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High sequence variability among hemocyte-specific Kazal-type proteinase inhibitors in decapod crustaceans

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ABSTRACT

Crustacean hemocytes were found to produce a large number of transcripts coding for Kazal-type proteinase inhibitors (KPIs). A detailed study performed with the crayfish *Pacifastacus leniusculus* and the shrimp *Penaeus monodon* revealed the presence of at least 26 and 20 different Kazal domains from the hemocyte KPIs, respectively. Comparisons with KPIs from other taxa indicate that the sequences of these domains evolve rapidly. A few conserved positions, e.g. six invariant cysteines were present in all domain sequences whereas the position of P1 amino acid, a determinant for substrate specificity, varied highly. A study with a single crayfish animal suggested that even at the individual level considerable sequence variability among hemocyte KPIs produced exist. Expression analysis of four crayfish KPI transcripts in hematopoietic tissue cells and different hemocyte types suggest that some of these KPIs are likely to be involved in hematopoiesis or hemocyte release as they were produced in particular hemocyte types or maturation stages only.

1. Introduction

Kazal-type proteinase inhibitors (KPIs) are present in various organisms and there are nearly a thousand deposited sequences in public databases (http://merops.sanger.ac.uk/) [1,2]. The overwhelming majority of these are from different animals but a small number of sequences originating from bacteria, plants and fungi can be found. The KPI was originally isolated and characterized from bovine pancreas [3] where the inhibitor controls excessive proteolysis activities in the alimentary organs. Thereafter, related inhibitors were characterized and cloned from the arthropods *Rhodium prolixus* [4] and *Pacifastacus leniusculus* [5]. In blood-sucking animals such as the insect *R. prolixus*, KPIs assist in preventing blood coagulation and thus allow the insect to acquire more blood [4]. In other invertebrates, the physiological roles of these inhibitors have remained elusive although it is commonly speculated that they may interfere with processes using proteinases. A subtilisin inhibitor, Bumps, isolated from the silkworm *Bombyx mori* may function to inhibit the microbial proteinases to protect the pupae from infection by pathogens [6] and a similar suggestion was made for a KPI isolated from a hydra that was found to be active against *Staphylococcus aureus* [7]. For the human parasite, *Toxoplasma gondii*, a KPI is produced to inhibit the digestive enzymes during its residency in small intestine [8]. The reproductive process in the fresh water prawn *Macrobrachium rosenbergii* requires the presence of a KPI to inhibit the sperm gelatinolytic activity [9]. The involvement of KPIs in immunity is implicated by their up-regulation upon infection and bacteriostatic activity. In Zhikong scallop *Chlamys farreri*, the expression of a 12-domain KPI in hemocytes was up-regulated upon *Vibrio anguillarum* challenge [10]. In the oriental white shrimp *Fenneropenaeus chinesis*, the expression was up-regulated against the white spot syndrome virus (WSSV) [11]. In addition, a KPI from the black tiger shrimp *Penaeus monodon* was found to be bacteriostatically active against *Bacillus subtilis* [12].

The KPIs are characterized by the presence of one to several tandem domains that typically contain three intra-domain disulfide bridges formed by six conserved cysteine residues. Inhibitory specificity is believed to mainly be due to the so-called P1 amino acid, positioned at the second residue after the second conserved cysteine [13]. The interaction of the proteinase with the reactive site loop and other parts of the inhibitor has been studied in great detail in several crystallographic studies [14,15]. Besides

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the P1 residue, a few other residues are involved in the interaction and responsible for the binding strength.

Taking into account the large number of serine proteinases that are produced in animals for a variety of reasons, the KPIs are potentially important for the well-being of the animals. They could potentially interfere with and regulate many physiological processes. In this study, we have, therefore, compared the KPI repertoire in the hemocytes of crayfish and shrimp as representatives for decapod crustaceans. Herein, the presence of a number of different KPIs in these cells is reported and the significance of this variation discussed.

2. Materials and methods

2.1. Animals

Freshwater crayfish, *P. leniusculus*, were purchased from lake Vättern and were kept in aquaria in aerated tap water at 10 °C. Animals weighing about 25 g were used.

