Antimicrobial peptides in sea urchins

Isolation, characterization and expression

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Abbreviations

AMP	Antimicrobial peptide
APD	Antimicrobial peptide database
BLAST	Basic local alignment search tool
CL	Cardiolipin
DNA	Deoxyribonucleic acid
EST	Expression sequence tag
GNBP	Gram negative binding protein
HBD	Human β-defensin
HNP	Human neutrophil peptide
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
NACHT	NAIP (neuronal apoptosis inhibitory protein), CIITA (MHC class II transcription activator), HET-E (incompatibility locus protein from *Podospora anserina*) and TP1 (telomerase-associated protein)
NLR	NOD like receptor
PAMP	Pathogen-associated molecular pattern
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PGRP	Peptidoglycan recognition protein
PMC	Primary mesenchyme cell
PRR	Pattern recognition receptor
PS	Phosphatidylserine
RNA	Ribonucleic acid
SM	Sphingomyelin
SMC	Secondary mesenchyme cell
SRCR	Scavenger receptor cysteine rich
TIR	TLR/interlukin-1 receptor
TLR	Toll-like receptor
tPMP	Thrombin-induced platelet microbicidal proteins

Abstract

Echinoderms are interesting animals for biodiscovery/bioprospecting as sources for novel compounds that have interesting activities. These compounds might be developed into potential drug candidates. In this work, two novel families of short proteins, referred to as antimicrobial peptides (AMPs) were isolated and characterized from the sea urchin, *Strongylocentrotus droebachiensis*. One of the functions of AMPs is that they act as antibiotics and thus have the ability to inhibit the growth of bacteria and other microorganisms. Even though sea urchins are evolutionary old animals, they have spikes and a hard shell that protect the internal organs suspended in coelomic fluid (blood). However, little is known about the functions and importance of the AMPs in the defence system of cells in adult sea urchins and larva.

The fist group of AMPs that were detected were the cysteine-rich peptides, named strongylocins, isolated from extracts of the coelomocytes (blood cells). They have a novel cysteine arrangement pattern when compared to other peptides. The mature strongylocins have activity against both Gram-positive and Gram-negative bacteria. The amino acid sequences for the precursor strongylocins contain a signal peptide, a prosequence and a native region. Their gene sequences indicate that strongylocins (except strongylocin 1b) have three introns and four exons. Similar genes were found in the sister species, *S. purpuratus.* Recombinant products of these genes inhibited growth of bacteria by a nonlytic, but presumably intracellular mechanism.

The other group of AMPs, named centrocins, are composed of two chains, one heavy chain and one light chain. The native peptides were very potent against bacteria. The gene sequences of centrocins also code for a signal peptide, two prosequences and a native region and contain one intron and two exons. A synthetic heavy chain had anti-fungi and anti-yeast activities in addition to being active against bacteria.

Of the four types of coelomocytes, strongylocin 1 was detected in phagocytes and the vibratile/colourless spherule cells, strongylocin 2 was found in phagocytes and red spherule cells while centrocin 1 was found only in phagocytes and was located in the granular vesicles of the cells.

Both the peptide families were detected in the important pluteus developmental stage (mid and late stage) of the sea urchin larvae. The distribution of centrocin 1 in the secondary mesenchyme cells around coelomic pouches and in some arms implies the importance of these molecules for immunity.

All together, this work gives novel insight to molecules that are believed to be important for protecting the animals against microbial infections. These molecules are regarded as important players in the host defence systems of both adult animals and larva.

中文摘要（Chinese abstract）

海洋生物作为丰富的天然资源库，正日渐成为人们寻找和发现新药物的重要来源之一。其中，棘皮动物已开始成为热点研究对象。本研究首次在海胆中寻找到抗菌肽，并且鉴定出两个新的抗菌肽家族。

第一类抗菌肽家族是从挪威绿海胆（*Strongylocentrotus droebachiensis*）体腔细胞中纯化提取的富半胱氨酸多肽，命名为 strongylocins。其氨基酸序列中具有独特的半胱氨酸分布模式。这类多肽包含有信号肽，前导序列和成熟多肽片断，其基因由四个外显子和三个内含子构成（strongylocin 1b 除外）。纯化的天然多肽具有广谱抗革兰氏阳性菌和革兰氏阴性菌的特性；另外重组表达来源于北美紫海胆（*S. purpuratus*）的多肽 SpStrongylocins 同样显示出抗菌活性。通过免疫细胞化学的方法，发现 strongylocin 1 在绿海胆的吞噬细胞，颤动细胞和无色圆细胞中表达；而 strongylocin 2 在吞噬细胞和红色圆细胞中表达。另外本研究比较了 Strongylocins 在绿海胆不同发育阶段的表达情况，发现 strongylocins 最初在长腕幼虫的早期阶段开始表达。

第二类抗菌肽家族是从挪威绿海胆体腔细胞中纯化提取的具有异二聚体结构的多肽，命名为 centrocins。Centrocins 家族同样包含有信号肽，前导序列和成熟多肽片断（一条长链和一条短链），其基因序列包含两个外显子和一个内含子。Centrocins 具有广谱的抗革兰氏阳性菌和阴性菌的能力；同时化学合成的长链多肽序列还具有抗真菌活性。Centrocin 1 表达于成体吞噬细胞中，并储存在颗粒小泡中。这些包含 centrocin 1 的颗粒小泡参与形成吞噬溶酶体，帮助清除吞噬进胞内的细菌。通过比较 centrocin 1 在海胆不同发育阶段的表达情况，发现 centrocin 1 在长腕幼虫的中期阶段表达，并且主要表达在幼虫的后期间叶细胞。这些细胞分布在早期消化道周围的腔囊以及部分长腕中。

综上所述，本研究首次从海胆中提取出两类新型抗菌肽，并对其分子特性，抗菌谱和细胞毒性作了研究。其广谱抗菌活性和低毒性表明 strongylocins 和 centrocins 可以应用于开发新的药物。同时，本研究证明了抗菌肽在海胆成体和幼虫的免疫系统中具有非常重要的作用。

List of papers

I. Chun Li, Tor Haug, Olaf B. Styrvold, Trond Ø. Jørgensen and Klara Stensvåg

Strongylocins, novel antimicrobial peptides from the green sea urchin, *Strongylocentrotus droebachiensis*. Developmental and Comparative Immunology 2008;32:1430-1440.

II. Chun Li, Hans-Matti Blencke, L. Courtney Smith, Matti T. Karp, and Klara Stensvåg

Two recombinant peptides, SpStrongylocin 1 and 2, from *Strongylocentrotus purpuratus*, show antimicrobial activity against Gram-positive and Gram-negative bacteria. (In press, Developmental and Comparative Immunology DOI: 10.1016/j.dci.2009.10.006).

III. Chun Li, Tor Haug, Morten K. Moe, Olaf B. Styrvold, and Klara Stensvåg

Centrocins: isolation and characterization of novel dimeric antimicrobial peptides from the green sea urchin, *Strongylocentrotus droebachiensis* (Manuscript).

IV. Chun Li, Hans-Matti Blencke, Tor Haug, Øyvind Jørgensen and Klara Stensvåg

Expression of antimicrobial peptides in coelomocytes and embryos of the green sea urchin, *Strongylocentrotus droebachiensis*. (Manuscript).

Introduction

Microbial life extends into every niche of our natural environment. We live in harmony with most microorganisms in our daily life. However, some microorganisms are pathogenic and cause problems both for our health and for our farming products. For instance, estimations of the economic losses in the finfish aquaculture suggest that Japan lost at least US$ 16.4 million due to diseases in 2004 alone [1]. Infectious diseases also threaten hard-won gains in health and life expectancy of human beings. It is estimated that 10% of worldwide mortality in the year 2007 was caused by only a number of severe infectious diseases [2]. In addition, infectious diseases appear to be emerging more quickly than ever before. Since the 1970s, newly emerging diseases have been identified at an unprecedented rate of one or more per year. Today there are nearly 40 infectious diseases that were unknown only a generation ago [3]. Another serious threat is the development of antibiotic resistance among these pathogens as a result of use and misuse. Since the first antibiotic, penicillin, was discovered in 1928, many antibiotics have been developed such as tetracycline, kanamycin and streptomycin etc. However, their wide application for treatment of infectious diseases both in humans and animals resulted in the development of resistance. Therefore the control of today's disease outbreaks by the use of conventional antibiotics remains difficult. The need for replacements or supplements to the conventional antibiotics will inevitably spur the search for novel antimicrobial drugs. Recently, the development of purification and characterization approaches has prompted the discovery of antimicrobial drugs from natural products.

1. Marine bioprospecting and new antimicrobial candidates

Natural products are naturally derived metabolites and/or by-products from microorganisms, plants, or animals [4]. Humans have exploited and used natural products as medicine for thousands of years. These natural-product-based compounds have had an

immense impact on modern medicine and about 40% of prescription drugs are based on them [5]. The process of searching for, isolation and characterization of novel natural products with commercial potential is generally called bioprospecting and plays an important role in drug discovery. Figure 1 gives a brief overview of typical steps in bioprospecting. Basically, the conventional method focuses on purification of the active extracts/compounds from organisms and a subsequent identification of the hits. Such information (i.e. molecular formula or amino acid sequences) aids to chemically synthesize the candidate molecules for further characterization. Alternatively, developments in the field of biotechnology provide a new strategy to discover interesting molecules. A wealth of genomic and metagenomic data is available for BLAST search thereby allowing for *in silico* screening of databases for peptide/protein sequences which are homologous to previously known active molecules. Those putative molecules may either be produced by bioengineering or by chemical synthesis.

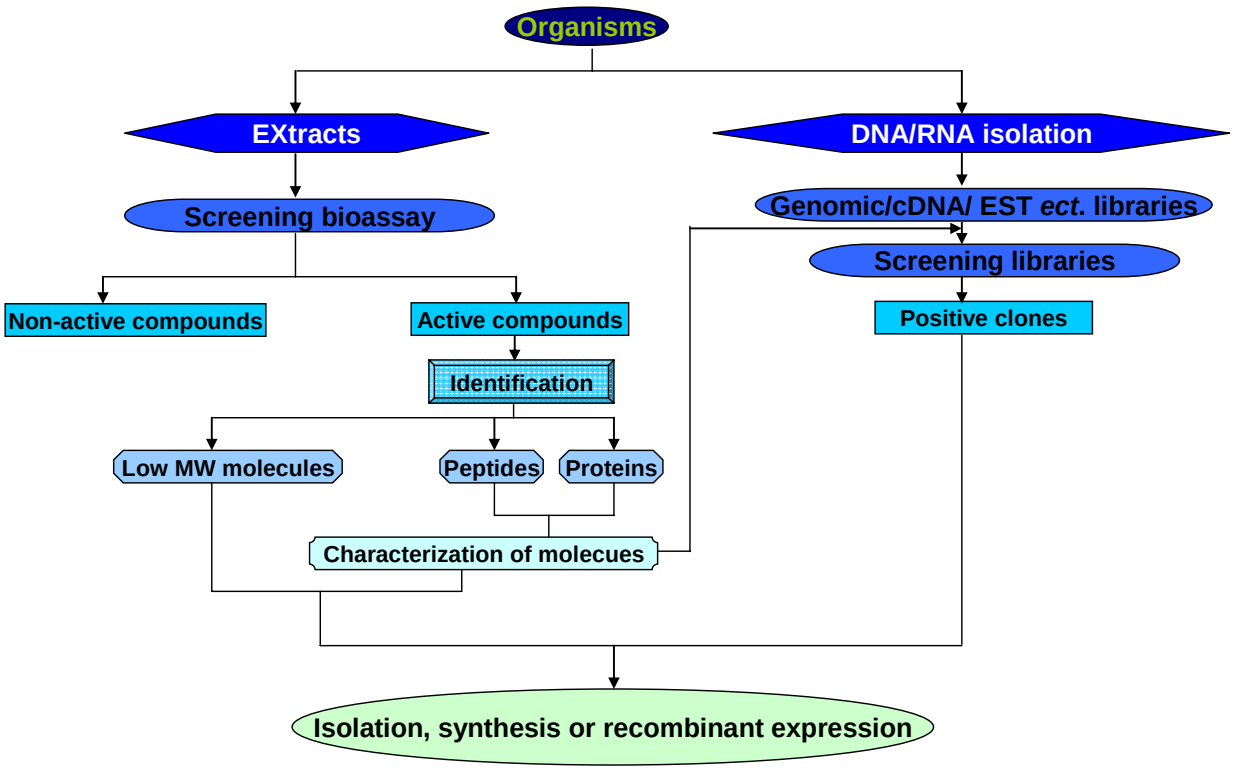

Figure 1 Brief flowchart of research on discovering bioactive molecules from organisms.

The traditional drug sources, especially terrestrial plants and microbes, have been examined extensively throughout the last century. The search for novel antimicrobial compounds heavily relies on complicated and time consuming programs [6]. Although the developments in analytical technology such as spectroscopy and high-throughput screening successfully aid to discover new natural products, these failed to deliver new drug leads in significant numbers during the past two decades [7, 8]. Oceans cover about 71% of the surface of the earth and contain approximately half of the total global biodiversity [9]. Bioprospecting activities in the marine environment have only begun; therefore, the oceans might turn out to be the new "Klondike" for bioprospectors around the world.

There are remarkable high hit rates during screening of marine compounds for potential drug leads. In 2007, it was reported that about 620 active compounds were isolated from marine microorganisms and phytoplankton, green algae, brown algae, red algae, sponges, cnidarians, bryozoans, molluscs, tunicates, echinoderms and true mangrove plants [10]. Marine bioprospecting has resulted in the discovery of an enormous variety of natural compounds with antifungal, antiviral, antitumor, anti-inflammatory and antibacterial functions. For example, compounds isolated from algae and sea sponges exhibit antifungal activity [11, 12], extracts and compounds isolated from algae exhibit antiviral activity [13-15], and compounds from sea sponges and soft corals show anti-inflammatory effects [16, 17]. In addition, Sandsdalen and Haug *et al*. isolated a compound from the brown alga [18] and several extracts from crustaceans [19], echinoderms [20], and shellfish [21] which show antibacterial activity. Among these marine natural products, antimicrobial peptides (AMPs) have attracted considerable interest due to their unique characteristics and promising bio-functions, for example arasin 1 [22], crustins [23] and hyastatin 1 [24] isolated from *Hyas araneus*.

2. Antimicrobial peptides

In 1956 Hirsch purified antimicrobial substances from phagocytic granule extracts [25]. But it was not until 1963, when enzymatic antimicrobial proteins from leukocytes were isolated [26], that research on AMPs really started. AMPs are evolutionarily conserved small molecular weight proteins of the innate immune responses, with a broad spectrum of antimicrobial activities against bacteria, viruses, and fungi. By October 2008, more than 1,200 AMPs had been discovered [27]. Although some peptides have anticancer and antiviral activities, most of the peptides seem to be antibacterial and antifungal (Table 1). AMPs widely appear throughout all three domains of life from unicellular to multicellular organisms [27-29].

Table 1 In total, there are 1518 antimicrobial peptides in the Antimicrobial Peptide Database (APD; http://aps.unmc.edu/AP/main.php).

Activity of peptides	Number of peptides	% in database
Anti-bacteria	**1168**	**76.94 %**
Anti-fungi	**442**	**29.211 %**
Anti-virus	**98**	**6.45 %**
Anti-cancer	**99**	**6.52 %**

2.1 The characteristics of AMPs

AMPs are characterized as having short amino acid sequences, usually less than 100 amino acids. The sequences usually contain many positive charged residues such as arginine and lysine, which help to form the net cationic AMPs. According to the Antimicrobial Peptide Database statistical information, the average length of all 1518 peptides is around 30 residues and the average net charge of them is 3.8 (http://aps.unmc.edu/AP/main.php). Nearly all antimicrobial peptides form amphipathic structures which reflect the relative abundance and polarization of hydrophobic and hydrophilic domains within a protein. The hydrophobicity enables water-soluble antimicrobial peptides to interact with the hydrophopic lipid bilayer of the membrane.

2.2 The diversity of AMPs

Although the research on antimicrobial peptides emerged only decades ago, hundreds of natural and synthetic AMPs have been discovered. Different standards have been applied to group peptides based on their amino acid composition, the target pathogens, or the conformational structure. Here antimicrobial peptides are classified into five groups according to their structure and composition since the function of AMPs is mainly determined by these two factors [30].

A) α-Helical AMPs

The group of α-helical AMPs is very diverse. The alpha-helix assists peptides to form an amphipathic structure. Well known examples for α-helical structure are magainins, cecropins and LL-37. Magainins (Figure 2A), isolated from the skin secretion of *Xenopus laevi* [31], have been shown to have α-helical structures when interacting with acidic phospholipid bilayers. This is supported by circular dichroism, Raman spectrosopy, Fourier transform infrared, and solid-state nuclear magnetic resonance studies (reviewed by [32]).

B) β-Sheet AMPs

Many AMPs contain a β-sheet structure and their conformational structure is commonly stabilized by one or two disulfide bridges. Polyphemusins (Figure 2B) and tachyplesins from horseshoe crab are composed of 17 to 18 residues. They adopt a conformation consisting of an anti-parallel beta-sheet connected by a beta-turn and two disulfide linkages. All five hydrophobic side groups are localized on one side of the molecule while the cationic side groups are facing the other side. This amphipathic structure is likely to favor the bactericidal activity [33, 34].

C) Cysteine-rich AMPs

Cysteine-rich AMPs have been found in vertebrates, invertebrates and plants. They contain six or more cysteine residues which form multiple disulfide linkages. The defensin

family contains well studied cysteine-rich AMPs. The defensins are divided into three types according to their structural difference: α-defensins, β-defensins and θ-defensins (Figure 2C). While the six cysteines of α-defensins are connected in a 1-6, 2-4, 3-5 pattern, the pattern of β-defensins is1-5, 2-4, 3-6. The structure of θ-defensins is circular without a free N- or C-terminus [35, 36].

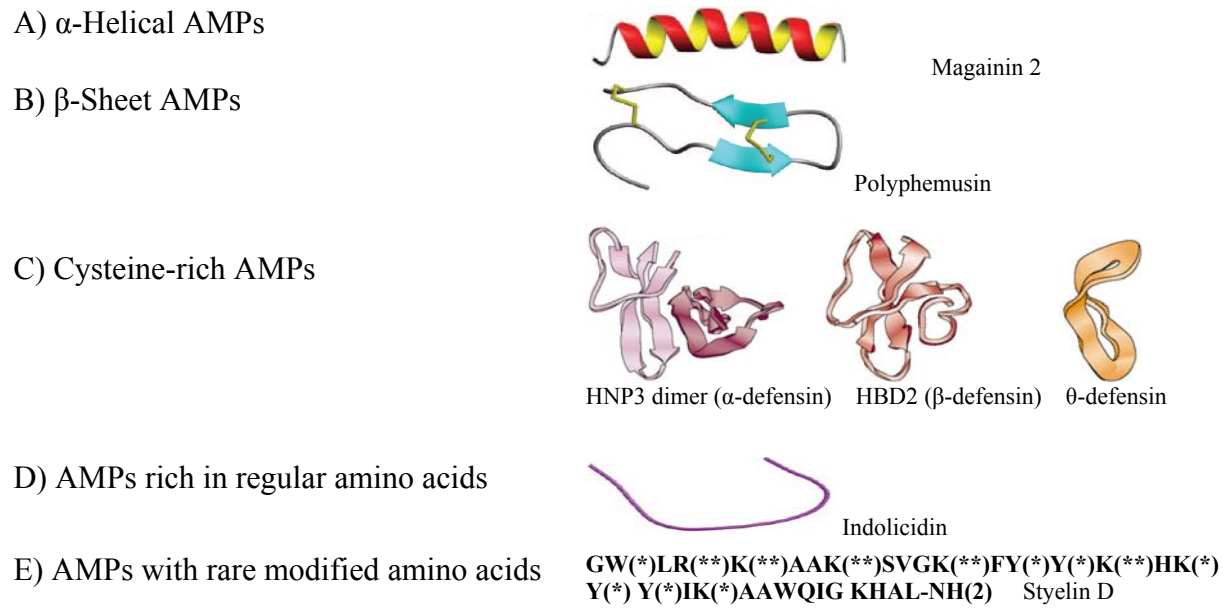

Figure 2 Classes of antimicrobial peptides. A) α-Helical AMPs, magainin 2 [37]; B) β-Sheet AMPs, polyphemusin [38]; C) Cysteine-rich AMPs, cartoon structures of defensins [35]. D) AMPs rich in regular amino acids, indolicidin [39]; E) AMPs with rare modified amino acids, styelin D where W (*) is 6-bromotryptophan, R(**) is dihydroxyarginine, Y(*) is 3,4-dihydroxyphenylalanine, K(*) is 5-hydroxylysine, and K(**) is dihydroxylysine [40].

D) AMPs rich in regular amino acids

Some AMPs are composed of high numbers of regular amino acids and have different structural conformations from the regular α-helical or β-sheet peptides. An example is indolicidin (Figure 2D) which is rich in tryptophan [39].

