Short sequence report

Molecular characterization of a crustin-like, putative antimicrobial peptide, Fi-crustin, from the Indian white shrimp, Fenneropenaeus indicus

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Antimicrobial peptides are important innate immune defense, especially in those animals which lack adaptive immunity [1–8]. Due to their small size, amphipathic structure and cationic character they can rapidly diffuse to the point of infection [9], a mechanism that presumably makes it easier to circumvent microbial resistance against the peptides [10]. Besides providing an immediate and broad-spectrum microbicidal activity, AMPs can kill bacteria in micromolar range, are promptly synthesized at low metabolic cost, and are easily stored in large amounts and readily available shortly after an infection [11–13]. Many AMPs show a remarkable specificity for prokaryotes with low toxicity for eukaryotic cells; a phenomenon which has favored their investigation and exploitation as potential new antibiotics [14]. AMP gene expression and distribution are regulated through haemocyte reactions [15]. Transcripts of crustin-encoding genes have also been observed in gills, heart and intestine [16–18] but as these tissues are highly vascularised, it is assumed the transcripts from these organs are due primarily to the haemocytes.

In penaeid shrimps, four main families of AMPs have been currently described and characterized from the haemocytes: penaeidins, crustins, anti-lipopolysaccharide factors (ALFs) and lysozymes. Penaeidins are mainly active against Gram-positive bacteria, filamentous fungi [19], viruses and protozoans [20] whereas ALFs have a broader antimicrobial spectrum including Gram-negative bacteria [21,22]. Conversely, crustins are reported to have a more-restricted activity spectrum, affecting mainly marine Gram-positive bacteria [17,23,24] Crustins, a widely distributed family of AMPs was first isolated from the shore crab, Carcinus maenas as an 11.5 kDa peptide [23]. Crustins are cationic, cysteine-rich antimicrobial AMPs having molecular weight of 7–14 kDa, with an isoelectric point in the range of 7.0–8.7, and contain one whey-acidic protein (WAP) domain at the carboxy terminus [25]. Crustins have been proved to be an important antimicrobial protein in the plasma and haemocyte granules of crustaceans and described as a component of the innate immune system [8]. These AMPs are dominantly synthesized and stored in haemocytes [4,8,16,18,23,24,26–30] and their release from haemocytes is induced by bacterial infection [15,27,31]. Crustin mechanisms of action and function are still largely unknown, although they contain a whey-acidic protein (WAP) domain common to proteinase inhibitory activities as well as antimicrobial activities [8].

Many full-length cDNA and several ESTs of crustins have been described in a wide range of penaeid prawns including Litopenaeus vannamei [8,24,30,32], Litopenaeus setiferus [24,32,33], Penaeus monodon [16,17,29,30,34–37], Marsupenaeus japonicus [17,28], Litopenaeus schmitti [33], Fenneropenaeus chinensis [17,29], Farfantepenaeus brasiliensis [33], Farfantepenaeus paulensis [33] and Farfantepenaeus subtilis [33]. However, no antimicrobial peptide sequences have been reported from Fenneropenaeus indicus. In the current study a crustin cDNA has been characterized from the Indian White Shrimp, F. indicus.

Healthy adult F. indicus (8–10 g body weight) were purchased from a local shrimp farm in Vypeen, Kochi. They were transferred to aquaria of 500 l capacity and acclimatized for one week under laboratory conditions. Prawns were fed with a standard feed (Higashimaru, India). Aeration was provided in all tanks during the experiment and bioreactor was set in all aquaria for the removal of ammonia and nitrate. Only shrimps in the intermoult stage were sampled during the study.

