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Defensin like peptide from *Panulirus argus* relates structurally with beta defensin from vertebrates

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ABSTRACT

Naturally occurring antimicrobial peptides take place in the first line of host defense against pathogen as part of the humoral innate immune response. β-defensins are among the most abundant antimicrobial peptides in mammals, and thought to be solely found in vertebrates until a recent report describing the cloning and sequencing of defensin like peptides in the spiny lobster *Panulirus japonicus*. In the current study, we cloned and sequenced two genes from the hemocytes of the spiny lobster *Panulirus argus* encoding for two isoforms of defensin-like peptides, thus confirming the presence of this protein in the *Panulirus* genus. The 44 amino acids mature peptides showed the conservation of cysteine pattern characterizing the β-defensins, as well as known amino acids residues critical to exert their antimicrobial activity. They are also amphipathics, hydrophobics, and display an overall positive charge (+1) located at the C-terminus. The tertiary structure obtained by homology modeling indicated that likely conformations of lobster peptides are highly similar to β-defensins from vertebrates. The phylogenetic study carried out by probabilistic methods confirmed the relation with ancestral β-defensin from vertebrates. The finding of a putative defensin-like peptide in the expressed sequence tag (EST) of the lobster *Homarus americanus* with high homology with those of *P. argus* described in this study, would indicate the presence of this peptides in *Palinuridae* family. Taking into account all similarities between these peptides with β-defensins from vertebrates, it is conceivable to further support the finding of a new family of β-defensins in invertebrate.

1. Introduction

Naturally occurring antimicrobial peptides (AMPs) are essential components of the innate immune response. They participate in the first line of host defense against pathogens [1]. These gene-encoded molecules have been isolated from a wide range of taxonomical groups and show a considerable variation in structure and size. Albeit, most of them are cationic and hydrophobic [2]. Depending on their tissue distribution, AMPs perform either a systemic, and/or a local protection of the organism. This mechanism is based on the interaction with the membrane of pathogens [1]. According to their structural conformation, they are classified into three major groups: (a) peptides with an α-helical conformation, (b) cyclic and open-ended cyclic peptides with pairs of cysteine residues, and (c) peptides with an over-representation of some amino acids (i.e. proline, histidine or glycine rich) [2].

Crustins [3] and penaeidins [4] are among the better characterized AMPs in crustaceans. The penaeidin family is described as cationic peptides of 5–7 kDa, distinguished by a proline-rich amino-terminal domain and a cysteine-rich carboxyl terminus domain. They seem to be confined to the *Dendrobranchiata* and have been reviewed elsewhere [4]. On the other hand, crustins are 7–14 kDa cysteine-rich peptides occurring more widely across decapods in both *Pleocyemata* and *Dendrobranchiata* [3,5].

Defensins are characterized as small (3–5 kDa) cationic peptides, belonging to the cysteine containing AMP subfamily. They may adopt a β-sheet conformation, a β-hairpin-like structure or a mixed α-helix β-sheet conformation [1,6]. The primary structure of defensins can differ considerably, but depending exclusively on the spacing patterns between cysteine residues. They have been

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classified into invertebrate, plant, and vertebrates α, β and θ subfamilies [2,6].

The arthropod defensins family comprises the major group of invertebrate defensins. They encompass insect and scorpion cysteine-rich antimicrobial peptides. They are characterized by a different topological arrangement among the cysteine patterns and the intra catenaries disulfide bridges, leading to a tertiary structure completely different to that from vertebrate defensins [7].

AMP in *Panulirus* genus has been poorly studied. Nevertheless, a PET-15 mRNA was observed to be expressed in the olfactory organs after damage in *Panulirus argus* [8]. This PET 15 crustin-like peptide was for a long time the only antimicrobial peptide described for this genus.

Recently, cDNAs of two new isoforms of defensin, from the Japanese spiny lobster *Panulirus japonicus*, were cloned and sequenced (using the hemocytes cDNA library [9]). Those sequences clustered with vertebrate defensins and were closely related to chicken β-defensin [9], although β defensins were thought to be exclusive of vertebrates' taxa, being α and β defensins the most abundant AMPs in mammals [2].

The present study confirms the occurrence of gene encoded defensin-like AMPs in the *Panulirus* genus. Additionally, a deduced sequence with high homology with *P. argus* peptide was found in silico in the Expressed Sequence Tag of *Homarus americanus*. Peptides from *P. argus* share a close structural relationship with vertebrate β-defensins, thus revealing a presumable new family of defensin-like antimicrobial peptides in arthropods. The conserved cysteine pattern and the three-dimensional (3D) structure model similarities of lobster defensin-like peptides with β defensins from vertebrates, would suggest an ancestral relation between defensins from vertebrate and the new invertebrate defensins described in the *Panulirus* genus. This suggestion is based as well on the result of the phylogenetic reconstruction described in this study.

