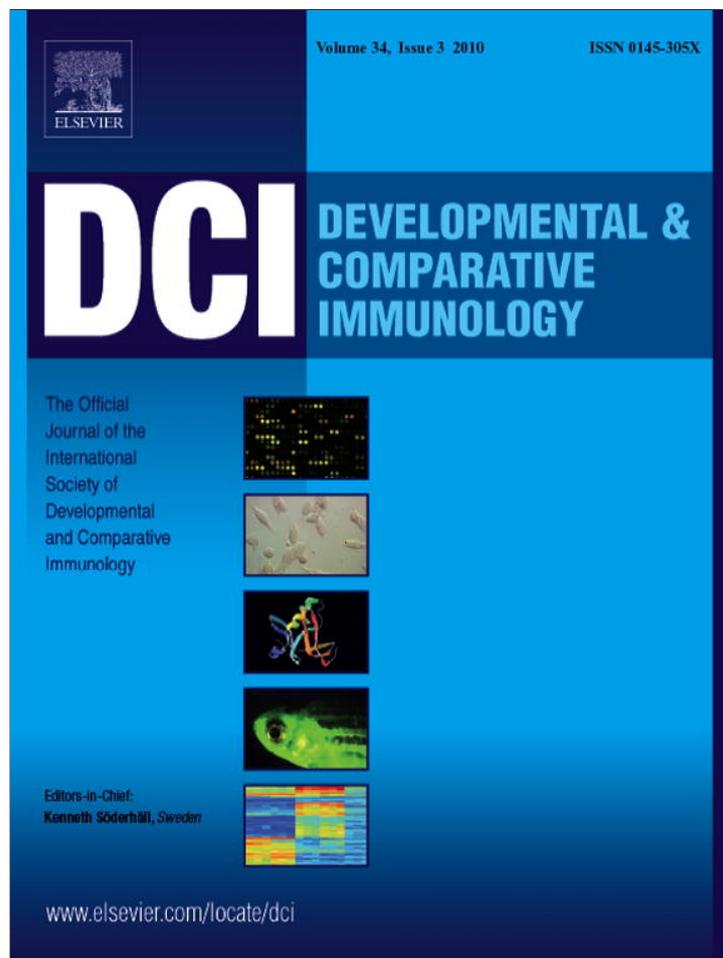


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## Two recombinant peptides, SpStrongylocins 1 and 2, from *Strongylocentrotus purpuratus*, show antimicrobial activity against Gram-positive and Gram-negative bacteria

Chun Li<sup>a</sup>, Hans-Matti Blencke<sup>a,b</sup>, L. Courtney Smith<sup>c</sup>, Matti T. Karp<sup>d,e</sup>, Klara Stensvåg<sup>a,b,\*</sup>

<sup>a</sup> Norwegian College of Fishery Science, Faculty of Biosciences, Fisheries and Economics, University of Tromsø, Breivika, N-9037 Tromsø, Norway

<sup>b</sup> Centre on Marine Bioactivities and Drug Discovery (MabCent), Faculty of Biosciences, Fisheries and Economics, University of Tromsø, Breivika, N-9037 Tromsø, Norway

<sup>c</sup> Department of Biological Sciences, George Washington University, 414 Lisner Hall, 2023 G Street NW Washington, D.C. 20052, USA

<sup>d</sup> Department of Chemistry and Bioengineering, Tampere University of Technology, P.O. Box 541, 33101 Tampere, Finland

<sup>e</sup> 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.

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### 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](mailto:Klara.Stensvag@uit.no) (K. Stensvåg).

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

**Table 1**  
Primers.

Name	Sequence
Sp6	5'CGATTAGGTGACACTATAG
T7	5'CAGTGAATTGTAATACGACTCACT
SpStrongylocin 1 forward	5' <b>GACGACGACAAGAT</b> CTTCAACTCGATCTATCATCG <sup>a</sup>
SpStrongylocin 1 reverse	5' <b>GAGGAGAAGCCCGGT</b> CACTAGGTTGATGGTCGGCAT <sup>a</sup>
SpStrongylocin 2 forward	5' <b>GACGACGACAAGAT</b> CTGGAACCCTTTAGGAAGCTCT <sup>a</sup>
SpStrongylocin 2 reverse	5' <b>GAGGAGAAGCCCGGT</b> CACTAACTGATGACGGTGCAT <sup>a</sup>

<sup>a</sup> SpStrongylocins 1 and 2 forward and reverse primers contain 5' sequences (in bold) employed in the ligation independent cloning technique.