Haemolymph was collected from the rostral sinus using specially designed capillary tubes (RNase-free) rinsed using pre-cooled anticoagulant solution (RNase-free, 10% sodium citrate, pH 7.0). Total RNA was extracted from the haemocytes using TRI...
and cagccgagagaaacactatcaagat/C0 for Amplifi
each primer and 1U Taq DNA polymerase (New England Biolabs).

reaction volume containing 5
a 25
mLV reverse transcriptase (New England Biolabs). The reaction was
by 35 cycles of 94
15 min. PCR ampli
HCl, 50 mM KCl, pH 8.3), 3.5 mM MgCl

0.25 -Cys

N          Y

V          V         V          Q           S

P          G           I           R          N         R            P          P           I

C          F          G            S         D          K

G         V            S          G          G         V           A          G         G          G           F          V

acg cca cgt at

acg cca cgt at

acg cca cgt at

acg cca cgt at

Fig. 1. Nucleotide and amino acid sequences of Fi-crustin from the haemocyte of the

DQ097703 Femoperenaeus chinesis crustin-like protein fc-1 mRNA, complete cds

94% 1e-78 91%

DQ334395 Penaeus monodon crustin-like antimicrobial peptide type 2 mRNA, complete cds

70% 2e-80 97%

E6fis4659 P. monodon crustin-like antimicrobial peptide gene, complete cds

70% 2e-75 97%

DQ097704 F. chinesis crustin-like protein fc-2 mRNA, complete cds

58% 2e-27 96%
Fig. 2. Multiple alignment of nucleotide sequence of the Fenneropenaeus indicus crustin-like antimicrobial peptide, Fi-crustin (GQ469987) with other shrimp crustins (Penaeus monodon GQ334395, Fenneropenaeus chinensis DQ097703, P. monodon EF654659, P. monodon EF654658, P. monodon FJ59177, P. monodon FJ59178, F. chinensis DQ097704, P. monodon FJ59174, P. monodon FJ59175; P. monodon FJ59176) obtained using GenDoc programme Version 2.7.0. Black and grey indicates conserved sequences.
amino acid sequences of crustins also showed that the deduced amino acids of Fi-crustin shared relatively high identities with those of *F. chinensis* and *P. monodon*. Multiple alignment of the nucleotide sequences of Fi-crustin and other shrimp crustins showed high similarity in the signal peptide region, major gaps could be observed in the ensuing region. The 58–70th nucleotide sequences were found to be absent in the Fi-crustin when compared to *P. monodon*. A similar gap could be observed for the *F. chinensis* crustins between the 64–70th position. Similarly, another missing sequence region could be observed for Fi-crustin between the 88–98th position that matches with a similar gap for the *F. chinensis* crustins at the 88–93rd position, when compared to *P. monodon*. Great variation between the sequences of *P. monodon* and *Fenneropenaeus* sp. could be observed at the 122nd–144th position. Other major missing sequences of the Fi-crustins were found at 182–202nd and also between 211th and 216th position. *F. chinensis* showed a major gap for the nucleotide sequences at 262–352nd position whereas *P. monodon* and Fi-crustin did not.

The phylogenetic relationships between Fi-crustin and other crustins with WAP domain are shown in Fig. 3. The tree topologies revealed the relationships of Fi-crustin with other invertebrate crustin-like peptides. The molecular phylogenetic tree based on amino acid sequences suggests that all the crustin members possess the same ancestral origin, which has subsequently diverged at different phases of evolution. Out of all the species, crustins of prawns are found to be evolutionarily distantly related to crustins of other decapod species. The tree could be broadly classified into three major groups, one major group (Group I) which included the crustins of prawns; another (Group II) with that of king crab/crayfish crustins and Group III containing the lobster/crab crustins. The bootstrap distance tree calculated for the crustin sequences clearly indicate that the Fi-crustin possessed great similarity to crustins isolated from *F. chinensis* and *P. monodon*. Great variability could also be noticed in the crustin sequences of various decapods.

This is the first report of an antimicrobial peptide from Indian white prawn, *F. indicus*. The reported AMP belonged to the class of crustins with its characteristic WAP domain and showed 91% similarity to *F. chinensis* crustins. The phylogenetic tree analysis showed that the crustins diverged from an ancestral sequence to three major groups i.e. Group I with prawns, Group II with Cray fishes/King crabs and Group III with Lobsters/Crabs. Under prawn — crustins, three sub groups were noticed 1) *L. vannamei* 2) *P. monodon* and 3) *Fenneropenaeus* sp. The wide distribution of crustins in crustaceans indicate the importance of these antimicrobial peptides in the innate immune system.

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