2. Materials and methods

2.1. Animal and sample collection

Caribbean spiny lobster *P. argus* were collected in the Batabanó gulf, at the south-western of the Cuban archipelago and kept in captivity in tanks with circulated seawater with a biological filter. The hemolymph was extracted using modified Alsever's solution (0.336 M NaCl, 0.15 M glucose, 0.027 M trisodium citrate, and 0.009 M EDTA) pH 7.5, as anticoagulant. Five milliliters of hemolymph were extracted from the fourth walking leg coxa, using a 10 mL pyrogen-free disposable syringe containing 5 mL of pre-cooled anticoagulant solution. The mixture was centrifuged at 700 *g* for 10 min at 4 °C. The cell pellet was washed two times with anticoagulant and placed in liquid nitrogen until RNA extraction.

2.2. Total RNA extraction and reverse transcription

Total RNA extraction was performed using the TRI-Reagent Kits (Sigma, St. Louis, MO) following the manufacturer's instructions. RNA concentration was determined at 260 nm. The quality and integrity of RNA were verified by $\text{Abs}_{260/280}$ and agarose (0.8%) gel electrophoresis, respectively. RQ1 RNase-free DNAse I (Promega, Madison, WI) was applied to remove possible genomic DNA contamination. First strand cDNA was synthesized by reverse transcription using oligo dT primer, and AMV reverse transcriptase following the manufacturer's instructions (Promega, Madison, WI). As an internal control of this cDNA, an elongation factor 1 alpha (EF1 α) was introduced in the PCR assay.

2.3. Cloning and sequencing

Defensin cDNAs sequences from *P. japonicus* [9] were procured from Nucleotide database at the GenBank/National Center for Biotechnology Information (NCBI at http://www.ncbi.nlm.nih.gov/nucleotide/). On the basis of similarity of these two sequences, a pair of degenerated primers were designed and synthesized: 10-373 Fw 5′-ATGAAGACGAAGGCCATTGTGATG-3′ and 10-374 Rv 5′-CTAA(A/C)CATAGGCA(T/A)GGCGGCAAC-3′. The primers were designed using GENRUNNER v. 3.05 (Hastings Software, Inc.). Primers from elongation factor of *P. argus* (EF1-α GGGAA-CATCTCAGGCCGACTGTGCTGTC forward and EF1-α CAACGGACTT-GACCTCAGTGGTGGG reverse) were constructed and used as control of PCR assay.

PCR amplification of 3 μL of cDNA was performed in 50 μL of reaction volume. This solution contained 2 pmol of each forward and reverse primers, 5 μL of 10× enzyme buffer, 1 mM $MgSO_4$, 5 μL of 10× enhancer, 0.3 mM dNTP, 0.4 μL of PlatiumPfx and H_2O to complete the total. The thermal profile was as follow: an initial denaturation cycle at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 1 min, primer annealing at 54° for 30 s, and elongation at 68 °C for 1 min. At the end of the last cycle, the PCR mixture was incubated at 72 °C for 5 min for overextension. It was cooled down at 4 °C for subsequent analysis. The PCR product was verified on 2.5% (w/v) agarose gel containing ethidium bromide and sized by the 100 pb Plus DNA Ladder (Invitrogen Corp., Carlsbad, CA). The desired size amplicon (190–200 pb) was purified from the gel using the QIAquick Gel Extraction Kit (QIAGEN GmbH, Germany), according to the manufacturer instructions.

The DNA obtained was cloned into pGEM-T easy vector (Promega, Madison, WI). TOP10 *Escherichia coli* cells were used for the transformation. Recombinant bacteria were identified by blue/white screening. Plasmids were purified and checked by restriction enzyme analysis (*Eco*RI) and PCR. Twelve clones containing inserts of expected size were sequenced at the sequences commercial service (Macrogen Inc., Korea).

2.4. Sequence analysis

Nucleotide sequences were analyzed for identification by BLASTN [10] at the website http://www.ncbi.nlm.nih.gov/. The deduced amino acid sequences were achieved with the Expasy Translate service, located at the Expert Protein Analysis System Server (http://www.expasy.org/tools/dna.html/). The protein motifs features were predicted with the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/) [11]. The theoretical signal peptide was predicted by SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/) [12]. Similar sequences searching against protein databases were performed using the FASTA and BLASTP programs (http://www.ebi.ac.uk/Tools/fasta33/index.html). The TBLASTN and TBLASTX programs were used to determine sequences homology with the complete *P. argus* nucleotide sequence, in an Expressed Sequence Tag (EST) database of invertebrates at NCBI.

2.5. Structural models calculation and analysis

Structural models were generated for defensin-like peptide from *P. argus* via homology modeling [13,14]. Homology searches for the mature peptide sequences were performed by PSI-BLAST and PSI-SEARCH using BLOSUM 80 as scoring matrix, in order to find a significant score and homologies (≥25%) with β defensins for which solved structures were available.

Datasets from **Protein Data Bank** (PDB) of six well resolved β defensins X-ray structures (accession codes: 1IJU,