Fish & Shellfish Immunology 37 (2014) 115–121

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Molecular characterization, expression and function analysis of a five-domain Kazal-type serine proteinase inhibitor from pearl oyster *Pinctada fucata*

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ARTICLE INFO

Article history:
Received 11 September 2013
Received in revised form
4 December 2013
Accepted 18 December 2013
Available online 27 December 2013

Keywords:
Pinctada fucata
Serine proteinase inhibitor
Microbial challenge
Quantitative real-time PCR
Inhibitory activity

ABSTRACT

Serine proteinase inhibitors represent an expanding superfamily of endogenous inhibitors that are regulate proteolytic events and involved in a variety of physiological and immunological processes. A five-domain Kazal-type serine proteinase inhibitor (poKSPI) was identified and characterized from pearl oyster *Pinctada fucata* based on expressed sequence tag (EST) analysis. The full-length cDNA was 737 bp with an open reading frame (ORF) 660 bp encoding a 219 amino acid protein a theoretical molecular weight (Mw) of 23.3 kDa and an isoelectric point (pI) of 8.40. A putative signal peptide of 19 amino acid residues and five tandem Kazal domains were identified. Four of the Kazal domains had the highly conserved motif sequences with six cysteine residues responsible for the formation of disulfide bridges. The deduced amino acid sequence of the poKSPI shared high homology with KSPIs from *Hirudo medicinalis*. The poKSPI mRNA could be detected in all examined tissues, the expression level of the poKSPI mRNA was the highest in mantle and gonad, while the lowest in haemocyte and intestine. After LPS challenge, the expression level of the poKSPI mRNA in digestive gland was significantly up-regulated at 4 h post-challenge and reached the peak at 12 h post-challenge, which was 4.23-fold higher than control group; the expression level of the poKSPI mRNA in gill was also significantly up-regulated at 8 and 12 h post-challenge, which were 4.48 and 2.26-fold higher than control group. After *Vibrio alginolyticus* challenge, the expression levels of the poKSPI mRNA in digestive gland were significantly up-regulated at 8, 12, 48 and 72 h post-challenge, which were 1.70, 1.79, 3.89 and 5.69-fold higher than control group, respectively; the expression level of the poKSPI mRNA in gill was significantly up-regulated at 24 h post-challenge, which was 5.30-fold higher than control group. The recombinant poKSPI protein could inhibit chymotrypsin and trypsin activities in dose-dependent manner, when the ratios of rpoKSPI to chymotrypsin and trypsin were 36:1 and 72:1, respectively, the proteinase activities of chymotrypsin and trypsin could be almost completely inhibited, but the rpoKSPI could not inhibit subtilisin.

1. Introduction

Serine proteinase inhibitors (SPIs) are found widely in all multicellular organisms and take part in controlling the various proteinase-mediated biological processes, such as digestion, complement system, blood coagulation, melanization, apoptosis, phagocytosis, cellular remodelling and reproductive processes [1–4]. Some SPIs also show the potent bactericidal or bacteriostatic activities [5,6] and play the important roles as part of the humoural defence of the innate immune system against the invading pathogens [7]. Several microbial pathogens and parasites use the SPIs to counter the protective proteinases of hosts. For example, the intracellular parasite of human *Toxoplasma gondii* secretes a serine proteinase inhibitor to protect itself from the digestive enzymes during its residency in small intestine [8]. *Rhodnius prolix*, *Dipetalogaster maximus* and *Triatoma infestans* can produce the potent proteinase inhibitors to prevent blood coagulation and make them easier to suck blood from their hosts [9–11].

SPIs are classified into at least 63 families on the basis of amino acid sequence similarities (http://merops.sanger.ac.uk/). Kazal-type serine proteinase inhibitors (KSPIs) are well known and grouped into family I1. All the KSPIs contain one or more Kazal domains and

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1050-4648/$ – see front matter © 2013 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.fsi.2013.12.011

each domain consists of 50–60 amino acid residues and contains six well-conserved cysteine residues forming three intra-domain disulphide bridges, which are responsible for the stability of three-dimensional structure [12]. The reactive site (P1 residue) is located at the second position after the second cysteine residue of the domain and believed to contribute mainly to the inhibitory specificity [13], a few other adjacent amino acid residues also influence the binding specificity [14,15]. Recently, a variety of KSPIs are found in crustaceans [4,6,7,16–18]. They were believed to be involved in innate immune responses, because their expressions were up-regulated after bacterial challenge. Meanwhile, several of KSPIs also had bacteriostatic activity against some pathogens [6,7]. However, until now, the KSPIs were only studied in few mollusks [19–22], these results preliminarily demonstrated that KSPIs were involved in innate responses of mollusk.

In contrast with the abundant knowledge on KSPIs in crustaceans, the information of KSPIs in bivalve mollusk is still poorly known. To further understand the molecular functions of KSPIs in the innate immune responses of bivalve mollusks, in this study, we cloned and characterized a Kazal-type serine proteinase inhibitor from pearl oyster *Pinctada fucata* (designated as poKSPI), which is the most important farmed bivalve mollusk for seawater pearl production in China [23,24], and investigated its tissue distribution and temporal expression profile after bacterial and lipopolysaccharide (LPS) challenge. This work would hopefully provide insight into the important functions in the innate immune responses of pearl oysters.