(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).

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<sup>600</sup> 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<sup>2+</sup> sepharose (GE Healthcare, 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, Billerica, MA).

## 2.6. Enterokinase cleavage

Enterokinase cleavage was conducted following the manufacturer's instruction (Sigma–Aldrich, St. Louis, MO). Briefly, each fusion protein was incubated with enterokinase in reaction buffer (500 mM Tris–HCl pH 8.0, 2.0 mM CaCl<sub>2</sub>, and 1% Tween-20) at 25 °C for 20 h. The cleaved hexahistidine tag and residual uncleaved fusion peptide on the mature peptide were removed by subsequent binding to Ni<sup>2+</sup> sepharose. The enterokinase was removed with a centrifugal filter device with an ultracel YM-10 membrane (Millipore).

## 2.7. Protein quantification

Protein concentration was measured using BCA protein assay kit (Pierce, Rockford IL) and the Nano-drop ND-1000 spectrophotometer (Nano Drop Technologies, Wilmington, DE).

## 2.8. SDS-PAGE analysis

Bacterial lysate and purified SpStrongylocin protein samples were mixed with protein sample buffer (0.1 M Tris–HCl pH 6.8, 24% glycerol, 1% SDS, 2% β-mercaptoethanol, 0.2% (w/v) Coomassie blue G-250), heated to 95 °C for 5 min, and analyzed by 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [21]. Protein bands were detected by Simple Blue SafeStain™ (Invitrogen, Carlsbad, CA) and imaged with the Bioimaging system, Syngene (Syngene, Cambridge, UK).

## 2.9. Antimicrobial activity assay

The antibacterial activity of the purified peptides was tested as previously described [22]. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of peptide that would fully inhibit bacterial growth as measured by optical density. Cecropin P1 and cecropin B, made synthetically as described by Kjuul et al. [23], were used as positive control peptides.

## 2.10. Membrane integrity assay

The effect of recombinant peptide activity on membrane permeability was determined by a whole-cell real-time assay employing *E. coli* that constitutively expressed a recombinant luciferase, as modified from Virta et al. [15]. Briefly, 50 μl of MH medium containing 1 × 10<sup>7</sup> *E. coli* cells (MC1061 [24]) and 2 mM D-luciferin (10 mM Tris–HCl buffer, pH 7.4) was mixed with 50 μl of a dilution of the peptide (15 μM) 25 °C. Luminescence was monitored using an Envision HTS microplate reader (PerkinElmer life and analytical sciences, Turku, Finland) and analyzed by the Wallac Envision Manager (Version 1.09, PerkinElmer) software. PR-39, cecropin P1 and polymyxin B (Sigma–Aldrich, St. Louis, MO) were employed as control peptides. All measurements were repeated at least three times.

## 3. Results

### 3.1. The sequences of strongylocins in *S. purpuratus*

Strongylocins were chosen for recombinant expression because they were the first AMPs to be identified and characterized from the green sea urchin (*S. droebachiensis*), which exhibit an activity against both Gram-positive and Gram-negative bacteria [6]. Strongylocin sequences were used for BLAST searches against the EST records of *S. purpuratus* in the GenBank, and eleven cDNAs similar to strongylocin 1 and seven cDNAs similar to strongylocin 2 (Table 2) were identified. From two coelomocyte cDNA libraries, two clones (accession numbers R61943.1 [17] and EC430627 [18]) were re-

**Table 2**  
SpStrongylocin matches from *S. purpuratus* ESTs<sup>a</sup>.

Sequences similar to strongylocin 1	E value	Sequences similar to strongylocin 2	E value
EC434986.1	0.0	EC436346	5e-07
EC430781.1	0.0	EC435792	8e-08
EC435495.1	0.0	EC436261	1e-07
EC435577.1	0.0	EC436113	1e-06
EC436002.1	0.0	EC435542	4e-07
EC436356.1	0.0	EC435655	4e-07
EC437492.1	0.0	<b>EC430627</b>	8e-08
EC429433.1	0.0		
EC436320.1	0.0		
R61957.1	5e-171		
<b>R61943.1</b>	6e-165		