E) AMPs with rare modified amino acids

Few peptides contain rare modified amino acids. Styelin D (Figure 2E), isolated from hemocytes of the solitary ascidian, *Styela clava*, has remarkably extensive post-translational

modifications. It contains several modified amino acids like dihydroxyarginine, dihydroxylysine, 6-bromotryptophan and 3, 4-dihydroxyphenylalanine [40].

2.3 The mechanisms of antimicrobial activity

AMPs have the surprising but clearly fundamental ability to distinguish the differences between targets and to carry out their antibacterial function by either directly damaging the pathogens membrane or by passing through to reach intracellular targets. The main component of all biomembranes is the phospholipid bilayer. However, the composition of prokaryotic and eukaryotic cell membranes differs considerably and is influenced by cell energetics. The membranes of bacterial pathogens are found to be predominantly composed of phosphatidylglycerol (PG), cardiolipin (CL), or phosphatidylserine (PS) which all have a negative net charge. In contrast, the mammalian cytoplasmic membranes contain the zwitterionic phosphatidylcholine (PC), phosphatidylethanolamine (PE), or sphingomyelin (SM) which are normally neutral in net charge [41]. These characteristic differences of membranes between prokaryotic and eukaryotic cells suggest that the net charges are very important for AMP activity against microbes. In addition, the variation in sterol content between mammalian and fungal cell membranes is likely to further distinguish the targets for antimicrobial peptides. [42].

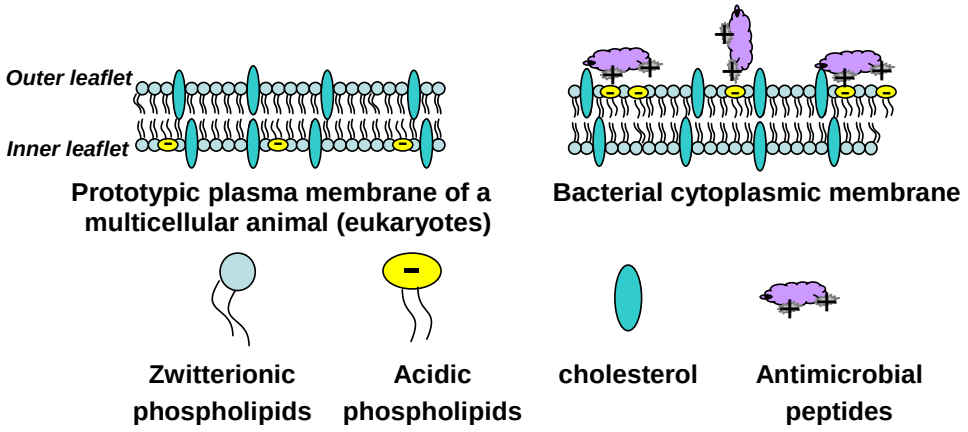

Figure 3 The membrane target of antimicrobial peptides of multicellular organisms and the basis of specificity. (Modified from [28])

The charged phospholipids of prokaryotic and mammalian membranes are not evenly distributed in the membrane layers (see Figure 3). The outer cytoplasmic membrane leaflet of bacteria is heavily populated by lipids with negatively charged phospholipid headgroups. In contrast, the outer leaflet of the membranes of plants and animals is composed of lipids with no net charge; most of the lipids with negatively charged headgroups are segregated into the inner leaflet [28]. For instance, PC or SM is generally the most abundant erythrocyte membrane phospholipid in mammals [43-45].

AMPs are likely to be attracted by and attach to negative charges on the surface of bacteria. The mechanism of passing the cell wall structures of Gram-negative and Gram-positive bacteria has rarely been addressed and is therefore not yet understood [46]. However, once the peptides get in contact with the outer leaflet of the cell membrane and the peptide/lipid ratio increases, the peptides start forming multimers or self-associating on top of the membrane [47]. At sufficiently high concentrations the peptides orientate perpendicularly and insert into the bilayer, thereby interfering with membrane integrity. Three models have been proposed to explain how AMPs damage cell membranes by interacting with the lipid bilayer. These models are based on the investigations on alaminthin, magainin 2, melittin, cecropin and other membrane active peptides.

The first is called the 'barrel-stave model' (Figure 4A). With increasing peptide/lipid ratios on the surface of the outer layer, transition of the peptides to an α -helical conformation occurs, which favors the hydrophobic regions to face the heads of the phospholipids, pushing them aside and thinning the bilayer. This process is likely to be driven by interactions between the peptides under aggregation and multimerization. The peptides insert into the membrane and form a barrel-like pore, where the hydrophobic regions face the lipid core of the membrane and the hydrophilic regions form the pore lining. This model is based on

investigations of the AMP alamethicin by X-ray scattering data [48, 49], neutron scattering [47] and oriented circular dichroism studies [47, 50].

The second is called the 'toroidal model'. Peptides acting in accordance to this model are thought to interact with the lipids on the target membrane, acquiring an α-helical conformation and shaping a complex, which induces the formation of membrane pores. However, there is a significant difference compared to the 'barrel-stave' model. Instead of simply integrating into the membrane, the outer lipid layer bends to the inside and forms a continuous top-to-bottom spanning connection with only lipid head groups and peptides facing the pore lumen. This bending is caused by the peptide solely interacting with the lipid head groups (Figure 4B). This model is suggested for the pore-formation by magainins [51-54], melittin [47, 55] and protegrin-1 [56] which all form α-helix structures when interacting with the membrane.

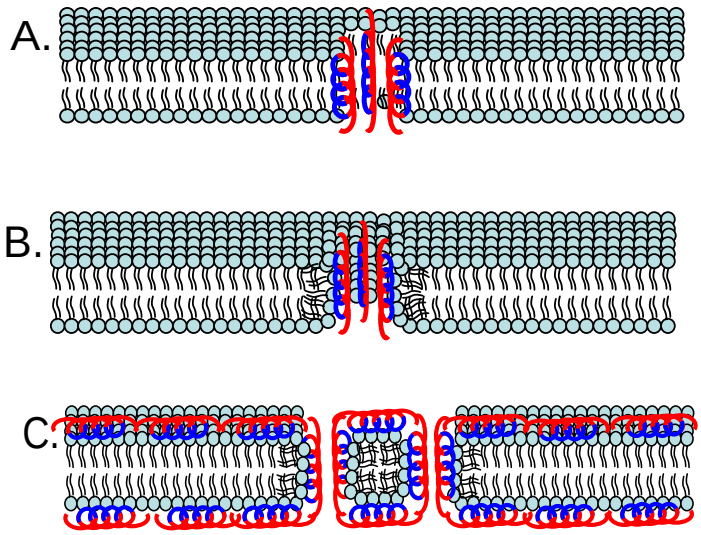

Figure 4 The models of antimicrobial-peptide-induced killing. A) The barrel-stave model, B) The toroidal model, C) The carpet model. (Modified from [46])

The third is called the 'carpet model'. This model differs considerably from both models mentioned above. Instead of pore formation, the peptides are supposed to break up the membrane in a detergent-like manner (Figure 4C). This detergent-like activity has been

documented for cecropins [57-59], caerin 1.1 [60] and melittin [61]. In this model, the peptides accumulate on the surface of the membrane orientated in parallel to the membrane. The peptides are electrochemically attracted by the phospholipid head groups and cover the surface of the membrane in a carpet like manner. When the peptide level reaches a critical concentration, phospholipids are displaced, and membrane fluidity changes and as a result the membrane integrity is reduced. This leads to micelle formation and a subsequently membrane disruption [41, 62].

The cytoplasmic membrane resembles one of the most important structures in prokaryotic cells as almost all bacterial metabolic processes depend on an intact plasma membrane [41]. Therefore it is not astonishing that the majority of AMPs seem to target the plasma membrane, thereby efficiently killing pathogenic bacteria. However, new paradigms imply that pore-forming is not the only mechanism of antimicrobial activity of peptides. Some peptides are also able to interact with intracellular targets without disrupting the membrane integrity. Intracellular targets of antimicrobial peptides vary from nucleic acids to enzymatic proteins. For example buforin II strongly binds both DNA and RNA after penetrating the cell membrane [63]. DNA and/or RNA synthesis is inhibited when bacterial cells are incubated in presence of thrombin-induced platelet microbicidal proteins (tPMP) for more than 30 minutes [64]. Similarly, experimental data indicate that microcin B17 is able to inhibit DNA replication in *Escherichia coli* by targeting gyrase [65]. PR-39, a proline-arginine-rich neutrophil peptide, is likely to kill bacteria by a mechanism that stops protein synthesis and DNA replication and results in degradation of these components [66]. The antifungal peptides, histatins, bind to a receptor on the fungal cell membrane and enter the cytoplasm where they attack the mitochondrion and induce the non-lytic loss of ATP from actively respiring cells [67]. To sum up, peptides penetrating the cytoplasmic membrane are able to carry out many different functions eventually resulting in cell death. Examples of such functions are

inhibition of enzymatic activity, inhibition of DNA, RNA and protein synthesis, inhibition of cell-wall synthesis, binding DNA molecules, etc (Figure 5).

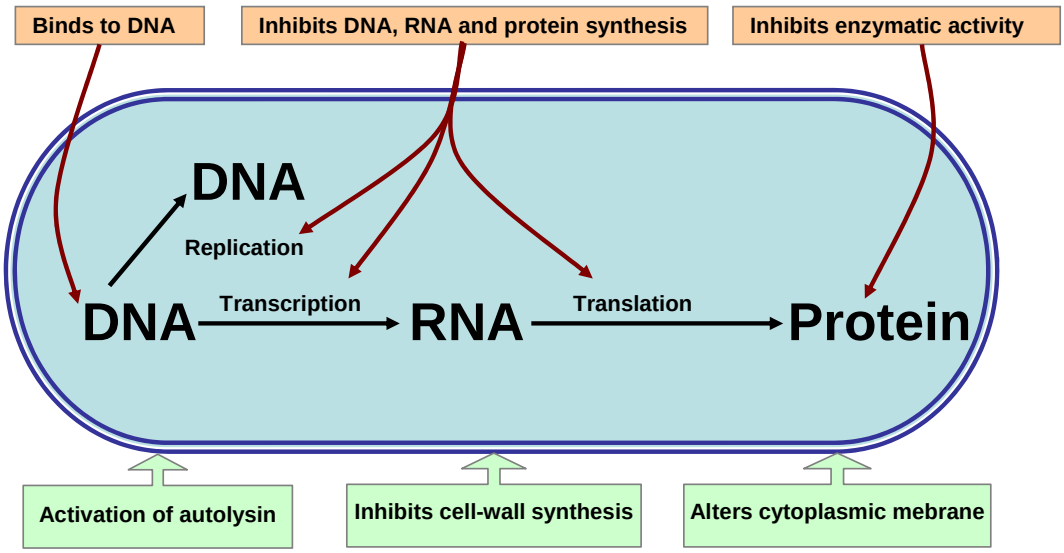

Figure 5 Mode of action for intracellular antimicrobial peptide activity. In this figure *E. coli* is shown as the target microorganism (Modified from [46]).

2.4 Production of AMPs

Although more than one thousand AMPs have been discovered, there still remain a lot to learn about AMPs. One of the biggest challenges when studying AMPs is to obtain enough peptide. In order to obtain large amounts of these peptides for different activity assays, three approaches can be employed: direct isolation of peptides from natural sources, chemical synthesis or recombinant expression of peptides in transgenic organisms. Although most AMPs are produced in their host organisms, the direct recovery of AMPs from host species is not economically feasible and might result in environmental issues. This applies especially for peptides isolated from species that occur in low numbers. Chemical synthesis of short amino acid sequences is economically viable. However, synthesis of sequences with more than 10 amino acids in length will become expensive. If the sequence of a peptide contains more than two cysteine residues for disulfide bridges, production costs will increase. The disulfide

bridges likely result in difficulties during synthesis. Therefore a recombinant expression system might be the most cost-efficient method for large-scale production of peptides.

Production of recombinant AMPs benefits from experiences in recombinant expression of proteins. Production of proteins, whether for biochemical analysis, therapeutics or structural studies, requires the success of three individual factors: expression, solubility and purification [68]. Although host organisms such as *Saccharomyces cerevisiae* [69], insect cells [70], mammalian cells [71], and even plants [72] have been used to express peptides, *E. coli* still remains a popular choice as the host organism for recombinant AMP production if no refolding and post-translation modification are required to restore protein activity [73-79].

The toxicity of AMPs to microorganism requires that the hosts are able to tolerate the toxic peptides and/or the toxicity of the recombinant peptides is masked. In order to successfully express toxic proteins, Miroux and Walker described two new mutant strains of *E. coli* BL21 (DE3) [80] which are frequently used to overcome the toxicity associated with overexpressing recombinant proteins [81-86]. In addition, strategies to cover the toxicity of AMPs were employed including the introduction of an anionic preproregion to neutralize the cationic charge of AMPs [79, 87] or tandem repeats of an acidic peptide-antimicrobial peptide fusion [88]. Another method uses other fusion carrier protein such as glutathione G-transferase [76, 77], *Pseudomonas aeruginosa* outer membrane protein, *Staphylococcus aureus* protein A, the duplicated IgG-binding domains of protein A [77], thioredoxin A [74], the green fluorescent protein [89, 90], bovine prochymosin [73] and the truncated protein PurF fragment F4 [91]. The following peptides have been produced using the methods described above: LL-37 [76], lactoferrincin [87], human neutrophil peptide 1 (HNP-1), cecropin-melittin hybrid [77], bombinin, indolicidin, melittin, tachyplesin I [91], sarcotoxin IA [89], designated P2 [73], humand β-defensin 5 (HBD5) and 6 [74].

Although the AMPs mentioned above have been produced by different recombinant expression systems, unfortunately, there exists no general protocol to express antimicrobial peptides yet since every peptide is different. Furthermore, a lot of experiments have been carried out in order to improve the expression conditions such as changing the expression host [80], reducing temperature [92], and modifying induction conditions [93] aiming to obtain a soluble recombinant product. This makes the recombinant expression an art of its own.

3. Immunity

Host defence mechanisms are classified into two types: adaptive immunity and innate immunity. The innate immunity forms the first defence line against pathogens. It is always present and ready to block the entry of microbes and to rapidly eliminate microbes that do succeed in entering host tissues. All jawed vertebrates, beginning with cartilaginous fish, not only have innate immune systems but also have evolved an adaptive immune system [94].

3.1 Adaptive immunity

Adaptive immunity is stimulated by microbes that invade tissues and adapts to the presence of microbial invaders [95]. The diversity of the antigen receptor from the lymphocytes repertoire results in the adaptive immune system possessing a remarkable specificity to millions of different antigens or epitopes. The adaptive immune system, owning memory of prior exposure to antigens, is able to mount larger and more effective responses to repeated pathogenic challenges. The adaptive immunity is composed of humoral immunity and cell-mediated immunity. Humoral immunity is mediated by antibodies, produced by B lymphocytes while cell-mediated immunity is mediated by T lymphocytes. Without question, adaptive immunity plays a crucial role in the vertebrate's immune system. However, in vertebrates the innate immunity is still involved in immune defence and provides the 'second signals' for adaptive immunity. Due to the fact that invertebrates like sea urchins lack an adaptive immune system [96-98], the focus of this work is innate immunity.

3.2 Innate immunity

The innate immune system consists of epithelial cells serving as barriers, cells in circulation and cells in tissues, as well as a number of plasma proteins. A continuous epithelia

forms a strong physical barrier to block the entry of pathogens into the host. In addition to production of AMPs to form an additional chemical barrier against infection [99]. AMPs synthesis is either constitutive or inducible by pathogens or cytokines. In addition, the stimulus-dependent degranulation of granulocytes enables to enhance the AMPs level. Once a pathogen breaches the protective barrier provided by epithelia, phagocytes are the first cell type to respond to this infection. They first ingest and then kill microbes, secrete molecules and recruit other immune cells [100-102]. The recruitment is done by specific soluble proteins called cytokines which enable the communication between immune cells. Therefore cytokines are crucial for a coordinated immune response involving both innate and adaptive immunity. Another important group of proteins involved in immune cell-communication is called the complement system. It consists of membrane-associated proteins which serve as chemoattractants. The complement system works by lysing and/or opsonising microbes and thereby promoting phagocytosis of the antigens.

The complement system is activated by three different pathways called the alternative, the classical and the lectin pathway [103]. The alternative pathway is triggered when a breakdown product of complement component 3 (C3) is deposited on the surface of a microbe. The classical pathway is triggered when antibodies are bound to microbes or other antigens. When this happens, the complex elicits to a breakdown product of C3 hydrolysis and other complement components. Thus it is a component of the humoral arm of adaptive immunity. The lectin pathway is activated when the plasma protein mannose-binding lectin binds to terminal mannose residues on the surface glycoproteins of microbes. Then the complex activates to the C3 hydrolysis and other complement components as well. Hence, all three pathways are initiated differently, but they share the common step of activating the central component C3 and therefore perform the same effector functions through polymerization of the membrane attack complex, poly-C9.

The receptors of the innate immune system that recognise antigens are not as specific as the receptors of the adaptive immune system. Thus they recognize structures shared by different pathogenic microbes, called pathogen-associated molecular patterns (PAMPs). For instance, Toll-like receptor 2 (TLR2) recognizes lipoproteins from Gram-negative bacteria, mycoplasms and spirochetes [104-108] as well as peptidoglycan and lipoteichoic acid from Gram-positive bacteria [109-112]. TLR3 recognizes double-stranded RNA [113]. TLR4 is able to recognize lipopolysaccharide (LPS) in association with the molecule CD14 [114, 115]. Flagellin, which is the protein subunit of flagella in pathogenic bacteria, can be detected by TLR5 [116]. TLR9 is essential for recognition of CpG DNA [117]. In addition to the TLR family, there are also many Non-Toll-like pattern-recognition receptors (PRRs). The mammalian intracellular Nod-like receptors (NLRs) are a family of sensors of intracellularly encountered microbial motifs and danger signals [118]. Like the mammalian NLR, plant nucleotide-binding site-leucine-rich repeat receptors detect pathogen-associated proteins, most often the effector molecules of pathogens responsible for virulence [119].

3.3 AMPs in the host defence system

AMPs seem to have at least two main functions. Not only do they inactivate bacteria *in vivo* and *in vitro*, thereby protecting host organisms against a wide variety of infections, but they also modulate immunity [28, 120, 121]. Numerous studies have documented that AMPs show a wide range of activities, such as antibacterial, antifungal, antiviral and even antitumor activities (reviewed by [122]). They are either expressed constitutively or the expression is induced by exposure to pathogens. AMPs have been identified both in plasma and in cells and their distribution in the host can be site-specific or systemic. It is worth mentioning that a few AMPs play a role as anti-endotoxins such as LPS [123, 124] and chemokins [125, 126]. AMPs can also induce production of cytokines and chemokines [127]. These

immunomodulatory functions do not directly kill microbes, however, recruitment and activation of immune cells and molecules improves the host defence system. Hence, AMPs are often referred to as host defence peptides [121, 128].

4. Immune system of sea urchins

The phylum Echinodermata (sea urchins, sea stars, sea cucumbers and others) lacks both immune memory and specificity in immune recognition which are characteristic for the adaptive immunity of jawed vertebrates. However by allograft rejection investigations, it was demonstrated that a few echinoderm species are able to differentiate self from non-self tissues [129, 130]. For many years, the immune system of echinoderms was considered as a simple form of innate immunity. More recent investigations on echinodermic immunity [131-135] and the publication of the purple sea urchin (*Strongylocentrotus purpuratus*) genome [97] suggest that sea urchins have a more complicated innate immunity than previously anticipated.

4.1 Coelomocytes

The adult sea urchins have a few major organs such as mouth parts (Aristotle's lantern), intestine, nerve ring, axial gland and gonad (Figure 6).

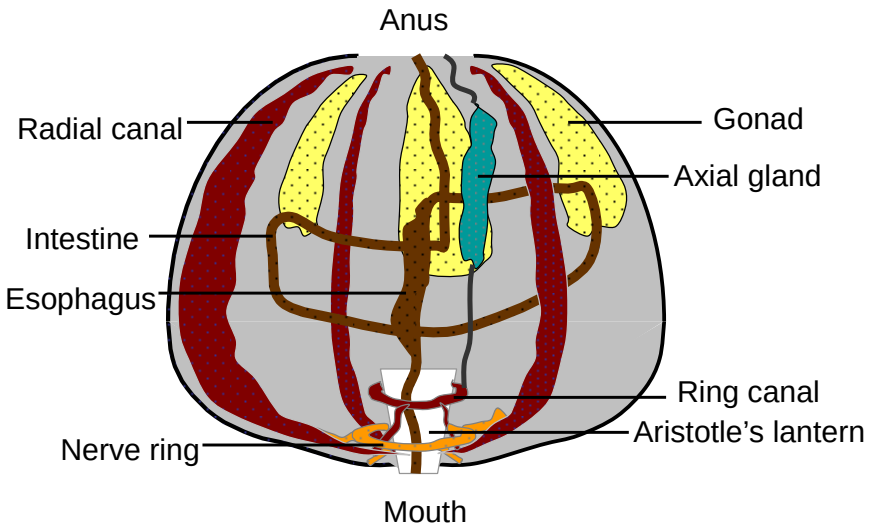

Figure 6 Anatomy of the sea urchin

These organs are located in and protected by a hard 'shell' which forms the coelomic cavity. The remaining space of the coelomic cavity is filled with coelomic fluid which

contains coelomocytes. Coelomocytes are classified into four subclasses: phagocytes, vibratile cells, colourless spherule cells and red spherule cells [136-140].

