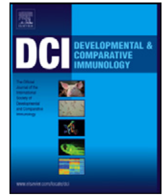
Developmental and Comparative Immunology 33 (2009) 583–591

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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



Characterization of crustins from the hemocytes of the spider crab, *Hyas araneus*, and the red king crab, *Paralithodes camtschaticus*

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ARTICLE INFO

Article history:
Received 29 August 2008
Received in revised form 20 October 2008
Accepted 23 October 2008
Available online 27 November 2008

Keywords:
Marine bioprospecting
Cationic antimicrobial peptides
Invertebrate immunology
Anti-fungal activity
WAP domain
Crustacea

ABSTRACT

Crustins are distributed across the decapods and are believed to play a significant part in the humoral defense system of their host. In this study, two crustin isoforms from *Hyas araneus* hemocytes were purified and tested for antimicrobial activity against selected microorganisms. They show both antibacterial and antifungal activity, with highest activity against the Gram-positive bacteria *Corynebacterium glutamicum*. Sequencing of the transcripts showed them to have a mature peptide of 90 amino acids and differing in three positions in the mature peptide. They were named Cru*Ha*1 and Cru*Ha*2. Real-time RT-PCR revealed that they mainly are expressed in hemocytes. Screening a cDNA library detected a crustin sequence in *Paralithodes camtschaticus* hemocytes, coding for a mature peptide of 98 amino acids. It was named Cru*Pc*. Based on phylogenetic inference and primary structure, Cru*Ha*1 and Cru*Ha*2 were placed within the Type I group of crustins, while Cru*Pc* belongs to the Type II.

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1. Introduction

Antimicrobial (poly) peptides (AMPs) comprise a diverse group of relatively small molecules (typically between 3 and 14 kDa) which often have an amphipathic structure, thus allowing them to interact with the lipid membrane of microorganisms. Among the some 900 AMPs characterized to date, some 70 are found within the crustacean group with most belonging to either the penaeidin or the crustin family [1,2]. The crustins are suggested defined as crustacean, Cys-rich, cationic antimicrobial polypeptides in the size of 7–14 kDa, containing a single whey-acidic-protein (WAP) domain in the C-terminal end [1]. The first member of this group was isolated from the granular hemocytes of the shore crab, *Carcinus maenas*. It is an 11.5 kDa peptide showing antibacterial activity against marine Gram-positive but not Gram-negative bacteria [3], and named carcinin after the genus from which it was isolated [4].

Crustin or crustin-like transcripts have also been characterized from numerous decapod species including shrimps, lobsters, crayfish and crabs. Within the Pleocyemata (crabs and lobsters), a carcinin-like sequence has been detected from the sand crab *Portunus pelagicus* [5], and crustin sequences have been found in the European and American lobster, *Homarus gammarus* and *H. americanus*, respectively [6,7]. An mRNA transcript encoding a crustin-like peptide, named PET-15, has been discovered in the epithelial cells in the olfactory organ of the spiny lobster *Panulirus argus* [8]. This is the only crustin-like peptide exclusively found in epithelial cells and not in circulating hemocytes, and the authors raised the hypothesis that PET-15 has other functions than being an antimicrobial substance [8]. Recently, three crustin-like sequences have been characterized from the hemocytes of the freshwater crayfish *Pacifastacus leniusculus* [9]. By genetic approaches, seven isoforms of crustin was found in the mud crab *Scylla paramamosain* [10]. Among the Dendrobranchiata (shrimps and prawns), several crustin sequences have been cloned from hemocytes in a variety of species, including *Litopenaeus vannamei* and *L. setiferus*, the Brazilian penaeid shrimps *L. schmiti*, *Farfantepenaeus paulensis*, *F. subtilis*, and *F. brasiliensis*, the Chinese shrimp *Fenneropenaeus chinensis*, the kuruma prawn *Marsupenaeus japonicus*, and the black tiger shrimp *Penaeus monodon* [11–17]. Isoforms have been detected at the genetic level in several of the species mentioned.

