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Molecular characterization of two kazal-type serine proteinase inhibitor genes in the surf clam *Mesodesma donacium* exposed to *Vibrio anguillarum*

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ABSTRACT

This study reports two kazal-type serine protease inhibitors (KPI) identified in a cDNA library from the surf clam *Mesodesma donacium*, and characterized through Rapid Amplification of cDNA Ends (RACE). The KPIs, denoted as *MdSPI-1* and *MdSPI-2*, presented full sequences of 1139 bp and 781 bp respectively. *MdSPI-1* had a 5′untranslated region (UTR) of 175 bp, a 3′UTR of 283 bp and an open reading frame (ORF) of 681 pb that encodes for 227 amino acids. *MdSPI-2* showed a 5′UTR of 70 bp, a 3′UTR of 279 bp and an ORF of 432 bp that encodes for 144 amino acids. Both sequences presented two kazal-type tandem domains. Phylogenetic analysis of *MdSPI-1* and *MdSPI-2* shows a main clade composed by other bivalve species and closely related crustaceans. Real time PCR analysis showed that *MdSPI-1* is mainly up-regulated in mantle, foot, gills and muscle tissues, while *MdSPI-2* is expressed principally in foot tissue. Moreover, to evaluate the immune response of *MdSPI-1* and *MdSPI-2*, infections with *Vibrio anguillarum* were performed. Herein, *MdSPI-1* and *MdSPI-2* transcription expression were significantly up-regulated at 2 and 8 h post-challenge. Our results suggest that *MdSPI-1* and *MdSPI-2* are important humoral factors of innate immunity in *M. donacium*.

1. Introduction

Serine protease inhibitors (SPI) are a super-family of proteins with physiological functions, such as blood coagulation [1], inflammation [2], metamorphosis [3], complement system [4] and innate immune response. In all these process, SPIs are mainly responsible for maintaining cellular homeostasis, inhibiting undesirable proteolytic cascades [5] or inhibiting exogenous protease secreted by pathogenic microorganisms that use the protease to penetrate the host and invade new tissues [6]. Among the families of serine protease inhibitors there is the I1, which is composed by proteins containing the kazal-type domain and therefore are called kazal-type SPIs (KPI) [7]. These have a single or multi kazal domain, which can inhibit different proteases [6]. The latter are characterized by a well conserved amino acid sequence, with six cysteines responsible of the formation of disulfide bridges (C1x (3) C2X (7) PVC3X (3) GX2YXNXC4X (6) C5X (12) C) [8]. The specificity of the inhibitor is determined by the P1 residue located in the second residue after the second cysteine conserved from the kazal domain [9].

Serine protease inhibitors have been identified in mollusks as humoral factors present in haemolymph and related to components of innate immune response. For instance, in species like the Pacific oyster *Crassostrea gigas* [10,11], soft-shell clam *Mya arenaria* [12], bay scallop *Argopecten irradians* [13] and Zhikong scallop *Chlamys farreri* [14], have been characterized as kazal-type inhibitors (KPI) capable of blocking the action of some proteases such as subtilisin A and trypsin. Furthermore, KPI immune response has been studied in the shrimp *Penaeus monodon* and the river crab *Procambarus clarkii*, evidencing the presence of kazal-type domains with inhibitory activity against subtilisin, trypsin and chymotrypsin [5,15]. Four serine protease inhibitors in *Fenneropenaeus chinensis* have the capacity to inhibit subtilisin and proteinase K and have been associated with the innate antibacterial and antifungal immune response system [6].

The surf clam *Mesodesma donacium*, the model species for our study, has socio-economic importance all along the Chilean coast. There have been few studies of this species at the molecular level, which has limited the development of DNA markers to assess this over-exploited population at genetic level [16]. The objective of this study was to characterize two EST sequences homologs to kazal-type SPI identified by 454 pyrosequencing from *M. donacium* transcriptome, and determine their transcription

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patterns in different tissues using qPCR and their role in antibacterial immune response.