<sup>a</sup> Searches were conducted by BLAST using the *S. droebachiensis* strongylocin sequences. The cDNAs listed in bold were re-sequenced and submitted as GU116566 and GU116567.

sequenced and submitted into the databank as GU116566 and GU116567 (Fig. 1). The cDNA of GU116566 contained 249 nt of coding region, 105 nt of 5' UTR and 293 nt of 3' UTR while GU116567 had 261 nt of coding region, 70 nt of 5' UTR and 333 nt of 3' UTR. The deduced amino acid sequences were called SpStrongylocins 1 and 2, respectively. *In silico* analyses by SignalP3.0 [25] and alignment of strongylocins and SpStrongylocins revealed that both SpStrongylocins 1 and 2 are composed of a pre-pro-region and a mature region of 48 and 52 amino acids, respectively. Interestingly, SpStrongylocins 1 and 2 share the same 22 amino acid signal peptide. Although the sequences of pro-regions of SpStrongylocins 1 and 2 were different, both had 13 amino acid residues with negative net charges. The mature region of SpStrongylocins 1 and 2 had six cysteines (Fig. 2). The alignment of amino acid sequences showed that SpStrongylocins 1 and 2 shared the same cysteine location pattern with strongylocins from the green sea urchin.

### 3.2. Expression, purification and activity of recombinant SpStrongylocins 1 and 2

SDS-PAGE analysis of the recombinant proteins showed that both SpStrongylocins 1 and 2 were present in the soluble fraction of the cell lysate and that both peptide bands matched the predicted molecular mass of 11 kDa (Fig. 3, lanes 2 and 6). Peptides purified on a Ni<sup>2+</sup> sepharose column had a molecular mass of 11 kDa, which was the expected size for the fusion peptide including the hexahistidine tags (Fig. 3, lanes 3 and 7). After cleavage by enterokinase, and removal of uncleaved peptides and cleaved tags by a second purification through the Ni<sup>2+</sup> sepharose column, the flow-through contained the mature peptides (Fig. 3, lanes 4 and 8). The mature recombinant SpStrongylocins 1 and 2 were resuspended in H<sub>2</sub>O and MIC assays were performed to investigate and quantify the antimicrobial activity. Results indicated that both recombinant peptides displayed a potent antibacterial activity against both Gram-positive and Gram-negative bacteria (Table 3). Both peptides showed activity against *E. coli* with a MIC value of 7.5 μM. *C. glutamicum* was slightly more susceptible to SpStrongylocin 2. The corresponding MIC values for SpStrongylocins 1 and 2 were 7.5 μM and 3.8 μM, respectively. The peptides showed MIC values of 15.0 μM against *L. anguillarum* and *S. aureus*. The control peptides, cecropins P1 and B, showed very potent activity against all bacteria, except as expected, there was no activity for cecropin P1 against *S. aureus*.

### 3.3. Mechanism of recombinant SpStrongylocins 1 and 2 antimicrobial activity

In order to examine whether the recombinant peptides inhibited bacterial growth by interference with membrane

## (A) SpStrongylocin 1 (GU116566)

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cggcacgaaggggctgtgttttttgagtgatctctcagtcagtagctgttcgcaagttt 60
ccttggaagttgttcaaagacaagtccattccaacccaatttcgaaatgggatctcaggag 120
                                     M D L R S 5
cgcatccttgggtcttcctgggtgtagtcatgggtgctatcatactccatggccgcacctct 180
A S L V F L V V V M V L S Y S M A A P L 25
tgatgcagataacgatgaagaaatggaggaaatcttcaactcgatctatcatcgcaaatg 240
D A D N D E E M E E I F N S I Y H R K C 45
cgtcgtaaaaaacagatgcaaaaccgtcagcggtcacaagacgtgcaaggacttaacatg 300
V V K N R C E T V S G H K T C K D L T C 65
ttgccgagccgtgatcttcaggcatgagcgccccgaagtatgccgaccatcaacctagac 360
C R A V I F R H E R P E V C R P S T 83
aggattcaccattcatgggaagatgaagctacccatctagagtgtatttagatcagtggc 420