The main population of coelomocytes consists of phagocytes which play a crucial role in the sea urchin defence system. Phagocytes participate in the graft rejection, form clotting, and are also capable of encapsulation, opsonisation and phagocytosis of invading particles [138, 139, 141-143]. In addition, phagocytes display chemotactic and antibacterial activities [136, 144].

Red spherule cells and colorless spherule cells are similar in size and are also referred to as amoebocytes [140]. The red spherule cells are much denser than the colourless spherule cells [139]. Echinochrome A (7 -ethyl-2,3,5,6,8-pentahydroxy-1,4-naphthoquinone), a red pigment, contributes the colour of the red spherule cells. This pigment has antimicrobial activity against both Gram-positive and Gram-negative bacteria and is released under the challenge of bacteria [20, 136, 144, 145]. Spherule cells have good motile ability (about 0.5 μm/s), which indicates that these cell types are likely to quickly respond to challenges of the immune system such as in the case of wound healing or the prompt attack of invading agents [140, 146, 147]. The phenomenon, where red spherule cells are trapped in clotting and encapsulation [148], seems to support this hypothesis.

Vibratile cells have a round shape and a single long flagellum which helps cells to move quickly in the coelomic fluid. These cells have also been discovered to associate with clotting reactions [137].

Although four populations of coelomocytes are documented in several urchin species [137, 149, 150], the proportion of each type of coelomocytes in the coelomic fluid can vary considerably. This variation does not occur between different species but also between individuals of the same species. This variability most likely results from different

physiological conditions such as size, the nutritional, immunological and homeostatic status of the individuals [98, 140].

4.2 Genes related to immunity of sea urchins

Although sea urchins have a simple anatomic structure, the number of innate immunity related genes is much higher than previously assumed. This holds especially true for recognition receptors. Altogether about 4 to 5 % of the genes of *S. purpuratus* are predicted to be directly related to immune functions [151].

The analysis of the genome of *S. purpuratus* shows that there are 222 TLR gene models. This remarkable TLR gene repertoire can be classified into two main groups according to the sequences of their Toll/interleukin-1 (TIR) domain [96]. One group of TLRs, including 211 genes, are similar to vertebrate TLRs which present the specialized N-terminal and C-terminal leucine-rich repeat (LRR) motifs capping both ends of the LRR domain. The rest of the 11 divergent TLR genes fall under the second group which are more similar to *toll* itself and other insects TLRs [152].

A similar expansive gene repertoire of recognition receptors is 203 NACHT domain-LRR genes [151]. These genes are similar to vertebrate NOD-like receptors. Most putative NACHT domain-LRR proteins are composed of a C-terminal LRR, a NACHT domain and an N-terminal Death-fold domain. The presence of an LRR domain suggests that these receptors likely recognize various PAMPs. The expression of a NACHT domain-LRR has been documented mainly in the gut of the sea urchin [96].

In addition, the analysis shows that there are genes coding for five putative peptidoglycan recognition proteins (PGRPs) and three putative Gram-negative binding proteins (GNBPs) in the genome of *S. purpuratus* [151]. A survey also reported that 218 putative genes encode the scavenger receptor cysteine-rich (SRCR) domains [135].

Several putative immune effector genes have been identified by analysis of the genome of *S. purpuratus*. There are three peroxidase genes, three Nitric Oxide Synthase genes and a gene family of cathepsin in the genome, which likely play a role of immune functions as it is the case in other bilaterians [96]. Another expended gene family, called 185/333, codes for proteins which also likely have immune-related functions. The 185/333 transcripts make up more than 60% of the expression sequence tags (ESTs) identified after challenge with LPS, and over 6% of the clones in a bacterially activated coelomocyte cDNA library [133, 153]. The predicted proteins have an identifiable signal sequence, an N-terminal glycin-rich region, a C-terminal histidine-rich region, and other numerous large tandems and interspersed repeats. The *185/333* sequences are extremely diverse. From 872 gene and message sequences isolated from 16 animals, 477 unique coding regions with 51 distinct element patterns have been identified [133, 153-156]. The function of the 185/333 proteins is currently unknown. However, the 185/333 transcripts increased significantly within 6 h of bacterial challenge [157]. The diversity of transcripts of 185/333 has varied considerably in response to different immunological challenges. For instance, the diversity of messages of the 185/333 increases in response to β-1, 3-glucan, double stranded RNA and injury, while diversity decreases in response to LPS [154, 158]. Since 185/333 proteins are primarily expressed by immune cells they are believed to play an immunological role in the sea urchin [159].

4.3 The complement components in sea urchins

The first identified complement components in sea urchins (*S. purpuratus*) were homologues of the vertebrate component C3, named SpC3 and the homologue component factor B, named SpBf [160]. The SpC3 protein contains a conserved cleavage site to generate a mature protein with two chains and a conserved thioester site. It has been demonstrated that SpC3 can bind both methylamine and yeast as an opsonin [142]. The investigations addressed that the expression of SpC3 in coelomocytes of sea urchins can be upregulated after a

challenge with LPS [161]. SpBf has a mosaic structure containing five short consensus repeats (SCRs), a von Willebrand factor domain, and a serine protease domain [160]. The function of the five SCRs is most likely to bind C3b during formation of the C3 convertase like other members of the Bf/C3 family do. In addition, two transcripts encoding mosaic proteins from *S. purpuratus*, a complement related long form (SpCRL) and a short form (SpCRS) were identified from coelomocytes. The two deduced amino acid sequences contain factor H and factor I. These domains were also discovered in other complement components like C6 and C7, and therefore they might participate in the terminal pathway of complement or act as complement regulatory proteins in sea urchins [162].

4.4 Other coelomic immune factors

Coelomic fluid helps coelomocytes circulation and supports a proper suspension of the organs in the cavity, but it also participates in immune activity. Clotting of coelomocytes is thought to be a mechanism to block the holes in the sea urchin's body wall and to encapsulate microbes and invading materials [163]. It has been documented that the clotting formation requires calcium and is associated with the presence of disulfide linkage [137, 164]. Recently a protein was identified in cell-free plasma of *S. purpuratus*. It was named amassin and mediates a massive clotting reaction by forming large disulfide-bonded aggregates that adhere coelomocytes to each other [149].

Echinochrome A is a naphthoquinone pigment and functions as a cofactor for a digestive enzyme [165]. In recent studies, echinochrome A was demonstrated to function as a bactericidal substance. Echinochrome A is active against both Gram-positive and Gram-negative marine bacteria. It is particularly active against *Pseudomonas* stain No.111 and *Vibrio fischeri* (NCMB 1281) [145]. In another report, non-lysozyme-dependent lysate from red spherule cells of *Paracentrotus lividus* showed remarkable bacterial growth inhibition [144]. Although little is known about its mechanism of release from red spherule cells,

echinochrome A is released in the presence of bacteria and red spherule cells accumulates around injuries and sites of infections. This suggests that echinochrome A and/or red spherule cells carry out important immune functions [136, 146, 147].

Several investigations have reported lysozyme-like activities in echinoderms. Such activity has been detected in the mucus of *Marthasterias glacialis* [166], coelomocytes of *Holothuria polii* [167] and *S. droebeachiensis* [20], and phagocytes of *P. lividus* [144]. A 60 kDa protein from coelomocytes of *P. lividus* was identified, which exhibits lyzosyme-like activity against cell walls of *V. alginolyticus*. The synthesis and degranulation of this 60 kDa protein continuously occurred under unchallenged conditions [168]. This activity suggests one a likely explanation why no bacteria are found in the coelomic fluid of healthy sea urchins [168, 169]. Further, it has been described that colourless spherule cells release the cytotoxic compound lysine, which exhibits cytotoxic activity against both erythrocytes and tumour cells [170].

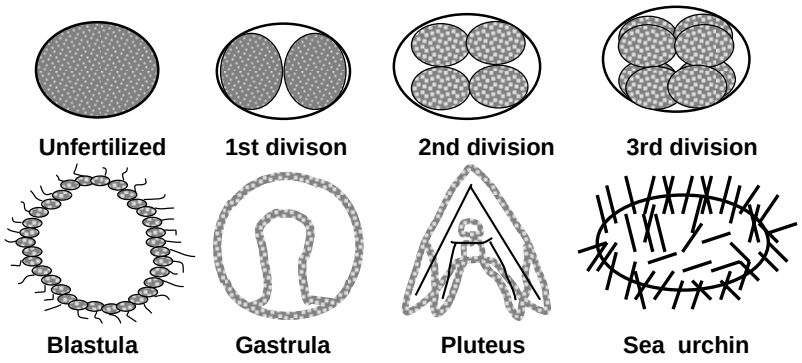

Figure 7 The developmental stages of sea urchins.
(Modified from http://www.stanford.edu/group/Urchin/dev.htm)

4.5 Embryonic and larval immunity of sea urchins

Both, embryos and adult sea urchins live in an environment teeming with microbes, many of which are pathogenic. During their developmental stages (Figure 7), there are likely to be at least two key time points when their immune system is challenged. The first crucial time is the pluteus larvae stage when embryos develop into feeding larvae. The immune system of

embryos/larvae has to protect two potential pathogen entry sites: ectodermal surfaces and endodermal surfaces [98]. The second key period occurs at metamorphosis when they settle to a substrate and form juvenile sea urchins [171]. Therefore, although embryos and larvae have a simpler morphologic structure than adult urchins, they may have their own mechanisms to commit immune defence.

It has been reported that larvae of the sea urchin *Lytechinus pictus* and the sea star *Patiria miniata* are able to pinocytose ferritin by the gut luminal cells [172]. In a recent study, it has been shown that the embryos of *L. variegatus* in mid-gastrula stage were first observed phagocytosing when they were microinjected with yeast [173]. The secondary mesenchyme cells (SMCs) contribute to this immune response. Mesenchyme cells are able to phagocytose more yeast cells during a longer incubation time [173]. In a later developmental stage, differentiated cells contain red pigment and may also have immune responses to pathogens [98]. An immunological function of mesenchyme cells in larval immunity has also been confirmed in the starfish *Asterina pectinifera*. In bipinnaria larvae of *A. pectinifera*, mesenchyme cells respond to the different foreign material in various manners, such as phagocytosis of a small bead, encapsulation of an oil droplet and formation of multinuclear cells in response to a relative large amount of foreign material [174]. The mesenchyme cells probably function as scavenger cells to 'sweep' cell debris, or polystyrene beads out of the blastocoel [174, 175]. Furthermore, it was documented that mesenchyme cells form a spatial network probably to manage physiological and pathological situations. Mesenchyme cells can shift their location in response to invading materials.

Complement C3 transcript is identified in the whole embryogenesis of *S. purpuratus* and the expression of SpC3 achieves a peak prior to and during gastrula stage [176]. In addition, when embryos were continuously incubated with heat killed *V. diazatrophicus*, the expression of SpC3 was significantly increased [176].

Aims of this study

The general aim of this study was to isolate and characterize antimicrobial peptides from adult sea urchins as well as to investigate their expression in coelomocytes and in larvae of *S. droebachiensis*.

The major aims were:

- Isolation and characterization of novel antibacterial peptides and their genes from sea urchins.
- Study the activity of these peptides against bacteria.
- Investigation of the localization of AMPs in coelomocytes of *S. droebachiensis*.
- Investigation of the temporal and spatial distribution of AMPs in the embryo and larva of *S. droebachiensis*.

Summary of papers

Paper I

Chun Li, Tor Haug, Olaf B. Styrvold, Trond Ø. Jørgensen and Klara Stensvåg

Strongylocins, novel antimicrobial peptides from the green sea urchin,

Strongylocentrotus droebachiensis

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 antimicrobial peptides isolated from sea urchins. The cDNA encoding the peptides and genomic sequences were isolated and sequenced. The two peptides (named strongylocin 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 strongylocin 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 the 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.

Paper II

Chun Li, Hans-Matti Blencke, L. Courtney Smith, Matti T. Karp, and Klara Stensvåg

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

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.

Paper III

Chun Li, Tor Haug, Morten K. Moe Olaf B. Styrvold and Klara Stensvåg

Centrocins: isolation and characterization of novel dimeric antimicrobial peptides from the green sea urchin, *Strongylocentrotus droebachiensis*

As immune effector molecules, antimicrobial peptides (AMPs) play an important role in the invertebrate immune system. Here, we present two novel AMPs, named centrocins 1 (4.5 kDa) and 2 (4.4 kDa), purified from coelomocyte extracts of the green sea urchin, *Strongylocentrotus droebachiensis*. The native peptides are cationic and show potent activities against Gram-negative and Gram-positive bacteria. The centrocins have an intramolecular heterodimeric structure, containing a heavy chain (30 amino acids) and a light chain (12 amino acids), which is linked by a single cysteine disulfide bond. The cDNA encoding the peptides and genomic sequences were cloned and sequenced. One putative isoform (centrocin 1b) was identified and one intron was found in the genes coding for the centrocins. The full length protein sequence of centrocin 1 consists of 119 amino acids, whereas centrocin 2 consists of 118 amino acids which both include a preprosequence of 51 or 50 amino acids for centrocins 1 and 2, respectively, and an interchain of 24-amino-acid between the heavy and light chain. The difference of molecular mass between the native centrocins and the deduced sequences from cDNA suggests that the native centrocins contain a post-translational brominated tryptophan. In addition, two amino acids at the C-terminal, Gly-Arg, were cleaved off from the light chains during the post-translational processing. The separate peptide chains of centrocin 1 were synthesised and the heavy chain alone was shown to be sufficient for antimicrobial activity. The genome of the closely related species, the purple sea urchin (*S. purpuratus*), was shown to contain two putative proteins with high similarity to the centrocins.

Paper IV

Chun Li, Hans-Matti Blencke, Tor Haug, Øyvind Jørgensen and Klara Stensvåg

Expression of antimicrobial peptides in coelomocytes and embryos of the green sea urchin, *Strongylocentrotus droebachiensis*

Antimicrobial peptides (AMPs) play a crucial role in innate immunity. We have previously reported the isolation and characterization of the AMPs, strongylocins 1 and 2, and centrocin 1, from extracts of coelomocytes of *Strongylocentrotus droebachiensis*. Here we show that these AMPs are expressed in phagocytes. In addition, the transcripts of strongylocin 1 were detected in vibratile cells and/or colorless spherule cells, while the transcripts of strongylocin 2 were found in red spherule cells. Results from immunoblotting and immunocytochemistry studies showed that centrocin 1 was produced by phagocytes and stored in granular vesicles. Co-localization of centrocin 1 and phagocytosed bacteria suggests that the granular vesicles containing centrocin 1 may be involved in the formation of phagolysosomes. We analyzed the temporal and spatial expression of AMPs throughout larval development. Strongylocins were expressed in the early pluteus stage, while centrocin 1 was expressed in the mid pluteus stage. The spatial expression pattern showed that centrocin 1 was mainly located in the secondary mesenchyme cells (SMCs) forming the coelomic pounches around the stomach and the esophagus. In addition, a few patrolling SMCs were detected in some larval arms. Together, these results suggest that AMPs are expressed in different types of coelomocytes and that centrocin 1 is involved in response against bacteria. Furthermore, the expression of AMPs in larval pluteus stage, especially in SMCs, indicates that AMPs and SMCs are engaged in the larval immune system.

General discussion

In this work, two families of AMP were isolated and characterized from the green sea urchin *S. droebachiensis*. The homologous of strongylocins from the sister species *S. purpuratus* were identified. The antimicrobial activity of native and recombinant peptides was investigated was well. In addition, the expression profile of these peptides in coelomocytes and different developmental stages of larvae was studied as well. The results are discussed in paper **I-IV**, but some selected topics will be discussed in the following sections.

Main characteristics of strongylocins and centrocins

In our study, we have isolated and purified four AMPs from the green sea urchin, *S. droebachiensis*, strongylocins 1 and 2 (paper **I**) and centrocins 1 and 2 (paper **III**). All of them show activity against both Gram-positive and Gram-negative bacteria. In addition, the centrocins show activity against fungi and yeast strains (Table 2).

Strongylocins 1 and 2 are both cysteine-rich peptides which share a novel cysteine location pattern in the APD database [27] (paper **I** and **II**). The other two AMPs, centrocins 1 and 2, are in their native form composed of a heterodimeric structure which is linked by a disulfide bridge (paper **III**). Interestingly, all peptides contain a signal peptide, a prosequence and a mature part. Centrocins 1 and 2 share an identical signal peptide. The signal peptide is considered to aid targeting the endoplasmic reticulum [177]. Our results have shown that centrocin 1 is located in the granular vesicles of phagocytes (paper **IV**). Therefore, we speculate that centrocin 2 may be located in similar organelles as centrocin 1. Strongylocins 1 and 2 have different signal peptides (paper **I**). Although transcriptomic data revealed that the transcripts of both strongylocins were found in phagocytes, the transcripts of strongylocin 1 were also present in the mixture of vibratile cells and colourless spherule cells while the

transcripts of strongylocin 2 were found in red spherule cells (paper **IV**). The different signal peptides of strongylocins may reflect different ways of translocation; however, more information is needed to illuminate how they relocate within phagocytes.

Table 2 Characters of strongylocins and centrocins

	Strongylocins (paper I and II)	**Centrocins (paper III)**
Structure of peptides	Monomer (cysteine-rich)	Dimer
Preprosequence	Yes	Yes
Isoforms	Two isoforms in both strongylocins 1 and 2	Two isoforms in centrocin 1, but centrocin 2 does not have isoforms.
Gene structure	Three introns and four exons but strongylocin 2 does not have an intron.	One intron, two exons
Antimicrobial activity		
Anti Gram-positive bacteria	Yes	Yes
Anti Gram-negative bacteria	Yes	Yes
Anti fungi	N.D.[1]	The synthesized heavy chain of centrocin 1 shows activity.
Anti yeast	N.D.	The synthesized heavy chain of centrocin 1 shows activity.

[1] N.D.=Not determined

In addition, strongylocins and centrocins were shown to undergo post-translational modifications (paper **I** and **III**). This is evident when the amino acid sequences of the purified peptides are compared to the sequences deduced from the genetic information coding for a precursor molecule. The anionic prosequences of these precursor molecules are cleaved off during maturation. It is known that prosequences help folding the mature portions of the peptide by acting as an intramolecular chaperone [178, 179]. It is also noted that prosequences in some precursors inhibit the activity of the mature part [180]. Strongylocin 2 and centrocins both contain a tryptophan which is likely to be brominated according to the molecular mass difference between the native peptides and the deduced ones. Several AMPs from other

marine species have bromotryptophan [40, 181, 182], which probably protects the molecules from proteolysis or increases their bioactivity [183]. The expression of recombinant SpStrongylocin 2 was conducted in the *E. coli* strain BL-21 (C43) (paper **II**). The recombinant SpStrongylocin 2 exhibited similar antibacterial activity against both Gram positive and Gram negative bacteria even though the recombinant peptide likely lacks brominated tryptophan. This suggests that bromotryptophan in strongylocin 2 might make the peptides less susceptible to proteolysis since it does not seem to affect the biological activity of the peptide.

The expression of both strongylocins and centrocins seems to be induced in different larval stages (paper **IV**). The RT-PCR results reveal that transcripts of strongylocins are present in the early pluteus stage, while centrocin 1 is mainly expressed in the mid pluteus stage. The expression of AMPs in the pluteus stage coincides with completement of the larval digestive tracts. In addition, centrocin 1 positive mesenchyme cells are located around the larval stomach and esophagus where they develop into the coelomic pouches. This indicates that AMPs are also involved in larval immune activity. They may be of particular importance for protection against pathogens colonizing the digestive tract.

AMPs in sea urchins may inactivate pathogens by different mechanisms

Although native strongylocins and centrocins both strongly affect Gram-positive and Gram-negative bacteria, they may employ a variety of mechanism to affect microorganisms. Strongylocins are cysteine-rich monomer. Recombinant SpStrongylocin seems to leave the membrane of bacteria intact, and is therefore likely to have intracellular targets but we still lack information with respect to which intracellular targets are affected (paper **II**). Centrocins are heterodimeric molecules. Assaying for antimicrobial activity of the centrocin 1 heavy and light chain, respectively, reveals that only the heavy chain is crucial for eliciting the

antibacterial function (paper **III**). In addition, pilot studies done in our laboratory indicate that the heavy chain of centrocin 1 might use a different mechanism of action against bacteria than strongylocins. All these taken together with the evident differences in amino acid composition between strongylocins and centrocins, make us to agree with the hypothesis that sea urchins have a much more complicated innate immune system than we imaged earlier. The analysis of the *S. purpuratus* genome shows a high diversity of receptor genes, signal transduction genes and immune effector genes [96, 151]. Although the signalling pathways for activating and regulating peptides are unclear, it is tempting to speculate that AMPs serve different purposes and are therefore probably regulated in different manners. Some peptides might be activated in order to quickly respond to and eliminate invading bacteria, while the others might be involved in constantly controlling microbial growth by inactivating intracellular targets, which affects microbial viability more slowly. Further studies of the activity of centrocins will give the answer whether these peptides would comprise two separated lines of antimicrobial defence inside the sea urchin immune system.