2. Materials and methods

2.1. Experimental design

Forty specimens with the same stage of gonadal maturation were obtained from Coquimbo, Chile (31°55′S, 71°30′W). The specimens were used to characterize the SPIs mRNA, and also in challenge trials against *Vibrio anguillarum*. Tissues from the foot, mantle, gills and muscle were fixed in RNAlater RNA Stabilization Reagent (Ambion, USA) and stored at −80 °C for subsequent RNA extraction. The remaining specimens were first acclimatized for a week in filtered seawater at 17 °C with constant airing. The specimens were then divided into a control group ($N = 18$) and a challenge group ($N = 18$). For the challenge trials, 100 μL of *V. anguillarum* (3.2×10^8 cell/ml) in Luria Bertani (LB) culture medium was injected into the adductor muscle of *M. donacium* specimens, while the control group was injected with 100 μl of LB medium. Both groups were sampled at different exposure times: 2, 4, 6, 8, 16 and 32 h post-injection. Around 300 mg of foot, mantle, gills and muscle samples were dissected and fixed in RNAlater RNA Stabilization Reagent (Ambion, USA), and stored at −80 °C for subsequent RNA extraction.

2.2. Extraction of total ARN

Total RNA was extracted from 100 mg of the previously described tissues using TRIzol Reagent (Invitrogen®, Life Technologies, USA) according to the manufacturer's instructions. The concentration and purity of the extracted RNA was determined by spectrophotometry in a Nanodrop ND-1000 (NanoDrop® Technologies, Inc) and its integrity was visualized by electrophoresis in denaturing agarose gels at 1.2% stained with ethidium bromide at 0.001%. Subsequently, from 200 ng of RNA of each sample, cDNA was synthesized using the RevertAidTM™ H Minus First Strand cDNA Synthesis kit (Thermo Scientific, Maryland, USA), in accordance with the supplier's instructions.

2.3. KPI identification and characterization

We identified two annotated contigs for KPIs from a cDNA library for *M. donacium* obtained by 454 pyrosequencing of gill and mussel tissue from adult individuals of *M. donacium* that are available for download at the Dryad Digital 162 depository (http://datadryad.org/) under the access http://dx.doi.org/10.5061/dryad.8jd18. For both sequences annotated as KPIs, specific primers were designed with Primer 3 in the software Geneious 5.1.3 (Biomatters, New Zealand) [17] (Table 1). The partial sequence of each contig was then amplified by PCR in a final volume of 12.5 μL of reaction. The amplification conditions were: 94 °C for 2 min and 30 s (holding stage), 35 cycles of 94 °C for 30 s (denaturing), 60 °C (T° annealing) for 30 s, 72 °C for 45 s and 72 °C for 5 min. The PCR product was visualized through electrophoresis in agarose gels at 1.2% and, then sent to Macrogen Inc. (Korea) for sequencing in an ABI 3730xl capillary sequencer (Applied Biosystems). Subsequently, the sequences were analyzed with Geneious 5.1.3 software, resulting in two partial sequences termed *MdSPI-1* and *MdSPI-2*. Based on these sequences, specific new primers were designed (Table 1) for amplification of the 3′ and 5′UTR ends, using the FirstChoise®RLM RACE kit (Ambion®, Life Technologies, USA) in accordance with the manufacturer's instructions. The fragments obtained for the 5′ and 3′UTR ends were ligated in the cloning vector TOPO TA Cloning kit (Invitrogen™, Life Technologies, Carlsbad, CA, USA) and transformed into electrocompetent bacteria of *Escherichia coli* JM109 in LB/amp/IPTG/Xgal plates overnight at 37 °C. The positive clones were selected and purified to obtain plasmids using the E.Z.N.A® Plasmid DNA Mini kit II (Omega Biotek, Doraville, GA, USA). The plasmids obtained were sequenced in both directions and their sequences were then assembled using the software Geneious 5.1.3.

Table 1
Sequence of the oligonucleotide primer used in the study.