tgaaacatggacctatacatgctgaaaccaatgctatactatgtcaaaccgtcgtatct 480
tgcgtatttcatttctcttttggtttttataatctttcagtgtttatattctgaaatttcg 540
aagatccctgaaatcaaacaactcaattttcttttgcaaaggcaataaacaataaatt 600

gatcattgtttatctttcttacatatacaaaactatacacccctttgta 648

```

## (B) SpStrongylocin 2 (GU116567)

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ccacgcgtccgcggttcgcaagttttcttaggagttgtccaaagactagttcaaacctact 60
caacttcaatatgggatctcagaagcgcacatccttgggtcttcctgggtgtagctatgggtgct 120
                                     M D L R S A S L V F L V V A M V L 17
atcatactccatgggtgaatctttcgaggatgatgatgccgtgatggaggaattatggaa 180
S Y S M A E S F E V D D A V M E E L W N 37
cccttttaggaagctctacagaaaggagtgtaacgacgtaacctcgtgacacccgctcag 240
P F R K L Y R K E C N D V T S C D T V S 57
cgggtggaagacgtgcacgaaaaaaattggtgtcatcgcaagttctttgggaagacaat 300
G V K T C T K K N C C H R K F F G K T I 77
cttaaaggcgcgccgaatgcaccgtcagtgaaaggctgaataccaatttctcgcagc 360
L K A P E C T V I S 87
tctggcaaatgaaccggcgctatgaggatgacagtggtttaccaagattataatcaatat 420

tattgcttcattatcttcggtacccttattattcaaaaaatgtaatttcttaagaatgc 480
acgaagcattaaagtagaaattaacatacgttggtgacaggggtactttttacagatcgtg 540
acatgatcatagaagtaagttctgttattaacagctgttttgcaagcggaaagtatattt 600

atcttcttgaaaacaacaagaacatccatattactacgaccaactaccagccccccc 660

tgta 664

```

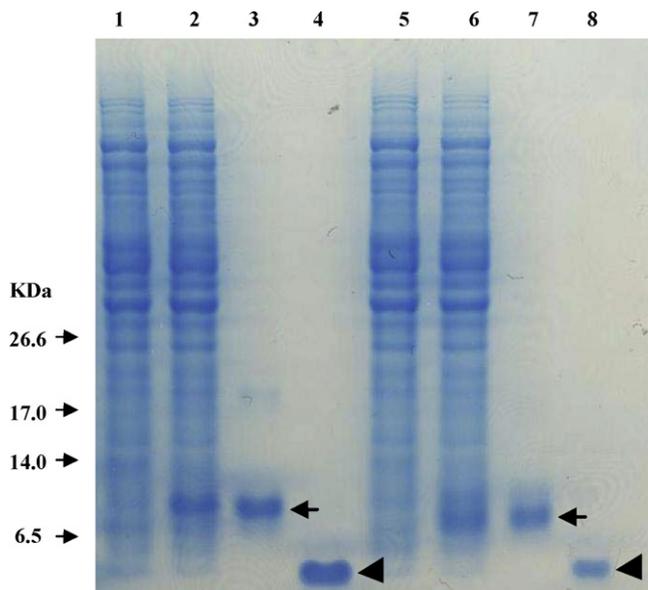
**Fig. 1.** The cDNA and deduced amino acid sequence of SpStrongylocins 1 and SpStrongylocin 2. (A) The cDNA sequence of SpStrongylocin 1 (GenBank accession number GU116566) and the deduced amino acid sequence. (B) The cDNA of SpStrongylocin 2 (GU116567) and the deduced amino acid. The start codon and stop codon are in bold font. The regions of the mature peptide are indicated with a single underline. The signal peptide regions are marked with double underlines. The numbers of nucleotides and amino acids are indicated to the right.