All types of coelomocytes are important in the immune activity

It is known that the immune response in sea urchins is mainly mediated by coelomocytes. In sea urchins, coelomocytes are classified according to their morphology and density at the centrifugation. Phagocytes, vibratile cells, and red and colourless spherule cells are four main morphologically distinct cell types in different sea urchins species [137-140, 163, 184-186]. Although red spherule cells are likely to be involved in immune activity due to the fact that they contain echinochrome A, very little information is available about other types of coelomocytes than phagocytes (reviewed by [98, 187]). Phagocytes are able to engulf foreign particles [188, 189] and exert encapsulation [144] and opsonisation [142]. Additional

evidence for red spherule cells being involved in immune activity was recently presented by Dheilly *et al.*, suggesting that the 185/333 proteins were present in red spherule cells [190].

On the other hand, the content of humoral factors in sea urchin coelomic fluid and extracts from coelomocytes has been in focus of research. It is known that coelomocytes lysate and cell-free coelomic fluid of the sea urchin *P. lividus* exhibits antibacterial activity against *V. alginolyticus* [168]. The unfractionated coelomocytes show cytotoxic activity against rabbit erythrocytes and the tumour cell line K562 [170]. It was also discovered that cell populations enriched in colourless spherule cells are highly cytotoxic as they are releasing lysins in the presence of phagocytes. This cooperative effect likely depends on soluble factors released by phagocytes. Our study shows that not only phagocytes and red spherule cells but also vibratile cells and/or colourless spherule cells are involved in immune activity (paper **IV**). For example, the transcripts of strongylocin 1 are found in both phagocytes, and vibratile and colourless spherule cells, while strongylocin 2 are detected in phagocytes and red spherule cells. One may speculate that signalling molecules are likely to mediate the expression and activation of these AMPs, even when distributed in different types of cells.

During bacterial clearance in *S. purpuratus*, the total coelomocyte counts declined by 93% within 90 min post injection [188]. All four types of coelomocytes declined. A similar phenomenon was reported for the total number of coelomocytes. Their number decreased significantly when examined 3-5 h after injection with *V. anguillarum* [191]. The disappearing coelomocytes are mainly phagocytes. Ten hours after injection, the number of coelomocytes is almost back to the same level as before the injection. Therefore, these cells must originate from a rapid division of circulating stem cells and/or from the recruitment of a 'stock' to make up for the 'lost'. The axial organ, which is thought to be an ancestral lymphoid organ, is likely the source of production and/or release of these coelomocytes [192]. Recently, Holm *et al.* reported that LPS and concanavalin A induced cell proliferation in the

axial organ, the coelomic epithelium and the Tiedemann body of the sea star *Asteria. rubens* (L.) [193]. In our study, AMPs were isolated from extracts of coelomocytes from adult animals. The expression of AMPs was detected in coelomocytes as well. In addition, SMCs, the progenitor cells forming the coelomic pouches, were also found to express AMPs. Therefore we hypothesize that there might be progenitor/stem cells in the axial organ expressing AMPs either constitutively or as a response to microbial infection.

In addition, to know the expression profile of AMPs, a constitutively expressed gene should be chosen as an internal control to determine whether equal amounts of cDNA template were loaded. Although *actin* is one of most widely used housekeeping genes [194], it has been reported that *actin* is not optimal for usage as an internal control for sea urchin transcriptional studies. It is known that phagocytes have more extensive cytoskeletons and therefore contain larger amounts of *actin* than the other types of coelomocytes [139]. In addition, it is reported that transcription of actin is low during early sea urchin development [195]. However, transcription of *actin* significantly increases at mid blastula stage [176]. Furthermore, the other commonly used internal control gene, ribosomal protein L8, has a similar variable expression profile during development [176]. Although the amplicon of 18S ribosomal RNA was used as an internal control in this study (paper **IV**), it should be kept in mind that a combination of several internal controls may be a solution for further use of quantitative PCR.

Embryonic and larval development and secondary mesenchyme cells

Sea urchin eggs are embedded in a jelly coat composed of polysaccharide and glycoprotein. This coat, in combination with maternal molecules attached to it, provides immune protection during early development [176, 196]. However, during later developmental stages, the embryo and the larva form a multi-cellular ectodermal body wall

facing the outside environment [197]. Also the digestive tract is developing during this period. Between the blastula stage and the late gastrula, the subdivision of the endodermal archenteron forms fore-, mid- and hindgut [198]. While the stomodeal invagination is becoming the larval mouth in the ectoderm, the blastopore remains unchanged and serves as the larval anus [199]. When the digestive tract is completed in the early pluteus stage, it is about to interact with the outside environment. The expression analysis of regulatory genes, called regulome, during these stages showed that 75% of the regulome has already been used at least once by the late gastrula stage. Another 20% of the regulome have been used at least once during the early pluteus stage [198]. Although we do not know yet how the larval immune system matures during the pluteus stage, we observe that the transcripts of for example strongylocins and centrocin 1, start to appear during this key developmental period (paper **IV**). Therefore, we postulate that there are regulatory genes specific for this stage, which are involved in 'turning-on' the expression of immune-related molecules and that these peptides must play an essential role in this stage.

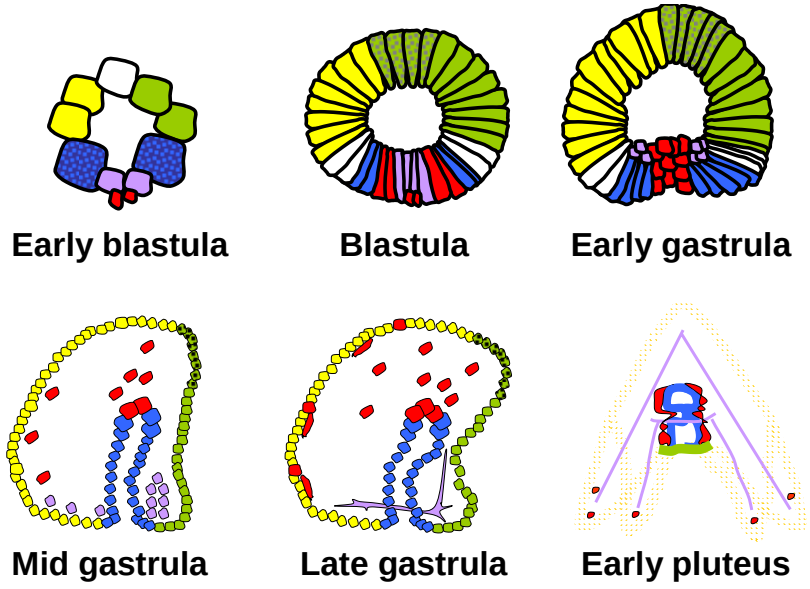

Figure 8 Specification of development of the sea urchin embryo. Colour-coded tracings from photomicrographs of the embryo of *S. purpuratus* are shown. Skeletogenic mesenchyme lineage, violet; endoderm, blue; secondary mesenchyme, red; oral ectoderm, green; apical oral ectoderm, dotted green; aboral ectoderm, yellow; unspecified cells, white (Modified from [198]).

Primary mesenchyme cells (PMCs) are a homogeneous population of cells, rigidly specified early in development, which differentiate exclusively as skeletogenic cells. SMCs are a heterogeneous population of nonskeletogenic cells (Figure 8). SMCs are originally located on the vegetal pole and on the top of the archenteron. They are mainly involved in forming the coelomic pouches but they also end up as pigmented cells, blastocoelar cells and circum esophageal muscle cells. It is possible that SMCs may play a crucial role in larval immune activity since cells from the coelomic pounches are considered as progenitor cells for the later development of coelomocytes [200]. It has been reported that SMCs are able to phagocytose microinjected yeast cells in *L. variegatus* [173] and phagocytically respond to almost all foreign material in the later bipinnaria larva, *A. pectinifera* [174]. Although it remains unclear how SMCs eliminate phagocytosed bacteria, the expression of AMPs in SMCs might imply that removal of bacteria in the pluteus stage might rely on the same mechanism as in coelomocytes. Furthermore, the location of centrocin 1 positive SMCs in the arms of larvae (paper **IV**) suggests that a portion of these cells is able to patrol actively for potential pathological situations [174]. Therefore, the apparent role of the SMCs might not only be for differentiating to form various tissues, but also be important for the larval immune defence.

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Conclusions and future directions

This study has focused on the discovery and characterization of antimicrobial peptides in sea urchins. The expression profile of the peptides was investigated in coelomocytes isolated from adult animals as well as embryos and larvae. The main conclusions are:

Strongylocins

- Strongylocins 1 and 2, contain 6 cysteine residues, are cysteine-rich antimicrobial peptides which are active against both Gram-positive and Gram-negative bacteria.
- Both strongylocins 1 and 2 have putative isoforms (strongylocins 1b and 2b). The genomic sequences show that strongylocins 1a, 2a and 2b have three introns and four exons, while strongylocin 1b has no intron.
- Strongylocin 1 is expressed in phagocytes and vibratile cells and/or colorless spherule cells. Strongylocin 2 is expressed in phagocytes and red spherule cells. Expression of strongylocins starts during the early pluteus stage of sea urchin larvae.
- The recombinant strongylocin homologues of *S. purpuratus* have antibacterial activity, but do not break the integrity of Gram-negative bacterial membranes and thus the peptides might have intracellular targets.

Centrocins

- Centrocins are heterodimeric antimicrobial peptides composed of a heavy and a light chain which are linked by a disulfide bridge. They show activity not only against bacteria but also against fungi and yeasts.
- Centrocin 1 has a putative isoform, centrocin 1b. The genomic sequences of centrocins 1a, 1b and 2 are composed of one intron and two exons. The amino acid sequence of centrocins is highly similar.
- Centrocin 1 is present in granular vesicles of phagocytes.

- Expression of centrocin 1 starts during the mid pluteus stage of sea urchin larvae and is located in SMCs.

There still remain a lot of open questions to be addressed in future follow-up studies.

The first question relates to the intracellular targets of strongylocins in bacteria. There are several possibilities such as binding DNA, inhibiting nucleotide or protein synthesis, inhibiting cell-wall synthesis, inhibiting enzymatic activity, and so forth.

The second question is which regulator molecules mediate AMP expression and activation in sea urchins. The promoter region of the AMP genes needs to be identified to clarify the transcription factors involved. This will at least aid to partially map pathogen responsive signaling pathways. We also wonder how such molecules control the expression of AMPs during development.

The third question is how progenitor cells proliferate and then differentiate into coelomocytes. Are AMPs promising candidates for bio-markers which could be used to trace this process, since different AMPs are located in the various types of coelomocytes?

In addition, peptides may be a useful tool, which might help monitoring the developmental condition of the sea urchin larvae.

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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

(Millipore Corp., MA, USA). After washing with acidified water, three stepwise elutions were performed with 10%, 40% and 80% ACN in acidified water. Based on previous results, showing potent antibacterial activity in the 40% SPE fraction, we focused the efforts on this fraction [24].

Reverse phase HPLC purification of peptides

The HPLC system (Waters Associates, Millipore Corp., MA, USA) consists of a 600E pump, a 717 autosampler and a 2996 photodiode array detector. The absorbance (200–400 nm) was recorded using the Millennium v4.0 (Waters) software program. The HPLC system was operated at 25 °C. The lyophilized 40% fraction from SPE was resuspended in 0.065% TFA and subjected to RP-HPLC on a SymmetryPrep C_8 (Waters; 90 Å, 7 μm, 7.8 × 150 mm) column. The mobile phase consisted of (A) 80% ACN in 0.05% TFA and (B) 0.065% TFA. Elution was performed with a linear gradient of 0–75% A for 120 min at a flow rate of 2 ml/min. Fractions (peaks) were collected manually, dried under vacuum, reconstituted with Milli-Q water and aliquots (50 μl) were tested for antibacterial activity against *L. anguillarum* and *C. glutamicum*.

Fractions were resuspended in 0.065% TFA and subjected to RP-HPLC on a Symmetry Shield RP_{18} (Waters; 90 Å, 5 μm, 4.6 × 250 mm) column. The mobile phase consisted of the same ingredients as described above and elution was performed with 25% A for 5 min and a linear gradient of 25–50% A over 45 min at a flow rate of 1 ml/min. Fractions were collected and tested for antibacterial activity. Active fractions were analyzed for purity using electrospray ionization mass spectrometry (ESI-MS). Impure, active fractions were rechromatographed on a Symmetry 300 C_{18} column (Waters; 300 Å, 5 μm, 4.6 × 250 mm) and eluted under the same experimental conditions as described in step 2, but with a flow rate of 0.5 ml/min. Fractions were collected manually and submitted to ESI-MS.

Antibacterial activity testing

The antibacterial activities of the HPLC fractions were determined by continuous monitoring of the bacterial growth using a Bioscreen C (Labsystems Oy, Helsinki, Finland) microbiology reader according to the method described by Haug et al. [26]. Cecropin P1 (0.5 μg/ml) was used as a positive control during these experiments. Antibacterial activity was determined when the optical density (OD) of the growth control (bacteria plus water) reached an absorbance of approximately 0.3. Fractions were regarded as active when the OD was less than 50% of the growth control.

The antibacterial testing of the purified peptide was performed as described above. The minimal inhibitory concentration (MIC) was set at the lowest concentration of peptide reducing the OD to less than 50% of the growth control. Polymyxin B (Sigma), Lactoferricin B [27], Cecropin P1 and Cecropin B [28] were used as positive control peptides.

Hemolytic activity testing

To determine whether the purified peptides are toxic to eukaryotic cells, the hemolytic assay was conducted as the modified method previously described by Haug et al. [26]. Briefly, the phosphate-buffered saline (PBS; 320 mOsm, pH 7.4) washed red blood cell (RBC) pellet was resuspended in PBS to a hematocrit value of 10%. The 50 μl test samples were incubated with 25 μl PBS and 25 μl RBC in 96-well U-shaped plates at 37 °C for 1 h with shaking. After incubation and 200*g* centrifugation for 5 min, the supernatant was transferred and the absorbance was measured at 550 nm. Baseline hemolysis and 100% hemolysis were defined as the amount of hemoglobin released in the presence of PBS and 0.1% Triton X-100 (Sigma), respectively. Mellitin B and Cecropin B were used as positive control peptides.

Mass spectrometry analysis and amino-acid sequencing

ESI-MS was performed with a Quattro-LC triple quadrupole instrument equipped with an electrospray LC interface (Micromass UK Ltd., Wythenshawe, UK). The analysis was performed in the ESI^+ mode with a cone voltage of 40 V. Samples were dissolved in 95% (v/v) methanol containing 0.02% formic acid and were pumped (Pump 11, Harvard Apparatus, Holliston, MA, USA) into the mass spectrometer at a flow rate of 10 μl/min. N_2 was used as desolvation (flow: 300 l/h) and cone gas (flow: 40 l/h). 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™ v4.0 (Micromass) software program. The data were recorded in the continuum mode of acquisition. Non-protonated average molecular masses were calculated from a series of multiple-charged protonated molecular ions.

Edman degradation of native peptides was performed at the Biotechnology Centre of Oslo (University of Oslo, Norway) using a protein microsequencer model 477A with a 120A PTH analyzer (Applied Biosystems, Perkin Elmer) and an HP 241 Protein Sequencer (Hewlett-Packard).

Genomic DNA and RNA isolation

Genomic DNA was extracted from coelomocytes from two animals using the Blood & Cell culture DNA mini kit (QIAGEN, Hilden, Germany). Total RNA was isolated from the pellets of coelomocytes from six animals using the QIAZol™ reagent according to the manufacturer's instruction (QIAGEN, Maryland, USA). Messenger RNA was extracted from total RNA by using the Oligo tex mRNA midi kit (QIAGEN, Hilden, Germany). The concentration and quality of DNA, total RNA and mRNA were measured using the Nano-drop ND-1000 spectrophotometer (Wilmington, DE, USA).

Reverse transcription-polymerase chain reaction (RT-PCR) and degenerate oligonucleotide primed PCR (DOP-PCR)

The RT-PCR reaction was performed using the Tagman® Gold RT-PCR kit (Applied Biosystems, New Jersey, USA). The following was added to the reaction: 5 μl 10 × reaction buffer, 70 ng mRNA, 2 μl (400 ng) modified oligo (dT) primer (5′TCTGAATTCTCGAGTCGACATCTTTTTTTTTTTTTTT), 1.25 μl

reverse transcriptase and 1 μl RNase inhibitor, 11 μl 25 mM $MgCl_2$, 1 μl dNTP 10 mM, and water to bring the reaction volume to 50 μl. The reverse transcription reaction was conducted in a thermocycler with the following thermoprofiles: 25 °C for 10 min, 48 °C for 45 min and 95 °C for 5 min for one cycle.

DOP-PCR was performed on a thermocycler in two separated steps using 2 μl first strand cDNA as a template, 5 μl 10 × Optimized DyNAzyme™ Buffer, 2 μl (400 ng) forward degenerate primer 5′AT(A/T/C)TT(T/C)GG(A/T/G/C)(A/T)(C/G)(A/T/G/C)AT(A/T/C)TA(T/C)CA (for strongylocin 1) or 5′CC(A/T/G/C)TT(T/C)AA(A/G)AAAAT(A/T/C)GC (for strongylocin 2), 0.5 μl (100 ng) reverse primer (5′TCTGAATTCTCGAGTCGACATCTT), 1 μl dNTP 10 mM, 0.2 μl (2 U/μl) DyNAzyme™ II DNA (Finnzymes, Espoo, Finland) and water to bring the reaction volume up to 50 μl. In the first phase, the DOP-PCR was carried out using 94 °C for 5 min, seven cycles at 94 °C for 25 s, 42 °C for 25 s and 72 °C for 1 min. The second phase was completed with 30 cycles of 94 °C for 25 s, 55 °C for 25 s and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The DOP-PCR products were analyzed by electrophoresis on a 1.8% agarose gel and documented with the Bioimaging system, Syngene.

The product of DOP-PCR was purified on a 1.0% agarose gel, cut from the gel, extracted using a gel extraction kit (OMEGA EZNA™, Duraville, USA) and cloned into pGEM®-T Easy Vector Systems (Promega, Madison, WI, USA). Transformants were selected on LB agar containing 100 μg of ampicillin/ml and 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)/ml. Plasmid DNA from white colonies was isolated using the plasmid mini prep kit (OMEGA EZNA™, Duraville, USA). The primers 5′CGATTTAGGTGACACTATAG (SP6) and 5′CAGTGAATTGTAATACGACTCACT (T7) were employed to sequence the plasmid inserts using the Applied Biosystems Big-Dye™ version 3.1 kit. Sequence data were collected on an Applied Biosystems model 3730 automated capillary DNA sequencer. The amino-acid sequences of cloned genes were deduced and aligned with the Edman degradation sequence of the isolated mature AMP.

cDNA library construction and screening

The ZAP-cDNA® Synthesis Kit and the ZAP-cDNA® Gigapack® III Gold Cloning kit (Stratagene) were used to construct cDNA libraries from the coelomocytes according to the manufacturer's instructions. After the amplifying step, the cDNA library was stored in 7% dimethyl sulfoxide at −80 °C.

The DNA fragment from a plasmid (25 ng) containing the target gene was used as a template to amplify the ^{32}P-labeled probes using Redi prime™ II, random prime labeling system (Amersham Biosciences) according to the manufacturer's instructions. After plating and growing the phages (5×10^4 pfu/plate at 37 °C for 8 h), the plaques were lifted in duplicate onto a nitrocellulose (+) membrane (Amersham Biosciences). Membranes were submerged into denaturation buffer (0.1 M NaOH, 1.5 M NaCl) for 1.5 min; neutralization buffer (0.2 M Tris–HCl, pH 7.4) for 5 min; and for 2 min in saline sodium citrate buffer (SSC) 2 ×, pH 7.0. DNA was cross-linked by baking at 80 °C for 2 h. Hybridization was performed for 12 h at 65 °C. Then, membranes were washed twice at 55 °C for 15 min each using a low stringency solution (0.1% SDS, 2 × SSC). Positive plaques were visualized by exposure on Biomax MS film (Kodak, USA). Positive clones were purified by two additional screenings. After the third screening, the positive λ-clones were excised into plasmids using a helper phage. Plasmids pBluescriptSK (−), containing the target inserts, were isolated and then sequenced on both strands using primer 5′AATTAACCCTCACTAAAGGG (T3) and T7, as described above.

