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Short sequence report

A novel clip domain serine proteinase (SPs) gene from the haemocytes of Indian white shrimp *Fenneropenaeus indicus*: Molecular cloning, characterization and expression analysis

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The Indian white shrimp, *Fenneropenaeus indicus* (formerly *Penaeus indicus*), is one of the major commercial shrimp species of the world. It is reported in the Indo-West Pacific from eastern and south-eastern Africa, through Malaysia and Indonesia to southern China and northern Australia [1,2]. The defense mechanism in *F. indicus* is poorly understood, but knowledge of this is a prerequisite for the development of intervention strategies to control diseases in Indian white shrimp culture. The immune related genes in arthropods have been discovered and characterized during the past few years and shown to participate in immunological processes. The proPO system plays an important role in the immune responses of invertebrates [3] and activated by an endogenous activating system and exogenous materials/agents such as lipids, detergents, organic solvents, and microbial elicitors like β-1, 3-glucan, lipopolysaccharide (LPS), and peptidoglycan (PG) [4–7]. The inactive proPO zymogen is converted to the active phenoloxidase (PO), by a clip domain serine proteinase (SPs) designated as a proPO activating enzyme (PPAE) [3,8]. The proPO cascade involves several proteolytic steps which are catalyzed by multiple SPs [9–13]. The terminal clip domain SP (PPAE) that carries out the proteolysis of the proPO, has been cloned and characterized from several insects and crustaceans, including, within insects, *Bombyx mori* [9], *Manduca sexta* [13], *Holotrichia diomphalia* [12] and *Tenebrio molitor* [10], and, within a crustacean, the crayfish *Pacifastacus leniusculus*, [11] Chinese shrimp *Fenneropeneaus chinensis*, [14], black tiger shrimp *Penaeus monodon*, [15–17] pacific white shrimp *Litopenaeus* (*Penaeus*) *vannamei* [18] and kuruma shrimp *Marsupeneaus japonicas* [19]. The serine proteinase consists of one or more clip domain(s) at the N-terminus and an SP domain at the C terminus, which is responsible for signal amplification by cleaving and activating the downstream SPs or proteins. The clip domain is ubiquitously found in SPs involved in the extracellular signaling cascades, especially in those that are locally amplified. The clip domain, usually composed of between 37 and 55 amino acid residues, is interlinked by three strictly conserved disulfide bonds [20] and connected to the SP domain by a disulfide linkage. However, shrimp cSPs that are involved in the proPO system have yet to be clarified. Research is hampered from a lack of knowledge of genomic data and genome organization in crustaceans including Indian white shrimp. Thus, the present study reports the identification and characterization of the full-length cDNA of clip domain serine proteinase from the Indian white shrimp *F. indicus*. We also examine the temporal response of its transcript levels in different tissue, and the haemocytes towards immunostimulants challenges and molting stage responses.

Indian white shrimp *F. indicus* ranged from 15.7 to 23.2 g, averaging 18.75 ± 3.60 g (mean ± SD) were obtained from the coastal area of Nagapattnam to Chennai, Tamil Nadu, India and were stocked and maintained in FRP tanks with flow-through sea water (35‰ salinity) at 28 °C and fed twice daily with a formulated shrimp diet. Haemocytes (500 μl) were withdrawn individually from the ventral sinus cavity of each shrimp into a 2 ml sterile syringe (25 gauge) containing 500 μl of precooled (4 °C) anticoagulant solution (10% trisodium citrate). The diluted haemocytes were centrifuged at 500 g at 4 °C for 20 min. Total RNA was isolated using the guanidinium thiocyanate method. First strand cDNA synthesis in RT (reverse transcription) was performed by using Promega MMLV reverse transcriptase (Promega, Madison, WI, USA). Full length of *F. indicus* serine proteinase (FISP) cDNA was obtained by the procedures of reverse transcription – polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends

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(RACE) method. The partial sequence of clip domain serine proteinase were obtained by degenerated primers synthesized based on the amino acid consensus sequence for the closely related species retrieved from NCBI GenBank. The gene specific primers FISPGR1 was used for the 5′-RACE reaction and FISPG F1 was used for the 3′-RACE reaction. The partial sequences were assembled into full-length sequence using the Contig Express program (part of Vector NTI version 10). The sequences of primers for amplification of clip domain FISP are shown in Supplementary Table 1.