integrity or by affecting intracellular targets, a membrane integrity assay was performed. Mid-logarithmic phase *E. coli* cells (MC1061) expressing firefly luciferase were incubated in the presence of 2 mM D-luciferin at pH 7.4. Recombinant SpStrongylocins 1 and 2 were added to a final concentration of 7.5  $\mu$ M (corresponding to the MIC) and changes in light emission were monitored. PR-39, which has intracellular antimicrobial activity [26] and cecropin P1, which inserts pores into membranes [27,28], served as positive controls. Results showed that SpStrongylocins 1 and 2 did not

enhance the permeability of the membrane, as they were not different from the water control (Fig. 4A). In contrast, a strong peak of light emission was observed after addition of cecropin P1, which is typical for AMPs that disrupt the membrane. On the other hand, PR-39, which served as a non-membrane active control, did not induce a peak of light emission. In order to make sure that the peptides do not inhibit luciferase activity, cecropin P1 was added after 5 min of incubation to the reactions conducted with SpStrongylocin, PR-39 and water. Thereby the assay system was



**Fig. 2.** The alignment of recombinant peptides with native strongylocins from *S. droebachiensis*. (A) Alignment of recombinant SpStrongylocin 1 with native strongylocin 1. (B) Alignment of recombinant SpStrongylocin 2 with native strongylocin 2. In the alignment, the identical amino acids are highlighted in black and similar amino acids are shown in grey. The cysteines are identified with an asterisk above the alignment. The extra isoleucine in recombinant SpStrongylocin 2, which is introduced by the ligation independent cloning technique, is marked by a diamond marker (◆).



**Fig. 3.** Recombinant proteins expressed from pET30-EK/LIC-SpStrongylocin 1 and pET30-EK/LIC-SpStrongylocin 2. Soluble protein from uninduced *E. coli* C43 cells harboring the plasmid pET30-EK/LIC-SpStrongylocin 1 (lane 1) and pET30-EK/LIC-SpStrongylocin 2 (lane 5). Soluble protein from induced cells with pET30-EK/LIC-SpStrongylocin 1 (lane 2) and pET30-EK/LIC-SpStrongylocin 2 (lane 6) after 4 h of induction with IPTG (expressed fusion SpStrongylocin 1 MW = 10.4 kDa and fusion SpStrongylocin 2 MW = 10.9 kDa). Purified fusion SpStrongylocin 1 (lane 3) and SpStrongylocin 2 (lane 7) using Ni<sup>2+</sup> sepharose columns (arrows). Purified peptide SpStrongylocin 1 (lane 4) and SpStrongylocin 2 (lane 8) (SpStrongylocin 1 MW = 5.6 kDa; SpStrongylocin 2 MW = 6.0 kDa) after removal of the histidine tag using enterokinase (arrow heads).

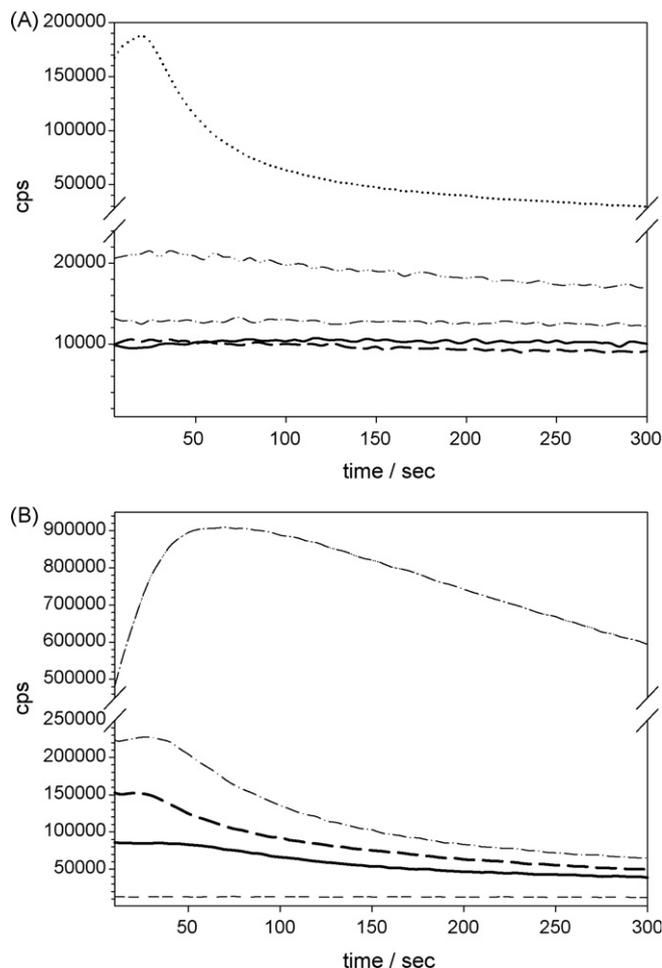
proven functional even after treatment with SpStrongylocins and PR-39. The resulting light peaks were comparable to the peaks of cecropin P1 alone, although the intensity of the peaks varied. The presence of SpStrongylocins 1 and 2 slightly reduced peak