Primers	Sequence (5′–3′)
MD_8873F(*MdSPI1*)	ATCCCAGAACGCTAAAAGCA
MD_8873R(*MdSPI1*)	TCTGCGATTCCCAGGACAGT
MD_3594F(*MdSPI2*)	GAGTCCGTCGCTCCAATGTA
MD_3594R(*MdSPI2*)	CTTGGTTGATCGTCCATGC
QMD_8873F	CGAATGGCAACAAGAACAAA
QMD_8873R	CAGGAGGAACAAATGGGAGA
QMD_3594F	TGGGTTTGAGATGCCACTTC
QMD_3594R	CATGCTGCCTCACATTCATT
MD_atub_1F	ATGTACCACGTGCCGTCTTT
MD_atub_1R	ACGGATTCTGTCGAGAACCA
3594_5′R inner	AAGCCTTGATTCTGCCGTCGCA
3594_5′R outer	TGATCGTCCATGCACTGTCGTGT
3594_3′F outer	GCTTGTGACGGACCTTGCCCAT
3594_3′F inner	GCATGGACGATCAACCAAGTA
8873_5′inner	CCAGCTCAAAAACCTGTTCC
8873_5′outer	GCCCAGCACGCCGAAATGAT
8873_3′inner	GTCTTACCCTCGCAAAGTGC
8873_3′outer	TGCAAATTGTGCGGCGGAAGGA
Adapter 5′	CTAATACGACTCACTATAGGGCAAGCA GTGGTATCAACGCAGAGT
Adapter 3′	CTAATACGACTCACTATAGGGCAAGCA GTGGTATCAACGCAGAGT

Blastn and Blastx searches were conducted against the non-redundant Genbank database (http://www.ncbi.nlm.nih.gov/) to identify the conserved sites of the kazal domain. Subsequently, a multiple alignment was made of the kazal domains of *MdIsp1* and *2* and the kazal-type domains publicly available for the species: *P. clarkii* (*hcPcSPI*, *hpPcSPI* and *PcSPI*), *P. monodon* (*SPIPm2* and *SPIPm*), *F. chinensis* (hpFcSPI1), *Litopenaeus vannamei* (*hcLvSPI* and *hpLvSPI*), *C. farreri* (*CfKZSPI*), *C. gigas* (*CvSI*) and *A. irradians* (*AISPI*). Finally, to determine clusters among the amino acid sequences of the kazal domains, a phylogenetic analysis was conducted using the Neighbor-Joining method adjusted to 1000 iterations, and including *Hidra magnipapillata* as outgroup.

2.4. Expression profiles of MdSPI in M. donacium

Sustainable primers were designed for qPCR reactions for the sequences obtained from *MdSPI-1* (QMD_8873F, QMD_8873R) and *MdSPI-2* (QMD_3594F, QMD_3594R) (Table 1). As well, the genes *α-tubulin*, *β-tubilin* and *GAPDH* were identified and assessed as endogenous controls for the relative expression qPCR assays. After an assessment of efficiency, *α-tubulin* was selected as an endogenous control. All the primers were designed to amplify ~100 bp of PCR products.

The qPCR runs were performed with StepOnePlus™ (Applied Biosystems, Life Technologies, USA) using the comparative ΔCt method [18]. This method requires efficiency values close to 100%, therefore we analyzed the dynamic range to calculate the reaction efficiency of every gene. To perform this, we conducted five cDNA dilution series, beginning with 80 ng of template and a dilution factor of 1:5. Each reaction was conducted with a volume of 10 μL using Maxima® SYBR Green/ROX qPCR Master Mix (Thermo Scientific, USA). The amplification conditions were: 95 °C for 10 min (holding stage), 40 cycles of 95 °C for 30 s (denaturation), T° (annealing) 30 s, 72 °C for 30 s (extension). To determine the presence of single amplicons, non-specific products, gDNA contamination and primer dimers, a melting curve was carried out following the amplification,