blood coagulation [4], inflammation [5], metamorphosis [6], complement system [7] and innate immune response. In all those physiological processes, SPIs are mainly responsible for maintaining cellular homeostasis, inhibiting undesirable proteolytic cascades [8] and inhibiting exogenous protease secreted by pathogenic microorganisms that use the protease to penetrate the host and invade new tissues, thus acting as antimicrobial agents [9]. Laskowski and Kato [10] have classified the serine protease inhibitors into ten major classes. Among them, four SPI family members such as Kazal, Kunitz, Serpin and α macroglobulin have been reported to serve as defense components [11–13]. Kazal type serine protease inhibitors (KSPIs) are one of the well-known family of serine protease inhibitors. They are found in both invertebrates and vertebrates [14,15]. It was named after Kazal et al. [16] who isolated KSPI from pancreatic secretory trypsin inhibitor.

KSPI belongs to peptidase inhibitor family 1, which inhibits S1 family serine proteases such as chymotrypsin, trypsin, plasmin, thrombin, elastase, proteinase K, or subtilisin A [10]. They also act against microbial serine protease and potassium channels [17,18].

* Corresponding author. Tel.: +91 44 27452270; fax: +91 44 27453903.
E-mail address: jesuaraj@hotmail.com (J. Arockiaraj).

http://dx.doi.org/10.1016/j.fsi.2014.11.027
1050-4648/

KSPI 3 and 6 are reported to act against human kallikrein related peptidases, which are trypsin and chymotrypsin like serine proteases [19,20]. KSPIs have been reported to have multiple biological and physiological functions including modulation of immune responses [11,15,21], blood coagulation [22–24] and regulation of the inflammatory response [25]. Additionally, KSPIs exhibit microorganism-binding activity which inhibit the activity of bacterial compounds like subtilisin and also exhibit strong antibacterial activity against many bacteria [21,26–28]. Thus, these properties lead serine protease inhibitor as a potential candidate in many pharmaceutical applications.

KSPI protein is secreted as a pre-pro peptide with a signal sequence. The pro-peptide sequence contains a putative O-glycosylation site-rich domain and a mature peptide sequence that includes a Kazal domain [29,30]. KSPI contains a Kazal proteinase inhibitory domain which inhibits the cognate proteinase based on the substrate-like standard mechanism [10]. A typical Kazal domain is composed of 40–60 amino acid residues including some spacer amino acids. The Kazal motif has a general amino acid sequence of C-X_a-C-X_b-PVCG-X_c-Y-X_d-C-X_e-C-X_f-C where the subscripts a, b, c, d, e and f are integral numbers of amino acid residues [31]. Though a few amino acid residues in the Kazal motif are relatively conserved, most of them are quite variable in vertebrates. Kazal domain contains six well-conserved cysteine residues which are capable of forming three intra-domain disulfide bridges between cysteine numbers 1–5, 2–4 and 3–6 resulting in a three-dimensional structure. Fritz and Krejci [32] and Nirmala et al. [33] reported a shorter Kazal domain which contains 36-40 amino acids and observed 2 missing cysteine residues in the inhibitor region. Jarasrassamee et al. [8] found that cysteine 5 and 6 were replaced by other amino acids and their functions remained unchanged in some KSPIs. The specificity of the inhibitor is determined by the P1 residue which is located in the second position after the second conserved cysteine in the kazal domain [34]. Generally, inhibitors with a Tyr, Pro, Met, Leu, or Phe in the P1 position tend to inhibit chymotrypsin, while inhibitors with an Ala or Ser at P1 inhibits elastase. Similarly, trypsin inhibitors tend to have an Arg or Lys at the P1 position [10,35]. The P1 residue is usually located in the most exposed region of this protease-binding loop and influences the specificity and binding strength [35–37].

In this study, we used freshwater striped murrel *Channa striatus* as a model organism to study the defense system of vertebrates. We constructed a cDNA library from the tissue pool of *C. striatus* and identified a Kazal-type serine proteinase inhibitor (named as *Cs*KSPI) during library screening. The identified *Cs*KSPI sequence was characterized using bioinformatics tools. Further, tissue distribution and expression profiles of *Cs*KSPI upon immune induction were reported. Moreover, to study the functional characterization of *Cs*KSPI, the coding region of *Cs*KSPI was cloned, overexpressed and obtained the purified the recombinant enzyme. Using the recombinant *Cs*KSPI, the following assays were performed: proteinase inhibition assay, kinetics of serine proteinase inhibition and bacteriostatic assay.