**Table 3**  
Susceptibility of bacterial strains to the recombinant antibacterial peptide SpStrongylocins 1 and 2 from *S. purpuratus*.

Peptide	Minimal inhibitory concentration (μM) <sup>a</sup>			
	<i>L. anguillarum</i>	<i>E. coli</i>	<i>C. glutamicum</i>	<i>S. aureus</i>
Recombinant SpStrongylocin 1	15.0	7.5	7.5	15.0
Recombinant SpStrongylocin 2	15.0	7.5	3.8	15.0
Cecropin P1 <sup>b</sup>	0.8	0.8	0.4	100.0
Cecropin B <sup>b</sup>	0.4	0.4	0.4	1.6

<sup>a</sup> Minimal inhibitory concentration (MIC) was determined as the lowest concentration of peptide causing 100% of the growth inhibition of the test organism compared to the growth control without any peptide present.

<sup>b</sup> Cecropin P1 and cecropin B were used as control peptides.



**Fig. 4.** Effect of SpStrongylocin 1 and SpStrongylocin 2 on bacterial membrane integrity. Perforation of the plasma membrane causes an influx of externally added *D*-luciferin into luciferase expressing *E. coli* cells and results in light emission. Light emission kinetics of *E. coli* cells treated with either SpStrongylocin 1, SpStrongylocin 2 or one of the controls at *t* = 0 is plotted as a function of time for 5 min starting 20 s after peptide addition. The lag time is due to plate handling and shaking inside the multi-plate-reader and is therefore excluded from the graph. The background noise due to intrinsic leakage of *D*-luciferin across the membrane without addition of a substance is measured in advance of each experiment and is in average 8696 ± 537 counts per second (cps). (A) The light peak after cecropin P1 addition (1 μM, dotted line) is the result of membrane permeabilization due to activity of the membrane-active control peptide cecropin P1. Absence of such a distinct peak after addition of SpStrongylocin 1 (7.5 μM, dashed line) and SpStrongylocin 2 (7.5 μM, solid line) as well as water (dash-dotted line) and PR-39 (4 μM, dash-double-dotted line), which serve as controls, indicates an intact plasma membrane. (B) Subsequently, cecropin P1 is added to the membrane-inactive samples and light emission is followed for another 5 min to exclude direct effects of the SpStrongylocin peptides on assay function. The light peaks indicate that the membranes are still intact and that the membrane assay is not inhibited. A sample only treated with water serves as the negative control (double-dashed line). Note the change in scale compared to (A) which is as a result of strong peak intensity in the presence of PR-39.

intensity, while the presence of PR-39 induced a strong increase in peak intensity (Fig. 4 B). To further investigate the effect of SpStrongylocin on assay activity, polymyxin B, which also disrupts the membranes [15], was added in the same manner as described for cecropin P1. The activity of polymyxin B was very strong (more than 300,000 cps) in spite of the low concentrations used (5  $\mu$ M, 2  $\mu$ M and 1  $\mu$ M) and seemed independent of the presence of SpStrongylocin. Due to the strong and rapid activity of polymyxin B, we were not able to record the emission peaks of these experiments (data not shown).

#### 4. Discussion

Both SpStrongylocin peptides contain six cysteine residues which are likely to form intramolecular disulfide bridges. Such disulfide links are crucial for cysteine-rich peptides to stabilize their tertiary structure which provides protection from proteolysis during biosynthesis and when bacterial proteases are present. In addition, correct disulfide bonding facilitates the molecular folding and activity [29]. Linearized human neutrophil  $\alpha$ -defensins are less active against viral or bacterial targets than the native molecule [30,31]. The recombinant production of HNP-1 has no detectable antibacterial activity presumably because of improper disulfide bonding during the synthesis in bacteria [32]. There is no information about how disulfide bridges are arranged in this cysteine-rich peptides group. However, our recombinant peptides show activity against bacteria suggesting that at least some of the disulfide bonds of the recombinant SpStrongylocin peptides may resemble the normal structure in the native peptides.