Gene cloning and sequencing

Based on these results, the primers 5′CCAAAGACCAGTTCTTATCAAC (for strongylocin 1, located in 5′UTR), 5′GTAGCTTCATCTTCCCATGCC (for strongylocin 1, located in 3′UTR), 5′CAGTGTTGTGTTCCTCGATCA (for strongylocins 2a and 2b, located in 5′UTR), 5′GAAGATCGTCAGAGGATGCCA (for strongylocin 2a, located in 3′UTR) and 5′CCAGATCGGTGTCTAACTCA (for strongylocin 2b, located in 3′ end and 3′UTR) were designed for studying the gene structure. Genomic DNA (100 ng) was employed as a template in PCR (25 cycles, 94 °C for 30 s, 58 °C for 30 s and 72 °C for 3 min). The PCR products were cloned into pGEM®-T vector and sequenced using the following primers: Sp6, T7, 5′CTTGGTCTTCCTGGTGGTA (for strongylocin 1), 5′ATCTATCATCGCAAATGCGTC (for strongylocin 1), 5′CCGTGATCTTTAGGCATGAAC (for strongylocin 1), 5′CGCAATTGTTATCCTAAAACAACTTG (for strongylocin 2), 5′CAAGTTGTTTTAGGATAACAATTGCG (strongylocin 2), 5′AGCACATCGCAATTGTTATC (strongylocin 2), 5′CTAAAACAACTTGCGAAACTGC (for strongylocin 2), 5′CTTGCCGAAGAGGACGATCT (strongylocin 2), 5′AGATCGTCCTCTTCGGCAAG (strongylocin 2), 5′CTCACCCTACACTTCGCACA (strongylocin 2) and 5′AACATGCACAATATATGGGCAA (strongylocin 2).

Sequence and data analysis

Sequence similarity searches were performed with the BLAST software on the NCBI homepage (http://www.ncbi.nlm.nih.gov/BLAST) and the sea urchin genome project homepage in the human genome sequence center (http://www.hgsc.bcm.tmc.edu/projects/seaurchin/). Sequence alignments were performed using BioEdit Sequence Alignment Editor Version 7.0.5.2 software. Peptide mass, amino-acid composition, and isoelectric points were predicted by the Expert Protein Analysis System (ExPASy) proteomics server of the Swiss Institute of Bioinformatics (http://www.expasy.org/). SignalP3.0 (http://www.cbs.dtu.dk/services/SignalP/) software was used to predict the potential cleavage site(s) of the signal peptides [29].

Results

Isolation of antibacterial peptides from *S. droebachiensis*

According to earlier bioactivity study [24] where activity was detected in a 40% SPE fraction of a coelomocyte extract from the green sea urchin *S. droebachiensis*, we hereby continued this work by RP-HPLC. Fractions showing growth-inhibiting

activity against the bacteria were eluted in a series of fractions ranging from 35% to 50% ACN (Figure 1). Of the two bacteria tested, *C. glutamicum* was the most sensitive. The active substances were further purified by analytical RP-HPLC. Two peptides, corresponding to the active fractions 14 and 16, were purified to homogeneity as monitored by RP-HPLC (data not shown) and named strongylocins 1 and 2 after the genus *Strongylocentrotus*. Mass measurement by ESI-MS (positive mode) of strongylocin 1 revealed multiple ions at *m/z* 510.28 $[M+11H]^{+11}$, 561.06 $[M+10H]^{+10}$, 623.20 $[M+9H]^{+9}$, 701.05 $[M+8H]^{+8}$, 801.00 $[M+7H]^{+7}$, 934.54 $[M+6H]^{+6}$ and 1121.10 $[M+5H]^{+5}$ (Figure 2A), indicating that the peptide has an average molecular weight of 5600.6 Da. ESI-MS (positive mode) of strongylocin 2 revealed multiple ions at *m/z* 643.13 $[M+9H]^{+9}$, 723.28 $[M+8H]^{+8}$, 826.42 $[M+7H]^{+7}$, 964.18 $[M+6H]^{+6}$ and 1156.49 $[M+5H]^{+5}$ (Figure 2B), indicating that this peptide has a molecular weight of 5778.5 Da.

Partial amino-acid sequences

The purified peptides were subjected to Edman degradation, yielding a partial NH_2-terminal sequences of 47 and 49 residues: IFGSIYHRKXVVKNRXETVSGHKTXKDLTXXRAVIFRHERPEVXXPQ (strongylocin 1) and XNPFKKIANRNXYPKTTXETAGGKKTXKDFSXXQIVLFGKKTXAKXTVV (strongylocin 2), respectively, where X corresponds to unidentified residues. Both peptides were characterized by several unknown residues. However, there are likely one or two missing amino acids at the C-terminal end of both of them after comparison of molecular mass and calculated ones. A more distinct UV absorbance at 280 nm (data not shown) suggested that strongylocin 2 contained tyrosine and/or tryptophan residues.

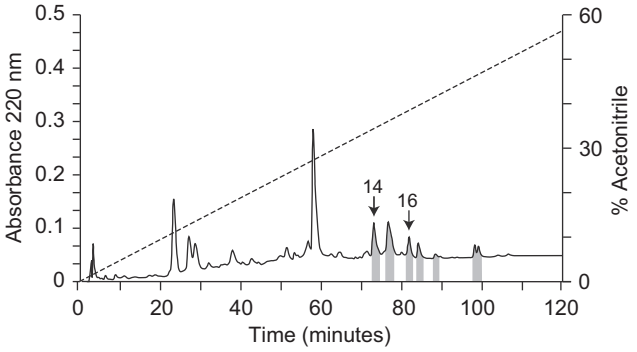

Figure 1 Purification of strongylocins from *S. droebachiensis* coelomocytes by reverse phase high-performance liquid chromatography (RP-HPLC). A coelomocyte extract was pre-purified by SPE using Sep-Pak C_{18} cartridges. The fraction eluted with 40% ACN was subjected to RP-HPLC using a semi-preparative C_8 column. Elution was performed with a linear gradient of 0–60% ACN for 120 min at a flow rate of 2 ml/min. Fractions with growth-inhibiting activity against *Corynebacterium glutamicum* and *Listonella anguillarum* are shadowed in gray. Two of these fractions (numbered peaks) were submitted to further purification on an analytical C_{18} column (data not shown). The absorbance at 220 nm and the concentration of acetonitrile in the eluting solvent are indicated.

Gene sequences of the strongylocins

Based on the partial peptide sequences, DOP-PCR was carried out using degenerated primers. A part of the cDNA sequences (about 670 bp for strongylocin 1 and 390 bp for strongylocin 2) was identified. To obtain the full length of the cDNA sequence, a cDNA library from a coelomocyte pool was constructed and screened. Two genes corresponding to strongylocin 1 and two genes corresponding to strongylocin 2 were isolated and sequenced (Figure 3). The full-length cDNA sequences of strongylocins 1a, 1b, 2a and 2b contain 917, 919, 558 and 556 bp of nucleotides, respectively. Strongylocin 1 includes a 5′-untranslated region (UTR) of 130 bp (1a) or 124 bp (1b), an open reading frame of 249 bp, and a 538 bp (1a) or 546 bp (1b) 3′UTR. Strongylocin 2a includes a 33 bp 5′UTR, an open reading frame of 270 bp, and a 258 bp 3′UTR, whereas the complete sequence of strongylocin 2b cDNA contains a 33 bp 5′UTR, an open reading frame of 273 bp, and 253 bp 3′UTR. A consensus polyadenylation signal sequence (AATAAA) is located 17, 15 and 17 bp upstream of the poly(A)$^+$tails in strongylocins 1, 2a and 2b, respectively (Figure 3).

The sequence analysis also showed that strongylocins 1a and 2 have three introns and four exons (Figure 4) whereas strongylocin 1b is intronless. In strongylocin 1a, intron 3 contains 964 nucleotides, while introns 1 and 2 have 329 and 440 bp, respectively. In strongylocin 2a, intron 2 contains 956 bp nucleotides whereas introns 1 (347 bp) and 3 (771 bp) are shorter ones. Intron 2 in strongylocin 2b contains 1279 bp nucleotides, while introns 1 and 3 have 349 and 800 bp, respectively. Exon 4 is the smallest element in strongylocins 1a, 2a and 2b, with only 8, 11 and 14 bp, respectively.

The BLAST analysis revealed that two putative proteins of the purple sea urchin, *S. purpuratus*, are similar to the strongylocins (Figure 5A). Strongylocin 1 shares 90% identity at an amino-acid level with the putative proteins gi:115757240, whereas strongylocin 2 shares 36% identity at the amino-acid level with the putative peptide gi:115736742 from *S. purpuratus* (GenBank). According to the BLAST analysis against *S. purpuratus* genome sequences and the alignment analysis, the data indicate that the two genes coding for putative strongylocins contain three introns.

Primary structure determination

The deduced peptide sequences of strongylocins 1a, 1b, 2a and 2b contain 83, 83, 89 and 90 amino acids, respectively (Figure 5A). Based on MS analysis and Edman degradation analysis, the mature peptides start from Ile-36 in strongylocin 1 and Trp-39 in strongylocin 2 and proceed with 48 (strongylocins 1a and b), 51 (strongylocin 2a) and 52 (strongylocin 2b) amino acids to the C-terminal end. Analysis, using SignalP 3.0, indicates that a cleavage site of a signal peptide is located between amino acid Ala-22 and Ala-23 for strongylocin 1 and Ala-22 and Ser-23 for strongylocin 2 using both the neutral network model and the hidden Markov model [29]. Thus, the precursor peptides contain a signal peptide, a prodomain region followed by the mature peptides.

According to the deduced amino-acid sequences, all peptides contain six cysteine residues which likely form

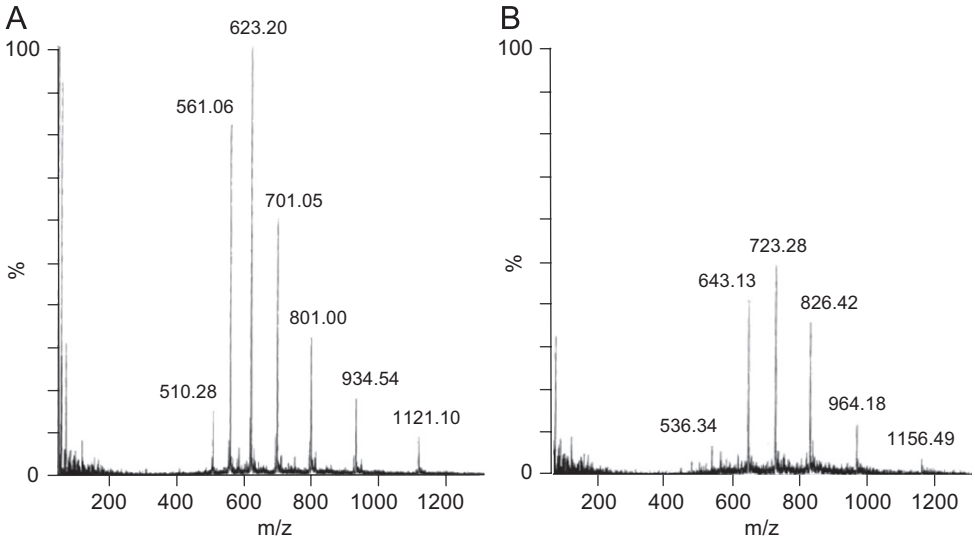

Figure 2 Electrospray ionization (ESI-MS) mass spectra of the purified peptides, strongylocins 1 (A) and 2 (B). ESI-MS (positive mode) of the peptides reveal multiple ions, giving average molecular masses of approximately 5600.6 and 5778.5 Da for strongylocins 1 and 2, respectively.

three disulfide bridges (data not shown [http://www.predictprotein.org/]). Thus the first six unknown N-terminal amino acids in strongylocin 1 (denoted as an X) and six of the last seven for strongylocin 2 are cysteines (Figure 5A). The last X in strongylocin 1 and the second last in strongylocin 2 correspond to an Arg, whereas the first X in strongylocin 2 corresponds to Trp (Figure 5A).

Alignment of the complete strongylocin 1 peptide sequences in Figure 5A shows that the two isotypes of the peptide differ in positions 8, 44, 47, 55, 57 and 62 possessing Leu, Lys, Val, Ser, His and Asp in strongylocin 1a, respectively, whereas in strongylocin 1b, these residues are Phe-8, Glu-44, Lys-47, Asn-55, Asn-57 and Glu-62. Except for position 57, all these amino acids are substituted by similar amino acids or amino acids with identical physiological properties. In strongylocin 2, there are eight amino-acid differences between the two molecules. The eighth amino-acid residue of strongylocin 2a is Leu-8, whereas Phe-8 in 2b. The 14th and 15th amino-acid residues of strongylocin 2a are both Met, whereas in 2b, these residues are Ile-4 and Val-5, respectively. The 47th, 54th and 55th amino-acid residues are Asn and two Thr in strongylocin 2a, but His, Gln and Glu in 2b. In contrast to Thr–Ser as the last two C-terminal amino-acid residues of strongylocin 2a, Met–Ser are the last ones in strongylocin 2b.

The isolated peptides have isoelectric points (p*I*) of 9.34 (strongylocin 1) and 9.65 (strongylocin 2). The calculated p*I* rises from 6.32, 5.63, 8.58 and 8.24 in the precursors to 9.34, 9.12, 9.65 and 9.51 in the mature peptides for strongylocins 1a, 1b, 2a and 2b, respectively, when the prosequences are cut off.

In addition, the six cysteines in all the peptides share the same location within the sequences. Comparison of the cysteine pattern in the primary structure of strongylocins with other AMPs containing six cysteine residues (sequences from *Antimicrobial Peptide Database* http://aps.unmc.edu/AP/main.html) revealed a novel and unique cysteine location pattern within the strongylocins (Figure 5B). The fourth and fifth cysteines are adjacent in the strongylocins, while in types III and IV thionins (from plants), the first and second cysteines are adjacent to each other. In tachystatin A (from *Tachypleus tridentatus*), the third and forth cysteine residues are adjacent, whereas in α- and β-defensins (from mammals) the fifth and sixth cysteine residues are adjacent.

Antibacterial activity

The antibacterial activity of the compounds was monitored during the purification procedure. Purified strongylocins 1 and 2 were tested against both Gram-positive and Gram-negative bacteria to evaluate their capability of inhibiting bacterial growth (Table 1). The data showed that both strongylocins display potent activity (MIC ranging from 1.3 to 5 μM) against all bacterial strains tested, and are even more potent than the reference peptides against *S. aureus*.

Hemolytic activity

The strongylocins showed no hemolytic activity at a concentration of 17.5 μM, which is in the range from 3.5 to 13.5 times higher than the MIC values. In contrast, the controls Mellitin B and Cecropin B showed 50% hemolysis (EC_{50}) at a concentration of 2 and 40 μM, respectively.

Discussion

Antibacterial activity has been previously described from a wide range of echinoderm species [30–34]. A screening for antibacterial activity in the green sea urchin, *S. droebachiensis*, showed that coelomocyte extracts contain components that possess antibacterial activity *in vitro* [24]. Here we describe the purification and characterization of two novel antibacterial peptides called strongylocins 1 and 2 from coelomocytes of the green sea urchin (*S. droebachiensis*). Both contain six cysteines and have isoelectric points

(p*I*) of 9.34 and 9.65, respectively. By screening a cDNA library from coelomocytes and using the degenerate PCR, several putative isoforms were also identified. To our knowledge, these are the first AMPs isolated from sea urchins and they share no significant identity to other known peptides. The native strongylocin 1 is 5600.6 Da and the native strongylocin 2 is 5778.5 Da. Both share more than 90% identical amino acids with the putative peptides of their isoforms.

According to ExPASy amino-acid modification page, the deduced amino-acid sequence of strongylocin 1 has a calculated mass of 5606.6 Da (http://au.expasy.org/tools/findmod/findmod_masses.html), which is 6 Da more than the purified peptide. This difference is likely due to intramolecular disulfide bridges between the six cysteine residues. Strongylocin 2 seems to contain a modified tryptophan in position 1, since no amino acid was determined by Edman degradation sequencing and the presence of tryptophan is deduced from the cDNA sequence. The calculated mass (5699.7 Da, assuming three disulfide bridges) of the cDNA sequence is 78.8 Da less than the mass measured by mass spectrometry (5778.5 Da) of the purified peptide. Assuming that Trp-1 is the only modified amino acid in the sequence, the discrepancy of 78.8 Da is probably due to bromination (+78.9 Da) since tryptophan lacks available hydroxyl groups needed for sulfation or phosphorylation. Furthermore, the

Strongylocin 1

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a   ATTATTCTGG TCTGCACCTT AACTTGTCGT TGCCAAAGGC TGTGTCGTCG AGTGATCTCT 60
b   ---ATTCCGG TCTGCACTTT AGCTTGTCGT TGCCAAAGGC TGTGTCGTTG AGTGATCTCT 57

a   CAGTCAGTAG CTGTTTGCAA GTTTCCTTGG AAGTTGTCCA AAGACAAGTC CATACTAACC 120
b   CAGTCACTAG CTGTTCGCAA GTTTCCTTGG AA---GTCCA AAGACCAGTT CTTATCAACC 114

a   CAACTTCAAG ATGGATCTCA GGAGCGCATC CTTGGTCTTC CTGGTGGTAG TCATGGTGCT 180
b   CAACTTCAAG ATGGATCTCA GGAGCGCATC CTTTGTCTTC CTGGTGGTAG TCATGGTGCT 174

a   ATCATACTCC ATGGCCGCAC CTCTTGATGC AGATAACGAT GAAGAAATGG AGGAAATCTT 240
b   ATCATACTCC ATGGCCGCAC CTCTTGATGC AGATAACGAT GAAGAAATGG AGGAAATCTT 234

a   CGGCTCGATC TATCATCGCA AATGCGTCGT AAAAAACAGA TGTGAAACCG TCAGCGGTCA 300
b   CGGCTCGATC TATCATCGCG AATGCGTAAA AAAAAACAGA TGCGAAACCG TCAACGGTAA 294

a   CAAGACGTGC AAGGATTTAA CATGTTGCCG AGCCGTGATC TTTAGGCATG AACGCCCCGA 360
b   CAAGACGTGC AAGGAATTAA CATGTTGCCG AGCCGTGATC TTTAGGCATG AACGCCCCGA 354

a   AGTATGCCGA CCACAAACCT AGACAGGATT CACCATGGCA TGGGAAGATG AAGCTACCCA 420
b   AGTATGCCGA CCACAAACCT AGACAGGATT CACCATGGCA TGGGAAGATG AAGCTACCCA 414

a   TCTAGAGTGT ATTTAGATCA GTGACTGAA- -------AGT CTGAAACCAT GGACCTACAT 472
b   TCTAGAGTGT ATTTAGATCA GTGACTGAAT CACTGAAAGT CTCAAACCAT GGACCTACAT 474

a   AGGCTGAAAC CAATGCTATA CTATGTCAAA CCGTCGTATC TTGCGTATTT CATTTCTCTT 532
b   AGGCTGAAGC CAATGCTATA CTATGTCAAA CCGTCGTATC TTGCGTATTT CATTTCTCTT 534

a   TGGTTTTTAT AATCTTTCAG TGTTTATATT CTGAAATTTC GAAGTATCCC TAAAATCAAA 592
b   TGGTTTTTAT AATCTTTCAG TGTTTATATT CTGAAATTTC GAAGTATCCC TAAAATCAAA 594

a   CAACTCAAGT TTCTTTTGCA AAGGCATTAA ACAAATAAAT TGATCATTGT TTATTTTCTT 652
b   CAACTCAAGT TTCTTTTGCA AAGGCATTAA ACAAATAAAT TGATCATTGT TTATTTTCTT 654

a   ACATATACAC AACTATACAC CCCTTTTACG TTAAAGCTTC CACTGCCTAG ACAGAATTCC 712
b   ACATATACAC AACTATACAC CCCTTTTACG TTAAAGCTTC CACTGCCTAG ACAGAATTCC 714

a   TAATTAAGTA AAGTTGAAGC CATCGATCTA GAGTGTATTT AGATCAGTAG CTGAAACCAA 772
b   TAATTAAGTA AAGTTGAAGC CATCGATCTA GAGTGTATTT AGATCAGTAG CTGAAACCAA 774

a   TGCTATACTA TGTCAAATCG TAGTTGCATG CCCATTTTTG TTTTCGTTGT CATAATCCTT 832
b   TGCTATACTA TGTCAAATCG TAGTTGCATG CCCATTTTTG TTTTCGTTGT CATAATCCTT 834

a   TTCGTTTTGA ATGAAATTGA TATGTTTTGA AATCTTAATA AATAGCATGG TTTAATGACA 892
b   TTCGTTTTGA ATGAAATTGA TATGTTTTGA AATCTTAATA AATAGCATGG TTTAATGACA 894

a   AAAAAAAAAA AAAAAAAAAA AAAAA                                       917
b   AAAAAAAAAA AAAAAAAAAA AAAAA                                       919
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Figure 3 Alignment of the gene sequences of strongylocins 1a and 1b (A) and strongylocins 2a and 2b (B) from the green sea urchin *S. droebachiensis*. The numbers of nucleotides are indicated on the right side. The differences in the nucleotides in the alignments are shaded in gray and gaps (dash) are introduced to maximize the alignments. The translation start codon (atg), stop codon (tag) and polyadenylation signal sequence (aataaa) are marked in bold. The sequences have been submitted to the GenBank with accession numbers EU122307, EU043119, EU043117 and EU043118.