2. Materials and methods

2.1. Identification of CsKSPI from a normalized cDNA library of C. striatus

A cDNA encoded *Cs*KSPI was identified from a normalized cDNA library of *C. striatus* during screening. The library was established using genome sequence FLX™ technology from the tissue pool (spleen, liver, kidney, muscle and gills) of *C. striatus*. The technical details of library establishment including total RNA extraction from tissue pool, mRNA purification, cDNA synthesis, sequencing, assembly and screening the library were described in our earlier findings [38,39].

2.2. Application of bioinformatics tools for CsKSPI characterization

The *Cs*KSPI cDNA sequence was analyzed on DNAssist (Ver. 2.2) to obtain its non-coding, coding and protein sequences. Homology of *Cs*KSPI was searched on BLAST program (http://blast.ncbi.nlm.nih.gov/Blast). Domain and motif were analyzed using PROSITE Database (http://prosite.expasy.org/scanprosite/). Cleavage site of the signal peptide was predicted by SignalP program (http://www.cbs.dtu.dk/services/SignalP/). Molecular mass, isoelectric point and instability index of the *Cs*KSPI putative protein was calculated using the Prot-Param tool on ExPASy program (http://www.expasy.org/tool/protparam/). Cellular location of the depicted *Cs*KSPI protein was predicted using MultiLoc tool (http://abi.inf.uni-tuebingen.de/Services/MultiLoc/). Transmembrane segments of the *Cs*KSPI protein was predicted using SACS MEMSAT2 tool (http://www.sacs.ucsf.edu/cgi-bin/memsat.py). Multiple sequence alignment of *Cs*KSPI was conducted using Bioedit (Ver. 7.0.0). Phylogenetic tree of *Cs*KSPI was established using Neighbor-Joining Method at MEGA (Ver. 5.05). A secondary structure of *Cs*KSPI protein was predicted using SOPMA program, followed by evaluation using Polyview program (http://polyview.cchmc.org). We also predicted various 3D structure of *Cs*KSPI protein using I-TASSER program (http://zhanglab.ccmb.med.umich.edu/I-TASSER). To obtain the best structure, the 3D structures were validated by applying Ramachandran plot analysis (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php). Further, the best structure was viewed and evaluated using PyMol tool (Ver. 0.99). Additionally, to obtain the *Cs*KSPI mRNA stability, we also established the mRNA structure of *Cs*KSPI using RNA fold server (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) program.

2.3. Immune challenge of C. striatus and tissue collection

Healthy *C. striatus* (average body weight = 50 g ± 10) were obtained from the Surya Agro Farms Ltd., Erode, Tamil Nadu, India. Fishes were maintained in flat-bottomed plastic tanks (100 L) with aerated and filtered freshwater at 29 ± 2 °C in the wet lab of Division of Fisheries Biotechnology and Molecular Biology, SRM University. All fishes were acclimatized for one week before being challenged to immune stimulants. During the acclimatization period, fishes were fed *ad libtum* with a commercially available pellet diet. The best water quality parameters were maintained by changing the water regularly. A maximum of 5 fishes were stocked in each tank during the experiments. For fungus and bacterial induced mRNA expression analysis, the fishes were intraperitoneally injected with *Aphanomyces invadans* (10^2 spores) and *Aeromonas hydrophila* (5×10^6 CFU/mL) suspended in 1X phosphate buffer saline (100 μL/fish). The isolation, identification and injection of fungus and bacteria were clearly described in our earlier findings [40,41]. For poly I:C [polyinosinic-polycytidylic acid sodium salt, a synthetic analog of double-stranded RNA (dsRNA), a molecular pattern associated with viral infection. Poly I:C is composed of a strand of poly(I) annealed to a strand of poly(C)] challenge, the fishes were injected with 150 μg poly I:C (γ-irradiated) per 50 g fish (100 μl/animal) (Sigma–Aldrich, USA). The tissues (blood, liver, spleen, heart, intestine, kidney, head kidney, muscle, skin, gill and brain) were collected before (0 h), and after injection (3, 6, 12, 24, 48 and 72 h) and were immediately snap-frozen in liquid nitrogen and stored at −80 °C until total RNA was isolated. Using a sterilized syringe, the blood (0.5–1.0 mL per fish) was collected from the fish caudal fin and immediately centrifuged at 4000 × g for 10 min at 4 °C to allow blood cell collection for total RNA extraction. PBS (1X)