Posttranslational modifications are known to be especially important for AMP activity and stability. For example bromination of tryptophan residues is found in many marine organisms. In the Atlantic hagfish, *Myxine glutinosa* [33] and the marine tunicate, *Styela clavata* [34], tryptophan bromination affects peptide activity and stability, respectively. In a previous study, we showed that strongylocin 2 from the green sea urchin contains a tryptophan residue which is likely to be brominated [6]. The deduced SpStrongylocin 2 sequence also contains tryptophan residue in the same position (Fig. 1B) although it is not known whether it is brominated. We assume that the recombinant SpStrongylocin 2 is not brominated, yet despite this, it elicits antimicrobial activity. Therefore the results suggest that posttranslational modifications may not be essential for activity. Interestingly, the tryptophan residue that is assumed to be brominated in strongylocin 2 is conserved in SpStrongylocin 2 while the overall similarity is only 40%. Therefore a similar posttranslational bromination may function in both peptides. In this context it is tempting to speculate that bromination of the conserved tryptophan residue of both peptides affects properties other than antimicrobial activity, such as to enhance stability.

The recombinant approach to produce large quantities of AMPs has been improved by many investigations, such as introducing different fusion tags for purification [32,35], carrier protein sequences for expressing small peptides [36–40] and an anionic pre-pro-region to neutralize the cationic charge of AMPs [41]. In this study, the recombinant peptides include a hexahistidine tag, an S-peptide fragment of RNase A tag (S•tag) and an enzymatic site which together introduce several anionic amino acids. Although these additions slightly neutralize the positive charges of the SpStrongylocin peptides, the recombinant expression by the *E. coli* strain BL21 (DE3) was not successful. However, when the *E. coli* BL21 (DE3) strain C43 was employed as expression host, we harvested sufficient amounts of peptide from the culture. This suggests that the use of toxic tolerant hosts is essential for recombinant expression of SpStrongylocin peptides at an elevated level to avoid toxic effects.

Although earlier studies suggested that AMPs affect bacteria mainly by disrupting membrane integrity, more recent observations suggest that some AMPs can translocate across the membrane and act on intracellular targets without affecting membrane structure or functions [5]. We determined that membrane pore formation is not the primary reason for the antibacterial activity of both SpStrongylocins 1 and 2. We cannot exclude that SpStrongylocins might interfere with membrane integrity at conditions different from our experimental setup or that pores are formed which only allow passage of molecules smaller than D-luciferin [15]. However, all peptides we have tested so far, which were previously described as membrane active, produced strong light peaks in our assay (cecropin P1, cecropin B, polymyxin B, data not shown). Although we have no direct evidence that the SpStrongylocin peptides directly affect membrane integrity, they may alter membrane properties such that the membrane is made less susceptible to the activities of cecropin P1 (Fig. 4B). However, the somewhat reduced peak intensity for cecropin P1 in presence of SpStrongylocin 2 (Fig. 4B) might as well be due to partial metabolic inhibition of the sensor bacteria and a resulting lack of ATP availability. In spite of that this second assay demonstrates that the membrane integrity assay is still functional in the presence of SpStrongylocins and therefore would result in light peaks if the membrane was perforated by the SpStrongylocins themselves. This strengthens the evidence that both SpStrongylocin peptides alone are able to inhibit bacterial growth without disrupting membranes. Therefore we propose that they have a mechanism of action different from peptides known to disrupt membranes in the same way as cecropin P1. Whether the targets are intracellular or important for the properties on the surface of the bacteria remains to be elucidated.

AMPs are well known as immune effector molecules which play an important role in marine invertebrate immunity [42]. Strongylocins are isolated from coelomocytes of *S. droebachiensis* [6,43], which are considered to mediate immune response comparable to hemocytes in other invertebrates. Although we lack information about the expression of strongylocins in *S. purpuratus*, the constant presence of strongylocins in circulating coelomocytes of *S. droebachiensis* suggests that these molecules may be involved in the first line of defence of the sea urchin immune system.

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