PCR products containing a single band was purified using a PCR purification Kit (Promega Corp., Madison, WI, USA) to remove excess primers and dNTPs. The PCR products were ligated into PCR^R II TOPO^R vector and the transformation of JM109 high-efficiency competent *Escherichia coli* cells were performed using the PCR^R II TOPO^R Vector Kit (Invitrogen). Recombinant bacteria were identified by blue/white screening and confirmed by PCR. Individual colonies grown on Luria Bertani (LB) agar plates were used as templates in standard PCR reactions with SP6 and T7 primers to amplify plasmid inserts. The sizes of the products were assessed on 1.5% agarose gels. Plasmids containing the insert were purified using plasmid isolation and purification Kit (Promega) and used as a template for DNA sequencing. The similarity analysis of nucleotide and protein sequence was carried out by using BLAST2.0 at NCBI (http://www.ncbi.nlm.nih.gov/blast). The deduced amino acid sequence was analyzed with the Expert Protein Analysis System (http://www.expasy.org/) and Vector NTI 10 Invitrogen software. Multiple alignments of the clip domain serine proteinase gene sequences were performed with the ClustalW Multiple Alignment program (http://www.ebi.ac.uk/clustalw/) and Multiple Alignment show program (http://www.bio-soft.-net/sms/index.html). Sequences of serine proteinase with clip domain registered in GenBank were used to construct the phylogenetic tree by Neighbor-Joining in Mega 3.1 (Molecular Evolutionary Genetics Analysis Version 3.1) with 100 bootstrapping steps [21]. The accession numbers of the template sequences used to construct phylogenetic tree are given in the Supplementary Table 2 RNA fold program (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi/) [22] was used to predict the secondary structure of full-length FISP mRNA and the structure was compared with the closely related species of *F. chinenesis* (ABC33918) and *P. monodon* (ACP19562).

Expression of FISP mRNA in tissues like haemocyte, heart, gill, muscle, hepatopancreas, intestine and eye was demonstrated by real-time (qRT-PCR) analysis. For the challenge test, there were three treatments *F. indicus* injected with β-glucan (*β*G), Peptidoglycan(PG) and saline combined with seven exposure times at 0, 3, 6, 12, 24, 36, and 48 h *β*G (G5011 from the yeast *Saccharomyces cerevisiae*, (Sigma–Aldrich, St. Louis, MO, USA) and PG (69 554 from *Bacillus subtilis*, Sigma–Aldrich) which had been dissolved in 0.85% NaCl solution to 1 mg ml^{-1} was used as test solution. *F.indicus* was injected at the abdominal side with βG & PG solution at a rate of 20 μl per 20 g shrimp to reach a dose of 1 mg kg^{-1}. Control groups were injected with 20 μl saline. For each treatment and each exposure time, haemolymph were sampled from three shrimp. Seawater was replaced daily. Five millilitres of haemolymph were collected into a 25 ml test tube containing 5 ml of anticoagulant [23,24]. The diluted haemolymph was centrifuged at 500*g* at 4 °C for 20 min. The resulting haemocyte pellet was used for total RNA isolation, and used for the FISP transcript analysis by PCR as described above. Quantitative RT-PCR was performed using the gene specific primers QFISP F with QFISP R. The primers β-actin F and β-actin R were used to amplify the β-actin fragment that was used as a positive control. The mRNA expressions of FISP in haemocytes from control saline, PG and βG injected *F. indicus* groups were measured using an SYBR green I qRT-PCR assay in an ABI PRISM 7500 Sequence Detection System (Model 7500, Applied Biosystems, Foster City, CA, USA), and details of the measurement procedures were previously described [25]. Specific primer pairs of QFISP F/QFISP R and β-actin F/β-actin R were used for the quantitative RT-PCR. After amplification, data acquisition and analysis were performed using the Sequence Detection Software (SDS vers. 2.1, Applied Bio systems). The $2^{-\Delta\Delta C_T}$ method was chosen as the calculation method [26]. The difference in the cycle threshold (C_T) value of the FISP gene and the control gene (β-actin), called ΔC_T, was calculated as: $\Delta\Delta C_T$ = (ΔC_T of immune stimulant or saline-injected *F. indicus* for the FISP gene at each time point) − (ΔC_T of the initial control).