Although there are many reports on crustin sequences, there is a dearth of studies describing the isolation of the native peptides and measurement of the antimicrobial activities of the purified proteins. Relf et al. [3] showed that carcinin from *C. maenas* is active against marine Gram-positive but not Gram-negative

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doi:10.1016/j.dci.2008.10.010

bacteria. Except for this report, the few activity spectra of crustins come from recombinantly expressed peptides, namely Cru*Fc* from the Chinese shrimp *F. chinensis* [16], and crustin*Pm*1 [15] and Crus-like*Pm* [17] from the black tiger shrimp *P. monodon*. Whereas recombinant Cru*Fc* and crustin*Pm*1 are consistent with carcinin from *C. maenas*, in which they show no or very low antibacterial activity against Gram-negative bacteria, recombinant Crus-like*Pm* exhibits inhibitory activity against some Gram-negative strains [17]. Thus, from the few *in vitro* activity studies undertaken, it seems that most crustins are more potent against Gram-positive bacteria.

Crustins are widely distributed across decapod species and not restricted to any of the suborders, as the only crustacean AMP described so far. It is constitutively expressed, and in some species at very high levels [6,9,18]. Furthermore, the WAP-domain is structurally conserved with three conserved blocks [19], and has been shown to be an important region for the biological activity of WAP-domain containing peptides/proteins [16,20]. Crustins might thus play an important role not only as immune effectors, but might also be involved in unidentified physiological processes. Purification and characterization of native molecules, or alternatively recombinant production of them, is therefore crucial for a better understanding of the biological activities these molecules have in crustaceans.

The hemocytes of the spider crab, *Hyas araneus*, and the red king crab, *Paralithodes camtschaticus*, have been shown to contain components exhibiting antimicrobial activity [21]. Recently a di-domain AMP named arasin 1 was isolated from *H. araneus*, characterized by a Pro-Arg rich N-terminus and a Cys-rich C-terminus [22]. In this report, we present the purification and characterization of two crustins from *H. araneus* hemocytes, and their activity spectra against selected bacteria and fungi. Screening of a cDNA library revealed the cDNA sequences of the peptides, identifying them as isoforms and belonging to the carcinin-like Type I crustins. The cDNA sequence of a putative crustin from the red king crab, *P. camtschaticus*, is also presented.

2. Materials and methods

2.1. Animal collection and peptide purification

Live specimens of *H. araneus* (average size: 70–120 g) and *P. camtschaticus* (average size: 1000–1500 g) were collected off the coast of Northern Norway, and kept alive in seawater tanks until sampling. Samples were collected only from male adults. Peptides were extracted from *H. araneus* hemocytes and tested for antibacterial activity against *Listonella anguillarum* and *Corynebacterium glutamicum* as previously described [22]. Briefly, 4.92 g freeze-dried hemocytes from 44 animals were extracted twice with 10 volumes (v/w) of 60% (v/v) acetonitrile (ACN; HPLC-grade, Peypin, France) containing 0.1% trifluoroacetic acid (TFA; Fluka Chemie AG, Buchs, Switzerland) for 24 h at 4 °C. The aqueous phase was dried and resuspended in 0.05% TFA, followed by solid phase extraction (SPE) as described by Haug et al. [21]. The lyophilized 80% ACN fraction from SPE was resuspended in 0.065% TFA and subjected to RP-HPLC and separated on a SymmetryPrep C8 (Waters; 90 Å; 7 μm; 7.8 mm × 150 mm) column using a linear gradient of 0–60% ACN containing 0.05% TFA. Fractions (peaks) were collected manually and tested for antibacterial activity. Active fractions were submitted for purity measurements by mass spectrometry. Non-pure, active fractions were re-chromatographed on a Symmetry 300 C_{18} column (Waters; 300 Å; 5 μm; 4.6 mm × 250 mm) and eluted under the same experimental conditions as described above, but with a flow rate of 0.5 ml/min. Fractions were collected manually, tested for activity, and submitted to mass spectrometry.