Strongylocin 2

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a   TCATTTTCAG TGTTGTGTTC CTCGATCATC AAGATGAATA TTCGGAAGGG ATCTTTGATG   60
b   TCATTTTCAG TGTTGTGTTC CTCGATCATC AAGATGAATA TTCGAAAGGG ATCATTCATG   60

a   CTCCTCATCA CCATGATGAT CCTGTCTCAA GCAGTGGCTT CACGATTCCT TGATGAAGCA  120
b   CTCCTCATCA CCATAGTGAT CCTGTCTCAA GCAGTGGCTT CACGATTCCT TGATGAAGCA  120

a   GACGAAGACG AACATCTCAT GGAGGCATGG AATCCGTTCA AAAAGATAGC AAATCGCAAT  180
b   GACGAAGACG AACATCTCAT GGAGGCATGG AATCCATTCA AAAAGATAGC ACATCGCAAT  180

a   TGTTATCCTA AAACAACTTG CGAAACTGCC GGAGGTAAAA AAACATGCAA GGACTTCAGT  240
b   TGTTATCCTA AACAAGAGTG CGAAACTGCC GGAGGTAAAA AAACATGCAA GGACTTCAGT  240

a   TGTTGTCAGA TCGTCCTCTT CGGCAAGAAA ACTCGCGCCA AATGCACAGT TGTTACAA--  298
b   TGCTGTCAGA TCGTCCTCTT CGGCAAGAAA ACTCGCGCCA AATGCACAGT TGTTACAATG  300

a   -GTTAGACAC CGATCTGGCA TCCTCTGACG ATCTTCATGC AGCAGCAAGT GAACTAATGC  357
b   AGTTAGACAC CGATCTGGTA TCCTCTGACG ATCTTCAT-- --CAGCTAGT GAACTAATGC  356

a   TATAACTTAT AAGACAACGA ACATCAACAT ACCCAGAGTT CTCTTTTCTC TTGATAATTT  417
b   TATAACTTAT AAGACAACGA ACATCAACAT ACCCAACGTT CTCTTTT-TC ATGATAATTT  415

a   AGTTGAACAT GTAAGAATCT AAAAATGAAA AACTAAGTGA GCAATGCAAC AAATAAGGAT  477
b   AGTTGAACAT ATAAGAATCT AAAA-TAAAA AATTAAGTGA GCAATGCAAC AAATAAGGAT  474

a   CATCAATTCC TACTAATGTA TGTTGTTGGC AAAATAACAA TAATAAACTG TTTTGAAGAT  537
b   CATCAATTCC TACCAATGTA TGTTGTTGGC AAAATAAAA TAATAGACTG TTTGGAAAAA  534

a   TGAAAA AAAAAAAAA AAAAA-                                          558
b   AAAAAA AAAAAAAAA AAAAAA                                          556
```

Figure 3 (*Continued*)

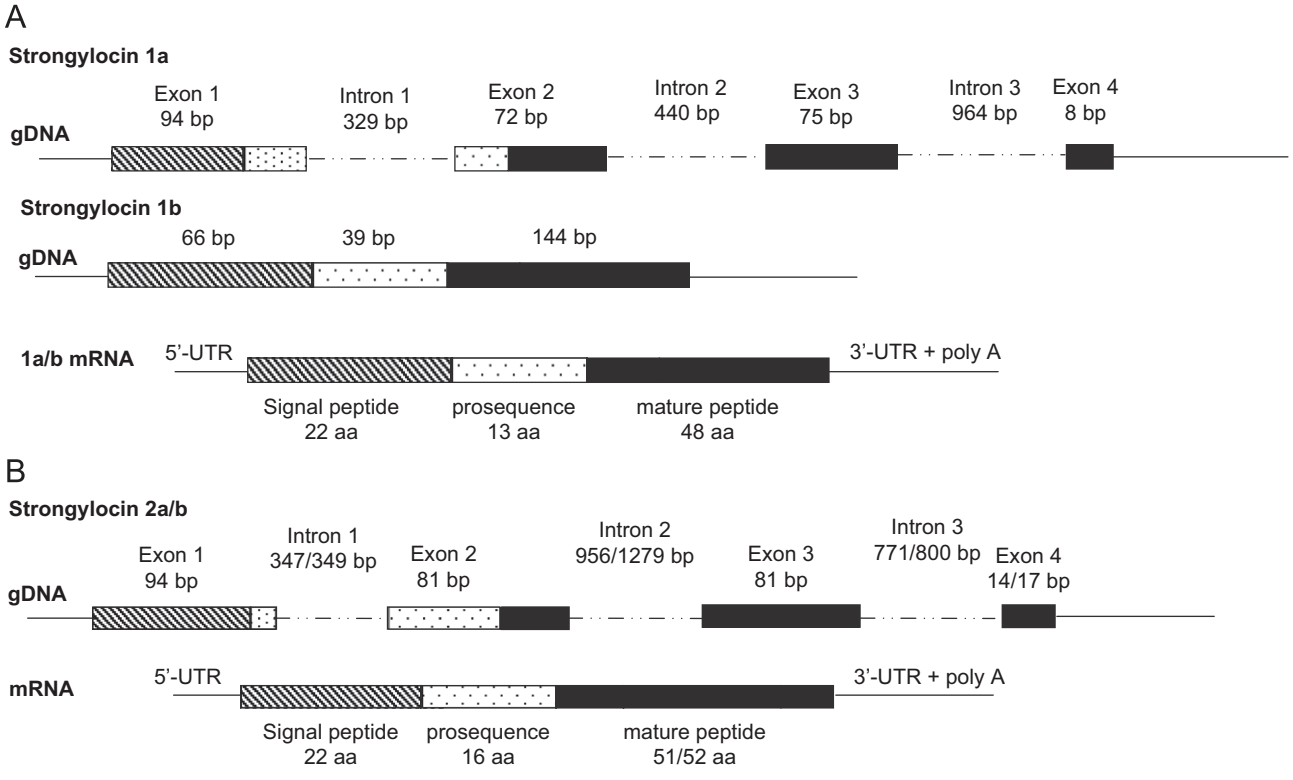
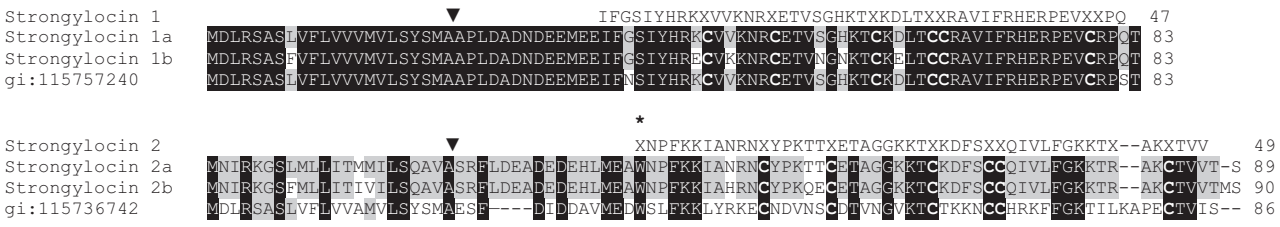


Figure 4 The structure maps of strongylocins 1a and b (A) and 2a and b (B). Strongylocin 1a, 2a and 2b genomic DNA contain the exon/introns organization (not to scale). The coding regions are indicated by boxes: striped boxes, regions encoding a signal peptide; stippled boxes, regions encoding a prosequence; and filled boxes, regions encoding mature strongylocin peptides.

A



B

Peptide family	Cysteine arrangements	Animals
Strongylocins	C – C – C – **CC** – C	Echinoderms
Beta-defensins	C – C – C – C – **CC**	Mammals, birds
Alpha-defensins	C – C – C – C – **CC**	Mammals
Tachystatins	C – C – **CC** – C – C	Horseshoe crab
Knottin-type AMPs	C – C – **CC** – C – C	Plants
Thionins type III and IV AMPs	**CC** – C – C – C – C	Plants
Insect defensins	C – C – C – C – C – C	Insects
Mytilus defensin	C – C – C – C – C – C	Molluscs

Figure 5 The alignment of strongylocin 1 and 2 from *S. droebachiensis*, with two putative AMPs of the purple sea urchin, *S. purpuratus*, genome databank (NCBI databank) (A). The predicted cleavage sites between the signal peptide and the proregions (highlighted in gray) are shown by a solid triangle (▼). The first amino-acid residue in the active strongylocin 2 is a modified tryptophan labeled by an asterisk (*). Identical residues are shaded in black, whereas similar residues are shaded in gray. Unknown amino acids are denoted X. Comparison of the cysteine location pattern in eight antimicrobial peptides containing six cysteine residues (B). Adjacent double cysteine residues are marked as boxes. Information regarding cysteine arrangements in the different peptides was obtained from the *Antimicrobial Peptide Database* (http://aps.unmc.edu/AP/main.html).

Table 1 Susceptibility of bacterial strains to the antibacterial peptide strongylocin 1 and 2 isolated from *S. droebachiensis* coelomocytes.

Peptide	Minimal inhibitory concentration (μM)			
	L. anguillarum	*E. coli*	*C. glutamicum*	*S. aureus*
Strongylocin 1	2.5	5.0	2.5	2.5
Strongylocin 2	1.3	5.0	2.5	2.5
Polymyxin B	0.8	0.8	1.6	6.3
Lactoferricin B	3.1	3.1	1.6	6.3
Cecropin P1	1.2	1.2	1.6	12.5
Cecropin B	0.6	0.5	1.0	9.4

Minimal inhibitory concentration (MIC) was determined as the lowest concentration of peptide causing an optical density less than 50% of the growth control. Polymyxin B, Lactoferricin B, Cecropin P1 and Cecropin B were used as reference peptides.

mass difference fits bromination better than phosphorylation or sulfation (both adding ca. 80 Da). There is a slight possibility that other residues in the C-terminal part could also be modified; residues not identified by Edman degradation (Arg-43, Thr-50 or Ser-51). Based on information at ExPaSy FindMod (http://au.expasy.org/tools/findmod/findmod_masses.html), we cannot find any combined modifications that add up to 79 Da. Altogether, these data strongly suggest that Trp-1 is the only modified residue, and that the amino acid is very likely a brominated tryptophan (bromotryptophan). Whether it is 5- or 6-bromotryptophan is unknown. AMPs containing bromotryptophan have been previously isolated from other marine organisms, like the Atlantic hagfish, *Myxine glutinosa* [35], the marine tunicate, *Styela clavata* [36], and the marine annelid, *Nereis diversicolor* [37]. In addition, carnivorous marine cone snails (*Conus* spp.) produce potent neurotoxic peptides containing bromotryptophan residues [38]. Bromination may make the peptides less susceptible to proteolysis, and/or increase the biological activity of peptides [39].

The gene organization of the strongylocins suggests that they contain four exons and three introns, with the exception of the intronless strongylocin 1b. Another group of cysteine-rich AMPs, the invertebrate defensins, is considerably diverse in genomic organization and sequence identity [40]. An insect defensin gene in fruit fly (*Drosophila melanogaster*) lacks intron [41], whereas the genomic DNA of a defensin in tick (*Ornithodoros moubata*) contains three introns [42]. Although the exon/intron organization of the invertebrate defensins is different, they share similarities in size, cysteine pattern and function. Intronless genes, not requiring post-transcriptional splicing, might be transcribed efficiently under the septic injuries. The diversity of gene structure of strongylocins may thus be due to selective pressure. However, we do not know how the intronless gene of strongylocin 1b and other strongylocin-coding genes respond to pathogens. Recently, a number of immune-related genes were found in the genome of *S. purpuratus*, for instance a vast repertoire of Toll-like receptors, a large family of cysteine-rich scavenger receptors and a highly variable family of immune-related genes called 185/333 [10]. The 185/333 sequences contain two exons and one intron. The second exon includes the variable element (blocks sequences) pattern; and the intron also exhibits sequence variability. The high diversity of the 185/333

sequences is a result of variation in element patterns, as well as point mutations and small indels [43]. Although little is known about the strongylocin gene family, the diversity of the strongylocin genes indicates the presence of a variety of genes and molecules related to defense in this animal.

According to the *in silico* analyses, the six cysteines present in the strongylocins are likely to be engaged in three intramolecular disulfide bridges in the native conformation. Cysteine-rich AMPs are widely distributed in animals and plants and it has been proposed that proteins that present the same location of cysteine residues also present similar disulfide pairing [2–4]. Although having the same number of cysteine residues, the strongylocins show a cysteine arrangement pattern different from any known cysteine-rich AMPs (Figure 5B). It has been suggested that the disulfide bridges are essential for the antimicrobial activity of defensins, based on the finding that linearized human neutrophil α-defensins were inactive against viral or bacterial targets that were effectively neutralized by the native molecules [5,44,45]. In addition, given the small size of defensins, disulfide bridges are likely to play an important role in stabilizing their tertiary structures. The main function of the disulfide bridges in strongylocins is not revealed, but they may protect the backbone from proteolysis during biosynthesis and intracellular trafficking within the coelomocytes, and/or secretion into protease-containing environments [5].

Comparison of peptide sequences of the purified strongylocins and the ones deduced from cDNA sequences indicates that there is a 35 or 38 amino-acid extension at the N-terminus, which may be a signal peptide and prosequence of strongylocin 1 or 2, respectively (Figure 5A). The present data suggest that the secretion signals are 22 amino acids both in strongylocins 1 and 2 while the prosequences are 13 and 16 amino acids, respectively. Many proteins contain a prodomain which is cleaved off during maturation of the precursor protein either at the terminus or even within the functional protein. It is also known that prosequences in some precursors inhibit the activity of the mature portions [9] and in many cases, mature proteins gain their functions only after the removal of these prosequences. In addition, the prosequences help folding of the mature portions by acting as an intramolecular chaperone [7,8]. According to the precursor molecule sequence, the net positive charges of the mature strongylocins might be neutralized by the prosequences containing seven (strongylocin 1) or six (strongylocin 2) negatively charged residues. Since the calculated p*I* in strongylocin 1a, 1b, 2a and 2b increased from 6.32, 5.63, 8.58 and 8.24 in the precursor forms to 9.34, 9.12, 9.65 and 9.51 in the mature peptides, respectively, the presence of an anionic prosequence may be crucial to keep the strongylocins inactive during biosynthesis and translocation within the intracellular compartments.

The SPE method separates compounds according to their hydrophobicity. As antibacterial activity was detected in both the 40% and 80% ACN fraction [24], it is reasonable to assume that multiple compounds/molecules in the coelomocytes are responsible for the antibacterial activity detected. Antibacterial testing of the HPLC purified fractions showed activity in more than fraction nos. 14 and 16 (Figure 1). However, we have not been able to isolate and identify peptides corresponding to the isoforms of strongylocins 1b and 2b, thus the other active fractions may contain other AMPs. All these antibacterial factors might have an important function as a first line of defense against pathogenic microorganisms. Furthermore, their potent antibacterial activities and the low hemolytic activity make them promising also as possible drug lead compounds.

In summary, strongylocins 1 and 2 are the first AMPs isolated from sea urchins. Their primary structure includes a signal peptide and a prosequence domain. The DNA organization of the coding genes is arranged differently as strongylocin 1b has no introns whereas strongylocins 1a, 2a and 2b have three introns. The active strongylocins are cationic and contain six cysteine residues, probably engaged in three disulfide linkages. The peptides have a unique cysteine location pattern and therefore seem to be a novel family of AMPs. Furthermore, one of the isolated peptides, Strongylocin 2, seems to be post-translationally modified by containing a putative brominated tryptophan. Further studies have to be done to reveal the mechanisms of action involved and eventually the importance of the brominated tryptophan.

Acknowledgments

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The strongylocin sequences have been deposited in the GenBank with accession numbers EU122307, EU043119, EU043117 and EU043118.

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

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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*.

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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).

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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 $CaCl_2$, 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^{2+} 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 1×10^7 *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-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.

Table 2
SpStrongylocin matches from *S. purpuratus* ESTs[a].

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	**EC430627**	8e-08
EC429433.1	0.0		
EC436320.1	0.0		
R61957.1	5e-171		
R61943.1	6e-165		

[a] 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.

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^{2+} 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^{2+} 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_2O 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

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(A) **SpStrongylocin 1** (GU116566)

```
cggcacgaaggggctgtgttttttgagtgatctctcagtcagtagctgttcgcaagttt  60

ccttggaagttgttcaaagacaagtccattccaacccaatttcgaaatggatctcaggag 120
                                                  M  D  L  R  S   5
cgcatccttggtcttcctggtggtagtcatggtgctatcatactccatggccgcacctct 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
cgtcgtaaaaaacagatgcgaaaccgtcagcggtcacaagacgtgcaaggacttaacatg 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

tgaaaccatggacctatacatgctgaaaccaatgctatactatgtcaaaccgtcgtatct 480

tgcgtatttcatttctctttggtttttataatctttcagtgtttatattctgaaatttcg 540

aagtatccctgaaatcaaacaactcaattttcttttgcaaaggcaataaacaaataaatt 600

gatcattgtttatttttcttacatatacaaaactatacacccctttgta             648
```

(B) **SpStrongylocin 2** (GU116567)

```
ccacgcgtccgcgttcgcaagttttcttaggagttgtccaaagactagttcaaacctact  60

caacttcaatatggatctcagaagcgcatccttggtcttcctggtggtagctatggtgct 120
          M  D  L  R  S  A  S  L  V  F  L  V  V  A  M  V  L  17
atcatactccatggctgaatctttcgaggtcgatgatgccgtgatggaggaattatggaa 180
  S  Y  S  M  A  E  S  F  E  V  D  D  A  V  M  E  E  L  W  N  37
ccctttaggaagctctacagaaaggagtgtaacgacgtaacctcgtgcgacaccgtcag 240
  P  F  R  K  L  Y  R  K  E  C  N  D  V  T  S  C  D  T  V  S  57
cggtgtgaagacgtgcacgaaaaaaaattgttgtcatcgcaagttctttgggaagacaat 300
  G  V  K  T  C  T  K  K  N  C  C  H  R  K  F  F  G  K  T  I  77
cttaaaggcgcccgaatgcaccgtcatcagttgaaaggctgaataccaatttcctcgacg 360
  L  K  A  P  E  C  T  V  I  S                                87
tctggcaaatgaacccggcgctatgaggatgacagtgtttaccaagattataatcaatat 420

tattgcttcattatcttcgttacccttattattcaaaaaatgttaatttcttaagaatgc 480

acgaagcattaaagtagaaattaacatacgttgtgacaggggtacttttacagatcgtg 540

acatgatcatagaagtaagttctgttattaacagctgttttgcaagcggaaagtatattt 600

attttcttgaaaacaaacaaagaacatccatattactacgaccaactacccagcccccc 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 μM (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

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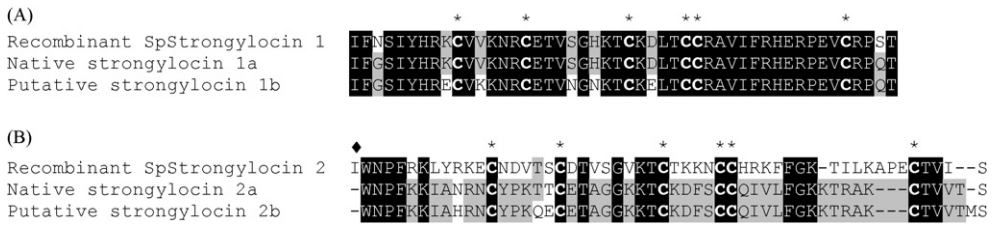

Fig. 2. The alignment of recombinant peptides with native strongylocins from *S. droebachiensis*. (A) Alignment of recombinant SpStrongylocin 1 with native strongylocin 1. (B) Alignment of recombinant SpStrongylocin 2 with native strongylocin 2. In the alignment, the identical amino acids are highlighted 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 (◆).

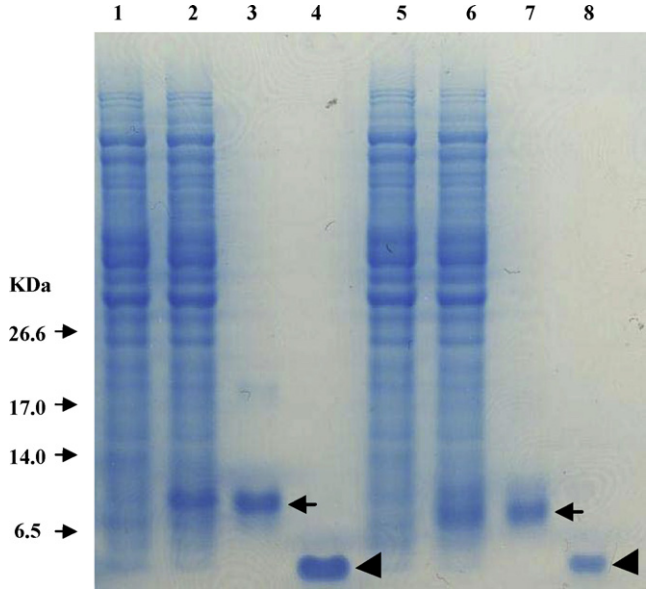

Fig. 3. Recombinant proteins expressed from pET30-EK/LIC-SpStrongylocin 1 and pET30-EK/LIC-SpStrongylocin 2. Soluble protein from uninduced *E. coli* C43 cells harboring the plasmid pET30-EK/LIC-SpStrongylocin 1 (lane 1) and pET30-EK/LIC-SpStrongylocin 2 (lane 5). Soluble protein from induced cells with 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^{2+} 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)[a]			
	L. anguillarum	*E. coli*	*C. glutamicum*	*S. aureus*
Recombinant SpStrongylocin 1	15.0	7.5	7.5	15.0
Recombinant SpStrongylocin 2	15.0	7.5	3.8	15.0
Cecropin P1[b]	0.8	0.8	0.4	100.0
Cecropin B[b]	0.4	0.4	0.4	1.6

[a] 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.
[b] Cecropin P1 and cecropin B were used as control peptides.

