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Two recombinant peptides, SpStrongylocins 1 and 2, from *Strongylocentrotus purpuratus*, show antimicrobial activity against Gram-positive and Gram-negative bacteria

Chun Li [a], Hans-Matti Blencke [a,b], L. Courtney Smith [c], Matti T. Karp [d,e], Klara Stensvåg [a,b,*]

[a] Norwegian College of Fishery Science, Faculty of Biosciences, Fisheries and Economics, University of Tromsø, Breivika, N-9037 Tromsø, Norway
[b] Centre on Marine Bioactivities and Drug Discovery (MabCent), Faculty of Biosciences, Fisheries and Economics, University of Tromsø, Breivika, N-9037 Tromsø, Norway
[c] Department of Biological Sciences, George Washington University, 414 Lisner Hall, 2023 G Street NW Washington, D.C. 20052, USA
[d] Department of Chemistry and Bioengineering, Tampere University of Technology, P.O. Box 541, 33101 Tampere, Finland
[e] BCC-Biosensing Competence Centre, P.O. Box 692, 33101 Tampere, Finland

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ABSTRACT

The cysteine-rich strongylocins were the first antimicrobial peptides (AMPs) discovered from the sea urchin species, *Strongylocentrotus droebachiensis*. Homologous putative proteins (called SpStrongylocin) were found in the sister species, *S. purpuratus*. To demonstrate that they exhibit the same antibacterial activity as strongylocins, cDNAs encoding the 'mature' peptides (SpStrongylocins 1 and 2) were cloned into a direct expression system fusing a protease cleavage site and two purification tags to the recombinant peptide. Both recombinant fusion peptides were expressed in a soluble form in an *Escherichia coli* strain tolerant to toxic proteins. Enterokinase was used to remove the fusion tags and purified recombinant SpStrongylocins 1 and 2 showed antimicrobial activity against both Gram-negative and Gram-positive bacteria. The results of membrane integrity assays against cytoplasmic membranes of *E. coli* suggest that both recombinant SpStrongylocins 1 and 2 conduct their antibacterial activity by intracellular killing mechanisms because no increase in membrane permeability was detected.

1. Introduction

Antimicrobial peptides (AMPs) have been isolated from a wide variety of organisms, including prokaryotes, plants, invertebrates, amphibians and mammals [1]. They are typically characterized as amphiphilic and positively charged short amino acid sequences that function as immune effectors and play a crucial role in the innate immune defence system. Some peptides are able to kill bacteria quickly, such as magainin 2, cecropin P1 and SMAP29, which kill within 15–90 min [2–4]. Many AMPs likely contribute to the formation of pores in the plasma membrane that lead to extensive membrane rupture eventually resulting in energy depletion and microbial lysis [5]. Although many AMPs have the capability of damaging the bacterial membrane, other bacteriostatic and bactericidal modes of action have been described in which AMPs can affect bacterial growth by binding DNA, inhibiting DNA replication, blocking gene expression or protein synthesis, as well as interfering with other enzymatic activity [5].

Strongylocins are the first AMPs to be isolated and characterized from green sea urchins (*Strongylocentrotus droebachiensis*) [6]. The active strongylocins 1 and 2 are cationic, cysteine-rich peptides and consist of 48 amino acids (5.6 kDa) and 51 amino acids (5.8 kDa), respectively. They display low haemolytic activity and activities against both Gram-positive and Gram-negative bacteria. The genome sequence of the purple sea urchin, *S. purpuratus*, indicates that the immune system, which includes a number of immune related genes such as Toll-like receptors, scavenger receptors and NACHT domain-leucine rich repeat (NLR) genes, is much more complex than was previously expected [7]. To date, there are very few immune effector genes identified [8] and only the putative immune effector genes called 185/333 have been studied [9–14]. In a previous study, two putative cDNAs from *S. purpuratus* showed high similarity with strongylocins [6]. Analysis of purple sea urchin expressed sequence tag (EST) records in GenBank showed several sequences that are highly similar to strongylocins. Therefore we questioned whether these strongylocin homologues in *S. purpuratus* would be able to carry out the same antibacterial functions as those from *S. droebachiensis*.

* Corresponding author at: Norwegian College of Fishery Science, Faculty of Biosciences, Fisheries and Economics, University of Tromsø, Breivika, N-9037 Tromsø, Norway. Tel.: +47 77 64 45 12; fax: +47 77 64 51 10.
E-mail address: Klara.Stensvag@uit.no (K. Stensvåg).

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Although the minimal inhibitory concentration (MIC) is commonly used as an indicator for peptide activity, a real-time measurement of cell permeabilization can be used to indicate whether peptides are capable of forming pores in biological membranes [15]. The cell permeabilization system is based on restricted import of firefly luciferase substrate, D-luciferin, into the cells at neutral pH. By making pores in the membrane the enzyme reaction is facilitated and light is produced. If a pore-forming compound is present, the reporter protein activity is enhanced by increased availability of the substrate inside the cell.

In this study, two gene sequences were identified from *S. purpuratus* with similarities to strongylocins. We subcloned the cDNA coding regions into the expression vector pET30-EK/LIC which includes the fusion tags for affinity purification and an enterokinase cleavage site. In addition a special strain of *E. coli* tolerating toxic proteins was employed for large-scale production. The fusion peptides were expressed in a soluble form, and after cleavage of the affinity tags, the purified recombinant peptides showed antibacterial activity against selected Gram-positive and Gram-negative bacteria. The results of the membrane integrity assay suggested that the mode of action for the SpStrongylocins is non-membranolytic.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The Gram-negative bacteria *Listonella* (*Vibrio*) *anguillarum*, serotype O2 (FT 1801 or AL 104/LFI 6004), *E. coli* (ATCC 25922 and MC1061), and the Gram-positive bacteria *Staphylococcus aureus* (ATCC 9144) and *Corynebacterium glutamicum* (ATCC 13032) were used for antimicrobial testing. All isolates were grown at room temperature in Mueller Hinton Broth, pH 7.4 (MHB; Difco Laboratories, Detroit MI).