The molting cycle of *F. indicus* was divided into five substages (A, B, C, $D_{0/1}$, and $D_{2/3}$) [26,27] .The criterion for determining molt stages were based on changes in the setae/setal base interface of the uropods examining under stereomicroscope and classified as: (1) A and B for the postmolt; (2) C for the intermolt; and (3) $D_{0/1}$ and $D_{2/3,}$ for the premolt. Ten shrimp from each stage were randomly sampled to inspect FISP mRNA expression in haemocytes by quantitative real-time PCR following measurement procedures and the calculation method as described above. The stage C was used for calibration in the molt cycle test. One-way analysis of variance (ANOVA) was used to analyse the data. When ANOVA identified differences among groups, Tukey's multiple-comparisons test (SAS Institute, Cary, NC, USA) was conducted to examine differences of FISP gene expression among treatments. A statistically significance level of $P = 0.05$ was chosen.

The full-length cDNA consisted of 1301 bp comprising an open reading frame (ORF) of 1143 bp (61 bp to 1203 bp) coding a mature protein of 380 amino acids, 60 bp of the 5′ untranslated region (UTR), 98 bp of the 3′ untranslated region, and with the putative initiation methionine codon (ATG) beginning at nucleotide 61 and stop codon beginning at nucleotide 1201. SignalP Server (EXPASY) predicts that the first 24 amino acids in the N-terminal region of the polypeptide chain form a signal peptide sequence. This putative translation product contained one N-terminal clip domain from residues 32–78 and the conserved serine proteinase catalytic traid domain (try-His domain) was found between amino acid from 157 to 162. The typical catalytic traid of serine proteinase required for functional activity (His^{161}, ASP^{231} and Ser^{286}) were conserved in the polypeptide sequence of Indian white shrimp *F. indicus*. The sequence around the catalytic residues also showed the highest sequence identity in the serine proteinase domain. The sequence showed twelve cysteine conserved residues (Cys^{33}, Cys^{39}, Cys^{62}, Cys^{77}, Cys^{78}, Cys^{146}, Cys^{162}, Cys^{254}, Cys^{302}, Cys^{316}, Cys^{325}, and Cys^{355}) at suitable position. The predicted molecular mass of the deduced amino acid sequence (380 aa) was 40.7 kDa with an estimated pI (isoelectric point) of 5.07. The cDNA sequence and deduced amino acid sequence are submitted to NCBI GenBank accession number HM368165 (Fig. 1).

Phylogenetic analysis of FISP cDNA along with its arthropod counterparts suggests that it has a high sequence similarity, and is homologues, to a serine proteinase of the crustaceans *F. chinensis* (93%, ABC33918), *P. monodon* (38%, ACP19562), *P. monodon* (30%, ACP19559), the insects *Nasonia vitripennis* (33%, NP_001155043), *Aedes aegypti* (30%, XP_001657092) *Anopheles quadriannulatus* (31%, ACN38223), *Culex quinquefasciatus* (29%, XP_001845622) and *M. sexta* (30%, AAC64004) respectively (Supplementary Table 2). Multiple sequence alignment of amino acid sequence of *F. indicus* using the ClustalW program indicated that the FISP shared common functional domains with serine proteinase (SP) which belongs to the super family of the trypsin-like serine proteinases, including six strictly conserved Cys residues corresponding to the three disulphide bridges (Cys^{146}–Cys^{162}, Cys^{302}–Cys^{316} and Cys^{325}–Cys^{355}). In the SP-like domains, the catalytic traid (His^{161}, ASP^{231} and Ser^{286}) residues were found conserved among the FISP