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Strongylocins, novel antimicrobial peptides from the green sea urchin, *Strongylocentrotus droebachiensis*

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Summary
Sea urchins possess an innate immune system and are regarded as a potential source for the discovery of new antimicrobial peptides (AMPs). Here we report the purification and characterization of two novel antibacterial peptides (5.6 and 5.8 kDa) from coelomocyte extracts of the green sea urchin, *Strongylocentrotus droebachiensis*. These are the first reported AMPs isolated from sea urchins. The cDNA encoding the peptides and genomic sequences was isolated and sequenced. The two peptides (named strongylocins 1 and 2) have putative isoforms (1b and 2b), similar to two putative proteins from the purple sea urchin *S. purpuratus*. The native strongylocins are cationic, defensin-like peptides (cysteine-rich), but show no similarity to other known AMPs concerning the cysteine distribution pattern. Strongylocin 1 consists of 83 amino acids that include a preprosequence of 35 amino acids, whereas strongylocins 2a and 2b are composed of 89 and 90 amino acids, respectively, where 38 amino acids represent a preprosequence. No introns were found in the cloned gene of strongylocin 1b, whereas three introns and four exons were found in strongylocins 1a and 2a/b. The latter gene organization was also found in genes coding for putative strongylocins in *S. purpuratus*. The molecular mass difference between the native peptide and the deduced strongylocin 2 suggests that the first amino acid is bromotryptophan. The native peptides display potent activities against Gram-negative and Gram-positive bacteria.

Introduction

The need to discover new antimicrobial agents is inevitable since progressively more bacteria develop resistance against the conventional antibiotics. Antimicrobial peptides (AMPs) have a significant role in the innate immune system in both vertebrates and invertebrates and have promising capacities

Abbreviations: AMP, antimicrobial peptide; CF, coelomic fluid; ACN, acetonitrile; TFA, trifluoroacetic acid; SPE, solid phase extraction; ESI-MS, electrospray ionization mass spectrometry; MIC, minimal inhibitory concentration.

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for drug development. They are characterized as short amino-acid sequences (10–100 aa), having a net positive charge and being amphiphilic in their active forms. The AMP structures obtained have allowed identification of five major classes of peptides: (1) α-helical, (2) cysteine-rich (defensin-like), (3) β-sheet containing, (4) peptides with an unusual composition of regular amino acids, and (5) bacterial or fungal peptides containing uncommon modified amino acids [1]. The cysteine-rich peptides are one of the best characterized groups of AMPs, including α- and β-defensins from mammals, the insect defensins, mytilus defensins, and tachystatin A. The location of cysteine residues within the peptides is important for the disulfide pattern of the molecule. It has been proposed that proteins that present the same locations of cysteine residues also present similar disulfide arrays [2–4]. The disulfide bridges play an important role in stabilizing the tertiary structures and in protecting the peptide backbone from proteolysis during biosynthesis and in protease-containing microenvironments [5]. In most cases, AMPs are initially synthesized as inactive precursors. The immature form consists of a signal sequence (presequence) that aids targeting in the endoplasmic reticulum, a prosequence at the N-terminus, C-terminus or even within the middle of the precursor proteins, and the mature cationic peptide that has the antimicrobial activities after it is cleaved from the primary protein [6]. The prosequence is known to help folding of the mature portions [7,8] or inhibit the activity of the mature portion as an intramolecular chaperone [9].

As an invertebrate, the sea urchin lacks a vertebrate-type adaptive immune system [10,11] with the capacity to defend itself against most invading organisms and infections through various mechanisms, including clotting reactions, phagocytosis and encapsulation [12–16]. The coelomocytes of the sea urchin, which are circulating within coelomic cavity, are considered to be responsible for most of the defense reactions [11].