2. Materials and methods

2.1. EST and poKSPI cDNA analysis

A cDNA library was constructed and analyzed in our previous studies [23]. These EST data were used to search for the Kazal-type serine proteinase inhibitors, and an EST (EST No. pmpca0_002189) of 453 bp was homologous to the KSPIs of *Chlamys farreri* (EU183309) and *Argopecten irradians* (AY830445), the plasmid of this EST was picked up and re-sequenced to get the full-length cDNA and ensure the correctness of the sequence. DNATool 6.0 software was used to deduce the poKSPI amino acid sequence. Searches for nucleotide and protein sequence similarities were conducted with BLAST algorithm at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). SignalP 4.0 software (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the putative signal peptide cleavage site [25]. The simple modular architecture research tool (SMART) 4.0 program (http://www.smart.emblheidelberg.de/) and ScanProsite (http://expasy.org/tools/) were used to predict the protein domain [26]. ClustalW (http://www.clustal.org/) [27] and MEGA 5.1 [28] were used to align poKSPI with other known KSPI sequences.

2.2. Pearl oysters and immune challenge

Pearl oysters *P. fucata* (shell length 6.15 ± 0.32 cm, body weight 23.8 ± 4.5 g) were obtained from pearl oyster culture base of South China Sea Fisheries Research Institute in Xincun village, Hainan province, China and maintained at 25–27 °C with the recirculating seawater for one week before experiment. The pearl oysters were fed twice daily on *Tetraselmis suecica* and *Isochrysis galbana*. To take account of individual variability, 15 individual pearl oysters, which were divided into three replicates, were used for each time point (0, 2, 4, 8, 12, 24, 48 and 72 h) in the LPS and bacterial challenge experiments, respectively.

The LPS challenge experiment was performed by injecting with 100 μL LPS (*Escherichia coli* 055:B5, #62326, Sigma–Aldrich, Munich, Germany, LPS 10 μg ml^{-1}) dissolved in phosphate-buffer saline (PBS) into the adductor muscles of each pearl oyster. The bacterial challenge experiment was carried out by injecting with 100 μL of *Vibrio alginolyticus* resuspended in PBS to OD_{600} = 0.4 (1 OD = 5×10^8 bacteria ml^{-1}) into the adductor muscles of each pearl oyster. While pearl oysters injected with 100 μL PBS were used as control group in the challenge experiments. The injected pearl oysters were returned into seawater tanks and 15 individuals were randomly sampled at 0, 2, 4, 8, 12, 24, 48 and 72 h in each group, respectively. The gill and digestive gland from the control and challenge groups were collected respectively and immediately stored in liquid nitrogen until used.

For tissue distribution experiment, three healthy pearl oysters were dissected to sample haemocyte, gill, digestive gland, mantle, gonad, adductor muscle and intestine tissues, respectively, and then they were stored in liquid nitrogen until used. Total RNA samples were extracted using the TRIzol reagent (Invitrogen) according to the manufacture instructions.

2.3. Quantitative real-time RT-PCR analysis of poKSPI mRNA expression

For the expression pattern of poKSPI in different tissues, total RNA was extracted from haemocyte, gill, digestive gland, mantle, gonad, intestine and adductor muscle of three healthy pearl oysters. For the temporal expression analysis, total RNA was extracted from gill and digestive gland sampled from the different time point in each group, respectively. The total RNA of each sample was treated with the DNase I (Promega) respectively, and then the single-strand cDNA was synthesized based on manufacture's instruction of PrimerScript™ 1st Strand cDNA Synthesis Kit (TaKaRa) using random primer. cDNA mix was diluted to 1:10 and stored at −80 °C for expression analysis.

Two poKSPI gene-specific primers, poKSPI-F (5′-TGGAAGATGTCCCTGCATC-3′) and poKSPI-R (5′-GTCCGCGCATTTAGCTGTA-3′), were used to amplify a product of 183 bp from cDNA template, and the PCR products were sequenced to verify the specificity of RT-PCR. A 97 bp fragment was amplified using two primers of pearl oyster β-actin to calibrate the cDNA template as an internal control [23]. The SYBR Green RT-PCR assay was performed in the Eppendorf Realplex^2 Detection System as described by Zhang et al. [23]. Dissociation curve analysis of amplification products was carried out at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. After the PCR program, data were analyzed with the Realplex^2 software (Eppendorf). The $2^{-\Delta\Delta CT}$ method was used to determine fold change for gene expression relative to controls [23]. All data were given in terms of relative mRNA expression as means ± S.D. the data were subjected to one-way analysis of variance (one-way ANOVA) followed by a multiple comparison with GraphPad Prism 5.0 Software, and the *P* value less than 0.05 were considered statistically significant.

2.4. Recombinant expression and purification of poKSPI

The fragment encoding mature protein of poKSPI was amplified with gene-specific primers poKSPI-F1 (5′- GGTGGATCCAGATCGCCACCCTACAAAC-3′) and poKSPI-R1 (5′- GCGAAGCTTTTATTCATACTTCCTACATGGACC-3′). For convenience of cloning, a *Bam* HI site was added to the 5′ end of poKSPI-F1 and a *Hind* III site was added to the 5′ end of poKSPI-R1 before the stop codon. The PCR products was cloned into pMD18-T simple vector (Takara), digested by completely by restriction enzymes *Bam* HI and *Hind* III, and then ligated into predigested expression vector pRSET. The recombinant plasmid pRSET-poKSPI was transformed into *E. coli* BL21 (Novagen). The positive transformant with pREST-poKSPI was