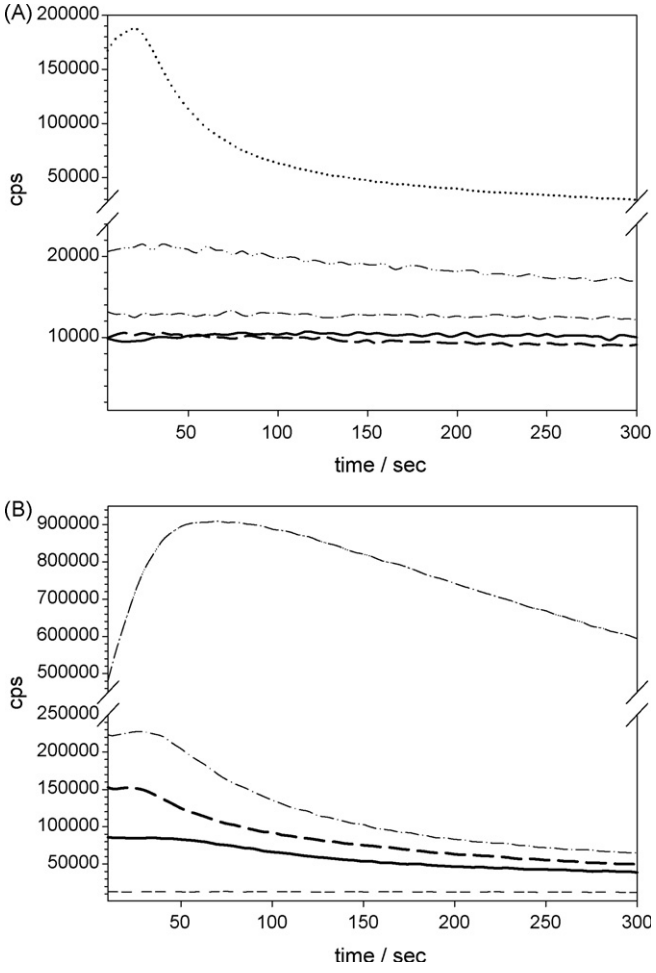

Fig. 4. Effect of SpStrongylocin 1 and SpStrongylocin 2 on bacterial membrane integrity. Perforation of the plasma membrane causes an influx of externally added D-luciferin into luciferase expressing *E. coli* cells and results in light emission. Light emission kinetics of *E. coli* cells treated with 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.

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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 disrupt 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 μM, 2 μM and 1 μM) 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 α-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.

Acknowledgements

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Centrocins: isolation and characterization of novel dimeric antimicrobial peptides from the green sea urchin, *Strongylocentrotus droebachiensis*

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Abstract

As immune effector molecules, antimicrobial peptides (AMPs) play an important role in the invertebrate immune system. Here, we present two novel AMPs, named centrocins 1 (4.5 kDa) and 2 (4.4 kDa), purified from coelomocyte extracts of the green sea urchin, *Strongylocentrotus droebachiensis*. The native peptides are cationic and show potent activities against Gram-negative and Gram-positive bacteria. The centrocins have an intramolecular heterodimeric structure, containing a heavy chain (30 amino acids) and a light chain (12 amino acids), which is linked by a single cysteine disulfide bond. The cDNA encoding the peptides and genomic sequences were cloned and sequenced. One putative isoform (centrocin 1b) was identified and one intron was found in the genes coding for the centrocins. The full length protein sequence of centrocin 1 consists of 119 amino acids, whereas centrocin 2 consists of 118 amino acids which both include a preprosequence of 51 or 50 amino acids for centrocins 1 and 2, respectively, and an interchain of 24-amino-acid between the heavy and light chain. The difference of molecular mass between the native centrocins and the deduced sequences from cDNA suggests that the native centrocins contain a post-translational brominated tryptophan. In addition, two amino acids at the C-terminal, Gly-Arg, were cleaved off from the light chains during the post-translational processing. The separate peptide chains of centrocin 1 were synthesised and the heavy chain alone was shown to be sufficient for antimicrobial activity. The genome of the closely related species, the purple sea urchin (*S. purpuratus*), was shown to contain two putative proteins with high similarity to the centrocins.

Key words: sea urchin, echinoderm extracts, antimicrobial peptides, innate immunity, marine bioprospecting, intramolecular heterodimer.

1. Introduction

Antimicrobial peptides play an important role in the host immune system as the first line of defence against invading pathogenic organisms. AMPs, commonly characterized as short cationic amino acid sequences, have been discovered and isolated from organisms belonging to a wide variety of animal phyla. Many AMPs are derived from inactive precursor molecules that include the mature active form, a presequence (signal peptide) and/or a prosequence. The preprosequence has a crucial function in the precursor before the AMPs are processed into mature products. In most cases, the signal peptide aids the translocation of the precursor within the cell [1, 2]. The prosequence, either located at the N-terminal, the C-terminal or within the mature sequence, may act as an intramolecular steric chaperone during the folding process. In addition, it may prevent interactions with other proteins or lipid membranes during intracellular trafficking [3, 4]. The prosequence has been proved to be necessary for proper protein folding [5], and has been shown to be essential for the production of recombinant *phormia* defensin A in a yeast heterologous system [6].

Sea urchins have a complicated immune gene repertoire that suggests markedly high diversity of immune molecules [7-10]. Analysis of the *S. purpuratus* genome has revealed the presence of a number of immune response receptors and immune mediators, including 222 Toll-like receptor (TLR) genes, 203 NACHT domain-LRR (NLR) genes, 218 scavenger receptor genes, and genes coding for key components of the complement system and peptidoglycan-recognition proteins [7, 10]. Furthermore, a large gene family, called 185/333, putatively encode proteins that likely have some association with immune function [8, 11].

Haug et al. detected antibacterial activity against Gram-positive and Gram-negative bacteria in coelomocyte extracts of the green sea urchin *S. droebachiensis* [12]. From a coelomocyte extract, we have isolated and characterized two AMPs named strongylocins 1 and 2, which contain six cysteine residues forming three disulfide bridges to stabilize the molecular structure [13]. Here we report the isolation and characterization of two other novel

AMPs, named centrocins, from the coelomocytes of *S. droebachiensis*. The combined information from the partial N-terminal amino-acid sequences (obtained by Edman degradation) and the coding sequences (obtained by constructing and screening a coelomic cDNA library) indicates that centrocins have a heterodimeric structure. Characterization of the gene was performed by sequencing the corresponding genomic DNA. The native centrocins showed potent activity against Gram-positive and Gram-negative bacteria. Furthermore, bioactivity studies of the synthesized peptides showed that only the heavy chain displayed the antimicrobial activity, not the light chain or the interchain that is located between the heavy chain and the light chain in the precursor molecule.

2. Materials and methods

2.1. Microbial strains

The Gram-negative bacteria *Escherichia coli* (ATCC 25922), *Listonella* (*Vibrio) anguillarum*, serotype O2 (FT 1801 or AL 104/LFI 6004), 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 (Difco Laboratories, Detroit, Il).

The yeast strains *Saccaharomyces cerevisiae* sp and *Candida albicans* (ATCC 10231) and the filamentous strains *Botrytis cinerea* 101 and *Penicillium rogveforti* were employed for antifungal activity assays [14].

2.2. Isolation and purification of antimicrobial peptides from coelomocytes of *S. droebachiensis*

Green sea urchins *(S. droebachiensis)* were captured off the coast of Tromsø, Norway, and cultured in fresh flowing seawater until sample collection. Antimicrobial peptides were extracted and purified from 54.7 g freeze-dried coelomocytes as previously described (Li et al., 2008 [13]). Briefly, coelomocytes from 66 sea urchins were extracted twice with 10 volumes (v/w) of 60% (v/v) acetonitrile (ACN; high performance liquid chromatography (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. [15]. The 40% ACN elute from SPE was subjected to reverse phase-HPLC (RP-HPLC) and separated on a SymmetryPrep C_8 (90Å; 7 μm; 7.8 x 150 mm; Waters, Milford, MA) column using a linear gradient of 0-60% ACN containing 0.05% TFA. HPLC fractions (peaks) were collected manually and tested for antibacterial activity against *L. anguillarum* and *C. glutamicum* as previously described [15]. Active fractions were further purified using a Symmetry Shield RP_{18} (90Å; 5 μm; 4.6 x 250 mm; Waters, Milford, MA) column.

2.3. Protein characterization

The molecular masses of the peptides were measured by electrospray ionization mass spectrometry (ESI-MS) as previously described [13]. Briefly, a Quattro LC triple quadrupole instrument equipped with an ESI ion source (Micromass, Wythenshawe, UK) was employed. Samples, dissolved in 95% (v/v) methanol containing 0.02% formic acid, were infused into the instrument at 10 μl min^{-1} and analyzed in positive ESI mode with a capillary voltage of 3.2 kV and a cone voltage of 40 V. 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.

Reduction and alkylation of peptides, proteinase digestion, purification of fragments by RP-HPLC, Edman degradation sequencing of alkylated peptides, and ESI-MS were performed at Eurosequence B.V. (Groningen, The Netherlands). Edman degradation analysis of the purified peptides revealed double signals of amino acids in each position, suggesting that these peptides were composed of more than one peptide chain. A portion of the peptide eluting in fraction 17 was therefore subjected to alkylation in the gas phase using 4-vinylpyridine. Two heterologous peptide chains (a heavy and a light chain) of the peptide were observed and partly separated by HPLC and subjected to Edman degradation. Since the pyridylethylated fragments of fraction 17 were not easily separated by HPLC, it was decided to alkylate the peptide with iodoacetamide, which converts Cys into carboxyamido-methylcysteine (CAM-Cys). Next, the alkylated sample was subjected to digestion with Endoproteinase Lys-C (ELC), an enzyme which cleaves at the carboxyl end of lysine residues. The resulting digest was separated by HPLC and analysed by Edman degradation. A portion of fraction 20 was directly subjected to alkylation using iodoacetamide. A heavy and light chain were separated by HPLC and subjected to Edman degradation.

To determine the unknown residue in position 2, a three-step manual Edman peptide degradation was conducted according to Zhang and Cockrill [16]. In brief, dried peptide was reconstituted in 10 µl water and incubated for 30 min. To this, 40 µl anhydrous pyridine, 5 µl PITC (phenyl isothiocyanate, 5% in heptane) and 5 µl *N*-methylpiperidine (25% in 15% water and 60% 1-propanol, v/v) was added, and the vial was filled with dry nitrogen and incubated for 5 min at room temperature. The mixture was then dried completely by vacuum centrifugation (<1 mbar at 30 °C and 2000 min^{-1} for 60 min). A volume of 10 µl anhydrous TFA was added to the dried PITC-coupled peptide and the mixture was incubated for 2 min at

50 °C. Finally, the amino acid was converted to its phenylthiohydantoin (PTH) derivative by adding 30 µl water and incubation at 64 °C for 10 min. This procedure was conducted twice. The final product was analysed by HPLC-ESI-MS, using a 2695 HPLC, equipped with a 2 mm inner diameter and 10 cm long SunFire column (particle diameter 3 µm) and a ZQ single quadrupole mass spectrometer, all from Waters (Milford, MA, USA). The PTH-amino acids were eluted by running a gradient of acetonitrile in water (both containing 0.1% formic acid) from 5 to 90% over 25 min. The mass spectrometer was operated in positive ESI mode and ions were recorded in full scan mode in the range *m/z* 200-500. The capillary and cone voltages were 3.3 kV and 35 V, respectively. Nitrogen was used as nebulizer (max flow), desolvation (1100 L h^{-1} at 350 °C) and cone (50 L h^{-1} at 110 °C) gas.

5- and 6-Bromotryptophan (Sigma-Aldrich, Oslo, Norway) were converted to their PTH-derivatives by the procedure outlined above.

2.4. Characterization of cDNA and gene sequences

The coelomocytes used for cloning experiments were collected and stored as previously described by Li *et al*. [13]. Total RNA was extracted from the coelomocytes by the QIAZol™ reagent (QIAGEN, Hilden, Germany), as recommended by the manufacturer. Messenger RNA was purified from extracted total RNA by Oligotex mRNA Midi kit (QIAGEN, Hilden, Germany).

Reverse transcription polymerase chain reaction (RT-PCR) was performed using Tagman® Gold RT-PCR kit (Applied Biosystems, Branchburg, NJ). Briefly, the single stranded cDNA was synthesized from a 70 ng mRNA sample using modified oligo (dT) primer G413 (Table 1), according to the method previously described [13].

Degenerate oligonucleotide primed PCR (DOP-PCR) was conducted for cloning partial cDNA sequences as previously described [13]. Briefly, the single stranded cDNA was employed as a template in PCR (7 cycles, 94 °C for 25 s, 42 °C for 25 s, 72 °C for 1 min; then 30 cycles, 94 °C for 25 s, 55 °C for 25 s and 72 °C for 1 min) with 400 ng of the forward degenerate primers 128F or 143F, and 1 μM of the reverse primer G479 (Table 1). The purified product from degenerative PCR was cloned into a pGEM®-T Easy Vector (Promega**,** Madison, WI). The primers SP6 and T7 (Table 1) were employed to sequence the plasmid inserts using the Big-Dye™ version 3.1 kit (Applied Biosystems, Foster City ,CA). Sequence data were analysed using an automated capillary DNA sequencer (Applied Biosystems, model 3730, Foster City, CA).

To obtain the full length of the cDNA sequence of the AMPs, a coelomic cDNA library was constructed and screened following a previously described method [13]. The ^{32}P labeled probes were transcribed from 25 ng linearized plasmid with the target gene as a template using the Redi prime™ II, Random prime labeling system (Amersham Biosciences, Buckinghamshire, UK). The positive clones were sequenced using the primers T7 and T3 (Table 1).

Based on the cDNA sequences, the primers 1512F/R (for centrocin 1) and 146F/144R (for centrocin 2) (Table 1) were designed for studying the gene structure. Genomic DNA (100 ng) was employed as a template in PCR (25 cycles, 94 °C for 30 sec, 58 °C for 30 sec and 72 °C for 3 min). The PCR products were cloned into a pGEM®-T vector and sequenced using the following primers: Sp6, T7, 172 F and 172R (Table 1).

2.5. Peptide synthesis

The proposed sequence of the light chain and the heavy chain (containing 5-Bromo-D/L-tryptophan) of centrocin 1 were synthesized at BIOMOL International, LP (Exeter, UK). The interchain of centrocin 1 corresponding to the 24-mer peptide (SPEEARVKILTAIPEMREEDLSEE) was synthesized at Thermo Biopolymer (Ulm, Germany).

2.6. Antimicrobial assays

Antibacterial activities of the HPLC fractions and purified and synthetic peptides were measured using a Bioscreen C microbiology reader (Labsystems Oy, Helsinki, Finland). Antibacterial activity was determined when the optical density (OD) of the growth control (bacteria plus water) reached an absorbance of approximately 0.3. Fractions were regarded as active when the OD was less than 50% of the growth control.

The fungi were cultivated on potato dextrose agar, supplemented with 2% glucose, at room temperature. Before antifungal testing, the fungi were dissolved in potato dextrose broth (Difco) at half-strength. Cell concentration was determined and adjusted and the test performed as described by [14]. Peptides, dissolved in 50 µl of distilled water, were tested at final concentrations (two-fold dilutions) ranging from 50 to 0.1 µM. The fungal cultures were grown in the dark (without shaking) and in a moist chamber at 25°C (37°C for *C. albicans*). Growth inhibition was determined microscopically after 48 h of incubation. The minimal inhibitory concentration (MIC) against bacteria, yeasts and fungi was determined as the lowest concentration of peptide resulting in 50% inhibition of visible growth compared to the control (fungal spores, yeasts or bacteria plus water).

2.7. Bioinformatics Analyses

Peptide masses, deduced amino acid sequences, and isoelectric points were predicted by the Expert Protein Analysis System (ExPASy) proteomics server of the Swiss Institute of Bioinformatics (http://www.expasy.org/). The BioEdit Sequence Alignment Editor software was employed for sequence alignments [17]. SignalP3.0 (http://www.cbs.dtu.dk/services/SignalP/) software was used to predict the potential cleavage site(s) of the signal peptides [18]. Sequence similarity searches were performed with the BLAST software on the NCBI homepage (http://www.ncbi.nlm.nih.gov/BLAST) and the sea urchin genome project homepage in the Human Genome Sequencing Center (http://www.hgsc.bcm.tmc.edu/projects/seaurchin/). A helical wheel modeling analysis was carried out on the HeliQuest server (http://heliquest.ipmc.cnrs.fr/) to predict hydrophobic and hydrophilic regions within the secondary structure of the centrocins [19].

3. Results

3.1. Isolation of antibacterial peptides from *S. droebachiensis* coelomocytes

In a previous study [12], we detected antibacterial activity in a 40% SPE fraction of a coelomocyte extract from the green sea urchin *S. droebachiensis*. Recently, we isolated two AMPs (named strongylocins) from this SPE fraction using RP-HPLC methodology [13]. A number of other active HPLC fractions were also observed in the previous study. In the present study, these fractions were further purified by analytical RP-HPLC. Two additional homologous AMPs, corresponding to the active fractions 17 and 20 (Fig. 1), were isolated and named centrocins 1 and 2 after the name of the genus *Strongylocentrotus*. Distinct UV absorbance at 280 and 297 nm (data not shown) suggested that both these components

contained tryptophan residues. Mass measurement by ESI-MS (positive mode) of centrocins 1 and 2 revealed that these peptides had average molecular weights of 4488.30 and 4396.27 Da, respectively (Fig. 2 A and B).

3.2. Partial amino acid sequences

Edman degradation analysis of the purified peptides revealed multiple signals of amino acids in each position (data not shown), suggesting that these peptides were composed of two or more peptide chains. The peptides were therefore subjected to alkylation and ELC treatment, followed by RP-HPLC purification of the peptide fragments and successive Edman degradation. Several fragments were obtained from centrocin 1 (fraction 17), and by peptide mapping, two peptide chains (one heavy chain and one light chain) were identified (Fig. 3A). Both peptide chains contain one cysteine residue, suggesting that they are connected by a disulfide bridge, thereby making a dimeric structure of the mature peptides. The fragment having the sequence of SGIHAGQRGCSALGF was further analysed by Nanospray ESI-MS, in order to establish its exact mass, and in order to investigate if the C-terminal residue of the heavy chain was indeed phenylalanine. The observed mass (doubly charged m/z ion at 759.38; spectrum not shown) was consistent with a C-terminal Phe-containing peptide structure (monoisotopic mass: 1517 Da), therefore unambiguously determining the structure of this Lys-C peptide was originated from the C-terminal part of the heavy chain of centrocin 1.

The heavy chain of centrocin 1 contained an unknown (probably post-translationally modified) residue at position 2. An N-terminal fragment having the sequence XXFKKTFHKVSHAVKS was characterised after proteinase treatment. The first amino acid was denoted unknown due to multiple signals, caused by impurities. Obviously, the lysine

residues were not cleaved by ELC. The unknown residue and perhaps somewhat bulky residue at position 2 prohibited efficient cleavage of the Lys residues. The calculated mass of the dimeric peptide (without the unknown residue) was 4222.86 Da, some 265.44 Da short of the measured mass (4488.30 Da). The missing mass was close to a brominated tryptophan residue, having a mass of 265.11 Da.

Centrocin 2 (fraction 20) was directly subjected to alkylation using iodoacetamide, and a heavy and light peptide chain were separated by HPLC and subjected to Edman degradation analysis. Both peptide chains contained one cysteine residue, which together probably formed a disulfide bridge, thereby making a dimeric structure of the mature peptide (Fig. 3B). The heavy chain, similar to centrocin 1, contained an unknown (probably post-translationally modified) residue at position 2. Furthermore, there were likely one or two amino acids missing at the C-terminal end of one of the chains after comparison of the measured molecular mass and the calculated one.

An effort to identify the unknown amino acids in the N-terminal position of centrocin 2 was made by subjecting the peptide to two rounds of manual Edman degradation. HPLC-MS analysis of the first degradation revealed an ion at *m/z* 223, which corresponds to PTH-serine [20]. The second round gave two equally abundant ions at *m/z* 400 and 402 (Fig. 4A), which support the presence of a brominated tryptophan. The mass spectrum of the PTH-derivative of 5-bromotryptophan, included here as a reference brominated tryptophan compound (Fig. 4B), also shows only peaks at *m/z* 400 and 402 in the recorded range of *m/z* 200-500. These results suggest that the N-terminal sequence of centrocin 2 is Ser-1 and (Br)-Trp-2.