2.2. RNA isolation and RT-PCR

Total RNA was isolated from the different tissues of crayfish, using TRIzol® Reagent (Invitrogen) and treated with RNase free DNase I (Ambion, Austin, TX, USA) treatment. To obtain isolated granular and semigranular hemocytes preformed Percoll gradients were used as described in [16]. Complementary DNA was synthesized with ThermoScript™ (Invitrogen, Carlsbad, CA, USA) transcriptase with oligo(dT) as primer using 1 μg of total RNA according to the manufacturer's instructions. The cDNAs were subjected to PCR in a total volume of 20 μl using the following primers for Kazal inhibitor sequences were as follows: for X79512 (product size 246 bp): forward primer 5′-AGTTTACT-GACCTGGATCACA-3′, reverse primer 5′-CACAGGATTATAGTC-CAAAGG-3′; CF542313 (product 231 bp): forward primer 5′-ACTGATGGCAATACCTACGAC-3′, reverse primer 5′-GTCGTAGG-TATTGCCATCAGT-3′; CF542312 (product 222 bp): forward primer 5′-ATAGCAACGACTGCGATTTTA-3′, reverse primer 5′-ATTCGCCTTTGTATGCTGTTT-3′; EU433325 (product 280 bp): forward primer 5′-GTGCAACTCTCCTGCTGG-3′, reverse primer 5′-GCATTCACCGTTATAGGCA-3′. For controls crayfish 40S ribosomal protein (Genbank accession number CF542417); the primer sequences were as follows: forward 5′-CCAGGACCCCCAAACTTCT-TAG-3′ and reverse 5′-GAAAACTGCCACAGCCGTTG-3′.

Amplification profile consisted of an initial denaturation step of 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s. All PCR products were analyzed by 1.5% agarose gel and stained with ethidium bromide.

2.3. The shrimp EST sequences

The shrimp KPI sequences were searched from Genbank and from the *P. monodon* EST database (http://pmonodon.biotec.or.th/home.jsp) [17]. All the KPI sequences were deposited in the Genbank with the accession numbers indicated in Table 2.

2.4. CDNA library construction and EST sequencing

Partial cDNA sequences were obtained from a ZAP Express cDNA library constructed from mRNA of crayfish hemocytes and the library was mass excised into the pBluescript SK(−) plasmid vector for analysis of transcripts via expressed sequence tag (EST) sequencing using an ABI PRISM dye terminator cycle sequencing ready reaction kit (PerkinElmer Corp.).

A total of 441 EST sequences were obtained and 389 of these sequences have been deposited in Genbank (accession numbers CF542269–CF542658).

2.5. SSH isolation

The suppression subtractive hybridization (SSH) study from the hemocytes of an individual animal before and after being subjected to experimental infection with WSSV was described in [18]. In short, total RNA was isolated from hemocytes collected from the animal pre- and post-WSSV-infection and used as driver and tester, respectively. The subtracted cDNAs were used for library construction into PCR®2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA). A total of 231 forward subtracted clones were sequenced. The sequences from the SSH study used here have the accession numbers FJ975048–FJ975053.

2.6. Sequence analysis

The sequences of the Kazal domains were aligned using Clustal W (http://www.ebi.ac.uk/Tools/clustalw2/) and then manually adjusted. The alignments were then used to perform the phylogenetic analysis using the online version of MAFFT version 6 [19] on http://align.bmr.kyushu-u.ac.jp/mafft/online/server/), using default parameters (blosum 62 matrix and gap penalty 1.53). A phylogenetic tree was built using the average linkage (UPGMA) algorithm, and visualized using ATV 4.05.

Sequence logos for the Kazal domains of *P. leniusculus* and *P. monodon* respectively were generated using the WebLogo 3 software (http://weblogo.berkeley.edu). The SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) and SIG-Pred (http://bmbpcu36.leeds.ac.uk/prot_analysis/Signal.html) were used to predict the signal cleavage sites.

3. Results

Crayfish hemocytes produce a large number of different KPI transcripts. Inspection of available EST sequences for *P. leniusculus* (see Table 1) revealed the presence of at least 26 different Kazal proteinase inhibitor domains (KPIDs). It is estimated that they represent at least 12 different transcripts (and possibly inhibitor molecules) although the exact number is difficult to establish since a majority of the EST sequences and all SSH sequences are partial transcripts. For the shrimp *P. monodon*, at least six transcripts accounting for 20 different KPIDs were found to be expressed by the hemocytes (Table 2).

As shown in Table 1 the earlier described PAPI I (accession number X79512) consist of four different KPIDs, one of which is present in several variants (KPID4a–g). The sequence polymorphisms recorded to some degree represent allelic variants but there are also separate KPI genes present in this species. Four different transcripts containing at least six different KPIDs were recorded from a single animal in the SSH study which may indicate that a single individual has the potential to synthesize a number of different Kazal inhibitors in their hemocytes, although other interpretations cannot be excluded.

Four different transcripts encoding 2-domain proteins composed of different KPIDs were detected in the hemocyte EST collection. Two of these, CF542272 and CF542345, share an identical putative signal peptide sequence (21 amino acids) and similar, but not identical KPIDs (KPIDs 17/18 and KPIDs 9/10, respectively) that together are sharing an 83% sequence identity. Two other transcripts, EU433325 and CF542313 have identical signal peptides (22 amino acids) but in this case there is only limited sequence identities between their respective KPIDs, i.e. KPID5 + 6 and KPID13 + 14, respectively. Interestingly, EU433325 as well as CF542313 contain KPIDs with a Gly_6 next to the P1 position at amino acid position 7 (Fig. 1A and B; KPID5, KPID6 and KPID13) that is unsual in reported Kazal domains.