2.2. Mass measurements, alkylation/reduction and peptide sequencing

Fractions showing antibacterial activity were submitted to purity and mass measurements by electrospray ionization mass spectrometry (ESI-MS). Analysis was performed with a Quattro-LC triple quadrupole instrument equipped with an electrospray LC interface (Micromass UK Ltd., Wythenshawe, UK), calibrated with bovine serum albumin. The analysis was performed in ESI+ mode with a cone voltage of 75 V. Samples were dissolved in water/methanol (50:50, v/v) containing 0.02% formic acid and were pumped into the mass spectrometer via a capillary using a microliter syringe at a flow rate of 10 $\mu l\ min^{-1}$. N_2 was used as desolvation (flow: 300 $l\ h^{-1}$) and cone gas (flow: 40 $l\ h^{-1}$), and the temperature in the ionization chamber was 100 °C. The quadrupole was scanned from *m*/*z* 200 to 2000 at 10 s/scan and the ion signals were recorded using the MassLynx^{TM} (Micromass) software program. The data were recorded in the continuum mode of acquisition. Non-protonated monoisotopic molecular masses were calculated from a series of multiple-charged protonated molecular ions.

Aliquots of the purified peptides were hydrolyzed in vacuum in 6 M HCl for 20 h at 110 °C and the amounts of amino acids were quantified on an amino acid analyzer (Model 421, Applied Biosystems, Perkin-Elmer). The peptides (ca. 5 nmol) were dissolved in 100 μl alkylation buffer (0.5 M Tris–HCl containing 1 mM EDTA and 6 M guanidine HCl, pH 8.4) and reduced with 5 μl 2.2 M dithiotreitol (DTT; Sigma, St. Louis, MO, USA). The samples were flushed with N_2 to prevent oxidation and incubated under oxygen-free conditions for about 16 h at 37 °C (over night). The reduced peptides (containing free cysteines) were alkylated by adding 5 μl pure (colorless) 4-vinylpyridine (Sigma) and subsequently flushed with N_2. The samples were further incubated for 20 min at 37 °C. The pyridylethylated peptides were then desalted on a Symmetry 300 C_{18} column (Waters) and eluted using a linear gradient of acetonitrile in acidified water (0.05% TFA) from 0 to 60% over 60 min and at a flow rate of 0.5 ml/min. Fractions were collected manually and submitted to ESI-MS. Edman degradation of natural peptides was performed at the Biotechnology Centre of Oslo (University of Oslo, Norway), and was performed on a protein microsequencer model 477A with 120A PTH analyser (Applied Biosystems, Perkin-Elmer) and a HP 241 Protein Sequencer (Hewlett-Packard).

2.3. Microbial strains and antimicrobial activity assays

The Gram-negative bacteria *L. anguillarum*, serotype O2 (FT 1801, also coded as AL 104/LFI 6004), originally isolated from Atlantic salmon by staff at the Norwegian Veterinary Institute (Oslo, Norway), *Escherichia coli* (ATCC 25922), and the Gram-positive bacteria *Staphylococcus aureus* (ATCC 9144) and *C. glutamicum* (ATCC 13032) were used as test bacteria. All isolates were grown at room temperature in Mueller Hinton Broth (MHB; Difco Laboratories, Detroit, USA). Antibacterial testing was performed and minimal inhibitory concentration (MIC) was determined as previously described [22], using a turbidometric method which measures the absorbance with a wide band filter (420–580 nm). The assay was done with duplicates in three independent experiments.

Antifungal activity was tested using the yeast strains *Saccaharomyces cerevisiae* and *Candida albicans* (ATCC 10231) and the filamentous fungus strain *Botrytis cinerea* 101 (*S. cerevisiae* and *B. cinerea* 101 were a gift from Professor Arne Tronsmo, The Norwegian University of Life Sciences, Ås, Norway). Fungal spores were dissolved in potato dextrose broth (Difco) and the cell

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