NovaBlue GigaSingles™ competent cells (EMD Biosciences, Madison, WI) and *E. coli* DH5α were used for molecular biology manipulations and for maintenance of recombinant plasmid DNA. *E. coli* OverExpress™ C43 (DE3) cells (Lucigen, Madison, WI) were employed for fusion peptide expression.

2.2. Bioinformatics analysis

Based on the cDNA sequences of *S. droebachiensis* strongylocins, sequence similarity searches were performed with the BLAST software from EST records in GenBank (http://www.ncbi.nlm.nih.gov/BLAST). Sequences were aligned in BioEdit software [16]. The potential cleavage site(s) of the signal peptides was predicted by SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) software.

2.3. Construction of pET30-EK/LIC-SpStrongylocin

The cDNAs coding for *S. purpuratus* strongylocins 1 and 2, named SpStrongylocins 1 and 2, are available from GenBank (accession numbers GU116566 and GU116567). The cDNAs originated from two *S. purpuratus* coelomocyte cDNA libraries that had been cloned into the pExCell vector and the pSPORT1 vector, respectively [17,18]. The inserts were re-sequenced using the primers Sp6 and T7 (Table 1).

The coding regions of SpStrongylocins 1 (48 amino acid residues) and 2 (52 amino acid residues) were cloned into pET-30EK/LIC vector (Novagen, Darmstadt, Germany) and called pET-30EK/LIC-SpStrongylocin 1 and pET-30EK/LIC-SpStrongylocin 2, respectively, following the manufacturer's instructions. The target insert sequences were amplified using primers for SpStrongylocins 1 and 2 (Table 1). Briefly, PCR was performed on a thermal cycler (Model 2720, Applied Biosystems, Foster City, CA) in two separated steps using 100 ng of each cDNA as a template, 1 μM of each primer, 0.5 mM of each dNTP, 3 units (U) of ExTaq polymerase (TaKaRa Bio, Otsu, Shiga, Japan), 1× company supplied buffer in a total volume of 50 μl. For the first phase, PCR was carried out using the following program: 94 °C for 5 min, 5 cycles at 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 1 min. The second phase was completed with 25 cycles of 94 °C for 30 s, 65 °C for 30 s and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The PCR products were analyzed by gel electrophoresis and imaged with a DC120 digital camera and 1D digital software (Eastman Kodak, New Heaven CT).

Table 1
Primers.

Name	Sequence
Sp6	5′CGATTTAGGTGACACTATAG
T7	5′CAGTGAATTGTAATACGACTCACT
SpStrongylocin 1 forward	5′**GACGACGACAAGAT**CTTCAACTCGATCTATCATCG[a]
SpStrongylocin 1 reverse	5′**GAGGAGAAGCCCGGT**CACTAGGTTGATGGTCGGCAT[a]
SpStrongylocin 2 forward	5′**GACGACGACAAGAT**CTGGAACCCTTTTAGGAAGCTCT[a]
SpStrongylocin 2 reverse	5′**GAGGAGAAGCCCGGT**CACTAACTGATGACGGTGCAT[a]

[a] SpStrongylocins 1 and 2 forward and reverse primers contain 5′ sequences (in bold) employed in the ligation independent cloning technique.

The PCR product was gel purified and treated with T4 DNA polymerase (Novagen, Darmstadt, Germany) which employed the 3′–5′ exonuclease activity of T4 DNA polymerase to create the specific single-stranded overhangs in the PRC product (Table 1) [19,20], according to the manufacturer's instructions. After annealing the target insert and the vector, NovaBlue GigaSingles™ competent cells were transformed with the vector. According to the ligation independent cloning strategy, the first nucleotide of the insert-specific sequence on the forward primer must complete the codon ATX resulting in Met or Ile. Therefore, a recombinant peptide SpStrongylocin 1 contained the exact mature peptide following the fusion fragment, whereas an extra amino acid (Ile) was introduced to the recombinant peptide SpStrongylocin 2 between the enterokinase cleavage site and the mature peptide. The sequences of the inserts encoding SpStrongylocins 1 and 2 were confirmed by sequencing using the T7 primer (done by MWG Biotech, Atlanta GA).

2.4. Expression of fusion SpStrongylocins 1 and 2

The SpStrongylocin constructs were transformed into *E. coli* C43 (DE3) cells and selected on LB plates with 50 μg/ml kanamycin. An over night culture was expanded in 1 l of LB medium with 50 μg/ml kanamycin and incubated at 25 °C with shaking at 200 rpm to an OD^{600} of ~1.0. The cells were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and were harvested 4 h after induction.

2.5. Purification of SpStrongylocins 1 and 2

The cells were resuspended in binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 40 mM imidazole, pH 7.4) and lysed by sonication. The lysate was cleared by centrifugation at 15,000 × *g* for 15 min at 4 °C and the fusion proteins were purified using Ni^{2+} sepharose (GE Healtheare, Uppsala, Sweden). The proteins were eluted with elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 250 mM imidazole, pH 7.4), desalted and concentrated using a Centriprep® centrifugal filter device with an ultracel YM-3 membrane (Millipore, Billeria, MA).