So far, only a few molecules isolated and characterized from the coelomocytes or the coelomic fluid (CF) of sea urchins have antibacterial and/or cytotoxic activities. For instance, Echinochrome A, a low molecular weight pigment which is released from the red spherule cells, has activities against Gram-positive and Gram-negative bacteria [17–19]. Antibacterial activity was also demonstrated in cell-free CF of *Echinus esculentus* [20] and in different types of coelomocytes of *Paracentrotus lividus* [19,21]. Coelomocytes of *Arbacia punctulata* mediated a non-specific cellular cytotoxicity against human and murine target cells *in vitro* when the phagocytic coelomocyte and target cell had membrane–membrane contact [22].

The evidence that coelomocytes and CF in the sea urchin show cytotoxic and antimicrobial activities has generated interest to identify the molecules responsible for these actions. Although many studies on the defense mechanisms in sea urchins have been conducted and the whole genome of *Strongylocentrotus purpuratus* has been sequenced [23], few studies have shed light on AMPs in these animals. In a previous study [24], we detected antibacterial activity against several bacterial strains in coelomocyte extracts of the green sea urchin *S. droebachiensis*. Sensitivity to protease treatment indicated that at least some of the active components were of protein nature. The aim of this study was to purify molecules with antimicrobial activity from the coelomocyte extracts and characterize the molecular features related to these activities. In this paper, we present the isolation and characterization of two novel AMPs, named strongylocins 1 and 2, from the coelomocytes of *S. droebachiensis*. Their partial amino-acid sequences were characterized by Edman degradation, and the coding sequences were obtained by construction and screening a coelomic cDNA library and by sequencing the corresponding genomic DNA. The strongylocins showed potent activity against both Gram-positive and Gram-negative bacteria. These molecules are the first purified AMPs from sea urchins.

Materials and methods

Animals and sample collection

Live green sea urchins *(S. droebachiensis)* were obtained off the coast of Tromsø, Norway, and maintained in fresh flowing seawater until sample collection.

The CF (1260 ml totally from 66 specimens) was collected by puncturing the calcareous body wall and was immediately centrifuged at 800*g* at 4 °C for 20 min to separate the coelomocytes from the plasma (cell-free CF). The coelomocytes were pooled, freeze-dried and kept frozen at −20 °C until extraction.

For cloning experiments, CF was immediately mixed with an equal volume of ice-cold calcium- and magnesium-free anti-coagulating buffer containing 70 mM EDTA and 50 mM imidazole according to Gross et al. [25]. All samples were centrifuged at 6500*g* for 5 min at 4 °C and the supernatant was discarded. The pellets of coelomocytes were stored at −80 °C until further use.

Bacterial strains and growth conditions

The Gram-negative bacteria *Listonella* (*Vibrio) anguillarum*, serotype O2 (FT 1801 or AL 104/LFI 6004), *Escherichia coli* (ATCC 25922), and the Gram-positive bacteria *Staphylococcus aureus* (ATCC 9144) and *Corynebacterium glutamicum* (ATCC 13032) were used as test organisms. All isolates were grown at room temperature in Mueller Hinton Broth (MHB; Difco Laboratories, Detroit, USA).

Extraction of antibacterial peptides from coelomocytes

Freeze-dried coelomocytes (54.7 g) were extracted twice with 10 volumes (v/w) of 60% (v/v) acetonitrile (ACN; HPLC-grade, SDS, Peypin, France) containing 0.1% trifluoroacetic acid (TFA; Fluka Chemie AG, Buchs, Switzerland) for 24 h at 4 °C. The combined supernatants were incubated at −20 °C for 1–2 h to allow the organic and aqueous phases to be partitioned. The aqueous phase was collected, dried in a vacuum centrifuge (Maxi Dry Lyo, Heto Lab., Denmark) and solubilized (100 mg/ml) in 0.05% TFA. Salt was removed from the extract by solid phase extraction (SPE) as described by Haug et al. [26]. Briefly, the extract was loaded onto a 35-cm^3 Sep-Pak C_{18} Vac cartridge (Waters Associates, MA, USA) equilibrated in acidified (0.05% TFA) Milli-Q water