3.3. Characterization of cDNA and gene sequences of the centrocins

The cDNA of centrocins were cloned using a combination of DOP-PCR and screening of

a cDNA library from the coelomocytes. Two genes corresponding to centrocin 1 and one gene corresponding to centrocin 2 were isolated and sequenced. The full length cDNA of centrocin 1 includes a 5'-UTR of 108 bp (1a) or 79 bp (1b), an open reading frame of 357 bp, and a 504 bp 3'-UTR. Centrocin 2 includes a 108 bp 5'-UTR, an open reading frame of 354 bp, and a 112 bp 3'-UTR (Fig. 5A). The genomic DNA sequence analysis showed that centrocins 1a, 1b and 2 have one intron and two exons (Fig. 5B). The intron in the centrocins contained 1338 bp (centrocin 1a), 1323 bp (centrocin 1b) or 760 bp (centrocin 2) nucleotides.

3.4. Characterization of the structure of centrocins

The complete deduced peptide sequences of centrocins 1a, 1b and centrocin 2 contain 119, 119 and 118 amino acids, respectively (Fig. 6A). According to MS and Edman degradation analysis, the mature peptides have a dimeric structure starting from Gly-52 in centrocin 1 and Ser-52 in centrocin 2 and proceed with 30 amino acids in the heavy chain (Fig. 6B). The light chain starts from Asp-105 in centrocin 1a, Gly-105 in centrocin 1b and Asp-104 in centrocin 2, and proceed with 12 amino acids. The deduced amino-acid sequence of centrocins 1 and 2 have the calculated mass about 4409.1 and 4317.0 Da including the formation of the dimeric structure by a disulfide bond (ExPASy amino-acid modification page). The deduced amino acid sequences indicate that the second amino acid residues of the native centrocins are tryptophans. Both peptides are about 79 Da less than the mass measured by mass spectrometry (4488.3 and 4396.3 Da, Fig. 2). It has been demonstrated that centrocin 2 has bromotryptophan (Fig 4). If the Trp-2 is the only modified amino acid in centrocin 1, the discrepancy of 79 Da is mostly due to bromination [13]. In addition, tryptophan lacks available hydroxyl-groups needed for sulfation or phosphorylation in its chemical structure.

Analysis by SignalP 3.0 indicated that a cleavage site of a signal peptide was located between amino acid Ala-20 and Lys-21 for centrocin 1 and 2 using both the neutral network model and the hidden Markov model [18]. Thus, a precursor peptide contained a signal peptide, a prosequence region followed by the heavy chain, another prosequence region (interchain peptide), the light chain and a C-terminal dipeptide (Gly-Arg). The calculated *pI* increased from 5.12, 5.30 and 4.95 in the precursors to 10.06, 10.06 and 9.69 in the mature peptides for centrocin 1a, centrocin 1b and centrocin 2, respectively, after the preprosequences have been cut off.

BLAST searches against the *S. purpuratus* genome database identified two putative proteins with similarity to the centrocins (Fig. 6A). The centrocins shared 75-80% identity at an amino acid level with the putative protein gi: 115924292, whereas they shared 74-80% identity at an amino acids level with the C-terminal region of the putative protein gi: 115772610. In addition, the alignment of centrocins and the putative proteins from *S. purpuratus* showed that there were three conserved regions of peptides, an identical signal peptide, the high similarity of the prosequence and the interchain regions as well.

Helical wheel modeling of the centrocin heavy chains (Fig. 7) showed that the polar and non-polar (hydrophobic) residues form two distinct clusters, which may give the peptides amphipathic structures. The hydrophobic side includes the conserved amino acids Trp-2, Phe-3, Val-10, Ala-13, Val-14, Ile-18, Ala-20, Cys-25 and Leu-28. All the charged residues are highly co-localized on the polar side of the molecules.

3.5. Antibacterial activity

Purified centrocins 1 and 2 were tested against both Gram-positive and Gram-negative bacteria, fungi and yeast to evaluate their capability of inhibiting microbial growth (Table 2).

The data showed that both centrocins display potent activity (MIC ranging from 1.3 to 5 μM) against all bacterial strains tested (Table 2). Due to limited amounts of purified native centrocins, we synthesized the heavy chain and the light chain of centrocin 1 and tested them separately for antimicrobial activity. The results showed that the heavy chain alone was responsible for the activity against bacteria, fungi and yeasts (MIC ranging from 0.8 to 50 μM), while the light chain displayed no activity against the microbes (Table 2). We also synthesized and tested the 24-mer interchain peptide (the second prosequence) of centrocin 1, but it did not show activity against the tested microbes.

Discussion

In our previous studies, we identified and characterized the cysteine-rich AMPs, named strongylocins, from *S. droebachiensis* [13] and *S. purpuratus* [21]. Here we present the isolation and characterization of two novel heterodimeric AMPs, named centrocins 1 and 2, from *S. droebachiensis*. *In silico* analysis of peptide sequences indicates that the first 20-amino-acid region in the precursor functions as a signal peptide. Signal peptides likely play an essential role for most secretory proteins, guiding them during translocation [1, 22]. Additionally, the data shows that the precursor molecules have the first prosequence region with 30 or 29 amino acids (centrocins 1 or 2) followed by the heavy chain sequence and the second prosequence region with 24 amino acids followed by the light chain sequence of the mature form. Some of the prosequences seem to have a function in folding of the mature portions as intramolecular chaperones [4]. For example, in the production of recombinant subtilisin E in a *E. coli* heterologous system, the prosequence seem to have an essential role in guiding appropriate folding of enzymatically active conformations [23]. Furthermore, the replacement of the insect prosequence by that of the yeast Mating Factor α-1 does not affect the production yield of the recombinant *phormia* defensin A, whereas a partial deletion of the

prosequence is deleterious to the secretion of the biologically active material [6]. The expression of the recombinant antifungal protein also supports the importance of prosequence for obtaining proper folding [5]. Two prosequences in centrocins may therefore be involved in the formation of their proper folding during their maturation. In addition, it is known that the prosequences in some proteolytic enzyme precursors inhibit the activity of the mature proteins [24]. The calculated *pI* in the centrocins (1a, 1b and 2) increases from 5.0-5.3 to 9.7-10.1 after the preprosequences have been cut off. This indicates that the prosequences of centrocins, composed of many acidic amino acid residues, introduce negative charges which might keep the precursors inactive before they are processed into mature cationic products.

The Edman degradation sequences of centrocins show that the light chain consists of 12 amino acids with Ala-Leu as the C-terminal amino acids. However, according to the deduced sequences from cDNA, there are two additional amino acids C-terminally, Gly-Arg, which are cleaved off during a post-translational modification. This phenomenon has been described in the tachyplesin precursor from the horse shoe crab (*Tachypleus tridentatus*) [25], aureins (excluding aurein 5.3) from *Litoria aurea* [26], astacidin 2 from the fresh water crayfish (*Pacifastacus leniusculus*) [27] and a cecropin-like peptide from *Galleria mellonella* [28]. The C-terminal part of their deduced precursors contains an amidation signal 'Gly-Lys-Arg' or 'Gly-Lys' that is cleaved off by a specific protease, leading to C-terminal amidation of the active peptides. In contrast to the above mentioned peptides, the centrocin precursors have a 'Gly-Arg' sequence C-terminally, which is cleaved off in the active, purified peptides. This cleavage does not introduce any C-terminal amidation of the active peptides, according the MS analysis. Similar results were also found in arasin 1 from *Hyas araneus* where a C-terminal dipeptide extensions (Arg-His) were cleaved off from the mature active peptide [29].

Comparison of the peptide sequences obtained by Edman degradation and the deduced sequences from cDNA, indicates that the only modified residue in the heavy chain of the

centrocins is a tryptophan in position 2. Although the MS data of centrocin 2 confirm that this post-translational modification happens at Trp-2 by bromination, we do not know the position of the bromine atom within the tryptophan residue. Tryptophan contains an indole group, and the MS analysis of chloroindoles gave identical spectra for all the six possible isomers [30]. Brominated tryptophans will presumably exhibit a similar behavior during mass spectrometric analysis. This is supported by the analysis of PTH-6-Br-Trp that only showed *m/z* 400 and 402 (results not shown). Therefore, the position in which the bromine is attached to the indole ring cannot be determined at this stage. Bromination of tryptophan or its derivatives appears to occur widely in marine organisms [31]. Several AMPs containing a brominated tryptophan have been isolated, like styelin D from the marine tunicate (*Styela clavata*) [32], cathelicidins from the Atlantic hagfish (*Myxine glutinosa*) [33], hedistin from the marine annelid (*Nereis diversicolor*) [34] and strongylocins from the green sea urchin (*S. droebaciensis*) [13]. Although the biological role of bromotryptophan in peptides is generally unknown, the presence of bromine could transfer the tryptophanyl side chain into a poorer substrate for endogenous proteolytic enzymes [35]. Therefore, bromotryptophan in position 2 of the heavy chain may protect the centrocins from proteolysis, and/or enhance their antibacterial activity.

Comparison and analysis of Edman degradation sequences, fragment sequences generated by endoproteinase digestion and deduced sequences suggest that native centrocins have a heterodimeric structure linked by an intramolecular disulfide bridge (Fig. 6B). The dimeric structure has also been found in distinctin isolated from the frog *Phyllomedusa distincta* [36], dicynthaurin and halocidin isolated from hemocytes of *Halocynthia aurantium* [37, 38]. Interestingly, the light chain in centrocins does not contributes to any substantial positive charge to the molecules, a character regarded to be an important feature of AMPs against microbes [39]. This is in agreement with our antimicrobial activity testing of the synthesized heavy and light chain of centrocin 1. Therefore, the formation of an intermolecular disulfide

bridge may stabilize the AMPs tertiary structures and protect the backbone from proteolysis during translation and translocation within the coelomocytes [40-42]. In addition, peptide dimerization have been found, not only to cause a longer lifetime than a monomer, but also to form a slightly larger pore diameter [43].

Helical wheel modeling of the centrocin heavy chains indicated that these sequences have an amphipathic structure, a feature common for most AMPs. However, it has to be noted that this hypothesis has to be confirmed by direct experimental observation, such as circular dichroism and nuclear magnetic resonance spectroscopic studies.

AMPs are important host defense molecules in invertebrate immune system. The *S. purpuratus* genome shows the remarkable amount of immune related putative molecules [7-11]. Our studies demonstrate that sea urchins have diverse effector molecules in their immune system such as identified two novel groups of AMPs, strongylocins and centrocins. However, it is worth noted that two groups of AMPs have their own conserved signal regions and prosequence regions [13, 21]. It is likely that strongylocins and centrocins respond differently to the diverse pathogens or have a variety of mechanisms to be activated and to intracellularly migrate. It has been reported that the diversity of transcripts of immune effector 185/333 has varied considerably in response to different immunological challenges [8, 44]. The strongylocins and the dimeric centrocins show a wide spectrum antimicrobial activity which throws more light on the sea urchin immunity. Further studies are needed to determine the expression of such AMPs in coelomocytes and whether AMPs are also involved in embryonic immunity, in order to advance our knowledge of immune effector molecules in sea urchins.

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collecting animals for this study and we appreciate the help with the Quattro LC MS analysis from the staff at the Department of Pharmacy, University of Tromsø.

The centrocin sequences have been deposited in the GenBank with accession numbers GU212784, GU212785 and GU212786, respectively.

Table 1 Primers

Name	Description	Sequence
Sp6	Plasmid primer	5′CGATTTAGGTGACACTATAG
T7	Plasmid primer	5′CAGTGAATTGTAATACGACTCACT
T3	Plasmid primer	5′AATTAACCCTCACTAAAGGG
G413	Olig (dt) adaptor primer	5′TCTGAATTCTCGAGTCGACATCTTTTTTTTTTTTTTTT
G479	Adaptor primer	5′TCTGAATTCTCGAGTCGACATCTT
128F	Degenerate primer (centrocin 1)	5′TT(T/C)AA(A/G)AA(A/G) AC(A/T/G/C)TT(T/C)CA(T/C)AA(A/G)GT
143F	Degenerate primer (centrocin 2)	5′TT(T/C)(A/T)(G/C)(A/T/G/C)(A/C)G(A/T/G/C)AC(A/T/G/ C)GT(A/T/G/C)CA(T/C)AA(T/C)GT(A/T/G/C)GG
1512F	Centrocin 1	5′TCATCTCACCCGCAACAAG
1512R	Centrocin 1	5′CACGATCCCTTCCGAGTCTA
146F	Centrocin 2	5′GCAGTTAGGAAAGGCATCCA
144R	Centrocin 2	5′CTAACGGCCAAGGGCATGTG
172F	Centrocin 1 intron	5′ACCTTTGGACCAGGTTCAATG
172R	Centrocin 1 intron	5′TCAGAATTTTAACGCCTAAGAT

Table 2 Susceptibility of the microbial test strains to antimicrobial peptides isolated from *S. droebachiensis* coelomocytes and synthesized peptides. Minimal inhibitory concentration (MIC) was determined as the lowest concentration of peptide causing an optical density less than 50% of the growth control.

Organisms	Minimal inhibitory concentration (μM)				
	Centrocin 1 native	Centrocin 2 native	Centrocin 1 Heavy chain[2]	Centrocin 1 Light chain[2]	Centrocin 1 Interchain[2]
Gram-negative bacteria					
L. anguillarum	2.5	2.5	0.8	>100	Nt
E. coli	1.3	2.5	1.6	>100	>100
Grame-positive bacteria					
C. glutamicum	1.3	1.3	0.4	>100	>100
S. aureus	2.5	5.0	3.1	>100	>100
Filamentous fungi					
B. cinerea	N. D.[1]	N. D.	50	>100	>100
P. rogveforti	N. D.	N. D.	6.3	>100	N. D.
Yeasts					
S. cerevisiae	N. D.	N. D.	6.3	>100	N. D.
C. albicans	N. D.	N. D.	6.3	>100	N. D.

[1] N. D. = not determined.
[2] The synthesized peptides

Figure 1 Purification of centrocins from *S. droebachiensis* coelomocytes by RP-HPLC. An extract from coelomocytes was pre-purified by solid phase extraction on Sep-Pak C_{18} cartridges. The fraction eluted with 40% acetonitrile was subjected to RP-HPLC using a semi-preparative C_8 column. Elution was performed with a linear gradient of 0-48% acetonitrile for 80 min at a flow rate of 2 ml/min. Two of these fractions (numbered peaks) with growth inhibiting activity against *Corynebacterium glutamicum* and *Listonella anguillarum* were submitted to further purification on an analytical C_{18} column (data not shown). The absorbance at 220 nm and the concentration of acetonitrile in the eluting solvent are indicated.

Figure 2 Positive ESI-MS of the purified peptides, centrocins 1 (A) and 2 (B). ESI-MS of centrocin 1 revealed multiple ions at *m/z* 562 $[M + 8H]^{8+}$, 642 $[M + 7H]^{7+}$, 749 $[M + 6H]^{6+}$, 899 $[M + 5H]^{5+}$, 1123 $[M + 4H]^{4+}$, indicating that the peptide has a molecular weight of approximately 4488 Da. ESI-MS of centrocin 2 revealed multiple ions at *m/z* 629 $[M + 7H]^{7+}$, 734 $[M + 6H]^{6+}$, 880 $[M + 5H]^{5+}$, 1100 $[M + 4H]^{4+}$, indicating that the peptide has a molecular weight of approximately 4396 Da.

Figure 3 Edman degradation sequencing of centrocins 1 and 2 after alkylation and proteinase treatment. N-terminal sequences were obtained after reduction and alkylation using 4-vinylpyridine (*S*-pyridylethylated) or iodoacetamide (CAM-Cys), followed by separation of the heavy and light chain by HPLC and Edman degradation of separate peptide chains. Peptide fragment sequences were obtained after Endoproteinase Lys-C (ELC) digestion, followed by separation of fragments by HPLC and Edman degradation of each fragment. X denotes unknown residues. One letter amino acid symbols represent centrocin 1 heavy chain and light chain (A) and centrocin 2 heavy chain and light chain (B), respectively.

Figure 4 Positive ESI-MS of the PTH-derivatives of (A) the amino acid in position 2 of centrocin 2 and (B) 5-Bromotryptophan. Both spectra show only two peaks at *m/z* 400 and 402, at equal abundance. This is in accordance with the presence of one bromine atom in the recorded ions, as Br naturally exists as two stable isotopes 79Br and 81Br, at respectively 50.5 and 49.5% relative abundance.

Figure 5 Alignment of the gene sequences of centrocins 1a, 1b and 2 from the green sea urchin *S. droebachiensis* (A) and their corresponding genomic DNA structure (B). The nucleotide numbers are indicated on the right side. The dot (·) below the sequence indicates positions where all sequences share the same nucleotide. The differences in the nucleotides in the alignments are shaded in gray and gaps (dash) are introduced to maximize the alignments. The translation start codon (atg) and stop codon (tag) are marked in bold. The genomic DNA of centrocins 1a, 1b and 2 contains two exons and one intron (not to scale). The regions encoding a signal peptide are indicated by striped box; the regions encoding prosequences with white color boxes; the regions encoding mature centrocin peptides with filled boxes; the intron region with gray color box. The sequences have been submitted to the GenBank with accession numbers GU212784, GU212785 and GU212786.

Figure 6 The alignment of the centrocins 1 and 2 from *S. droebachiensis* with two putative proteins obtained from the purple sea urchin, *S. purpuratus*, genome databank (A) and the proposed structure of centrocins 1 (B) and 2 (C). The predicted cleavage site between the signal peptide and the prosequences is shown by a solid triangle (▼).Identical residues are shaded in black, whereas similar residues are shaded in gray. The boxes indicate the heavy chain and the light chain regions. In the proposed structure of centrocins, the heavy chain and

the light chain are connected by disulfide bridges. The brominated tryptophan in position 2 in the active centrocins is labeled with Br on the top.

Figure 7 Helical wheel diagrams indicating amphipathic α-helical conformation of the heavy chain of centrocins 1 (A) and 2 (B). The hydrophobic residues are shaded in gray.

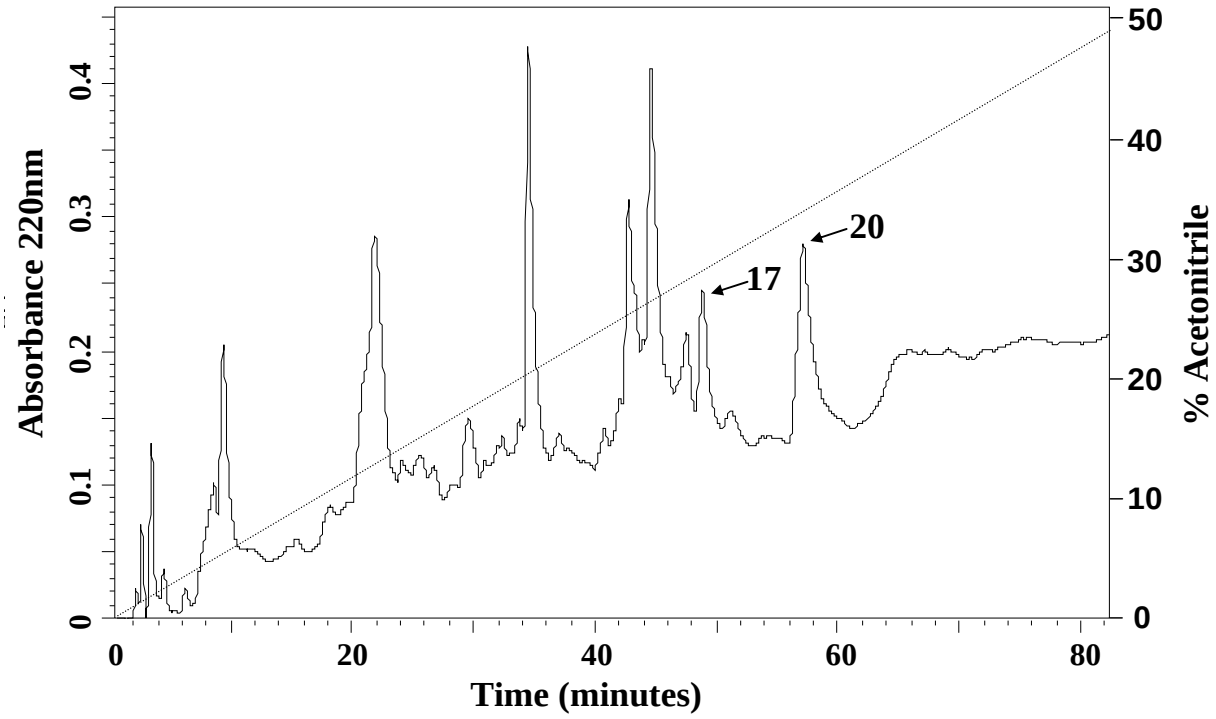

Absorbance 220nm
0
0.1
0.2
0.3
0.4
% Acetonitrile
0
10
20
30
40
50
0
20
40
60
80
Time (minutes)
17
20

Figure 1

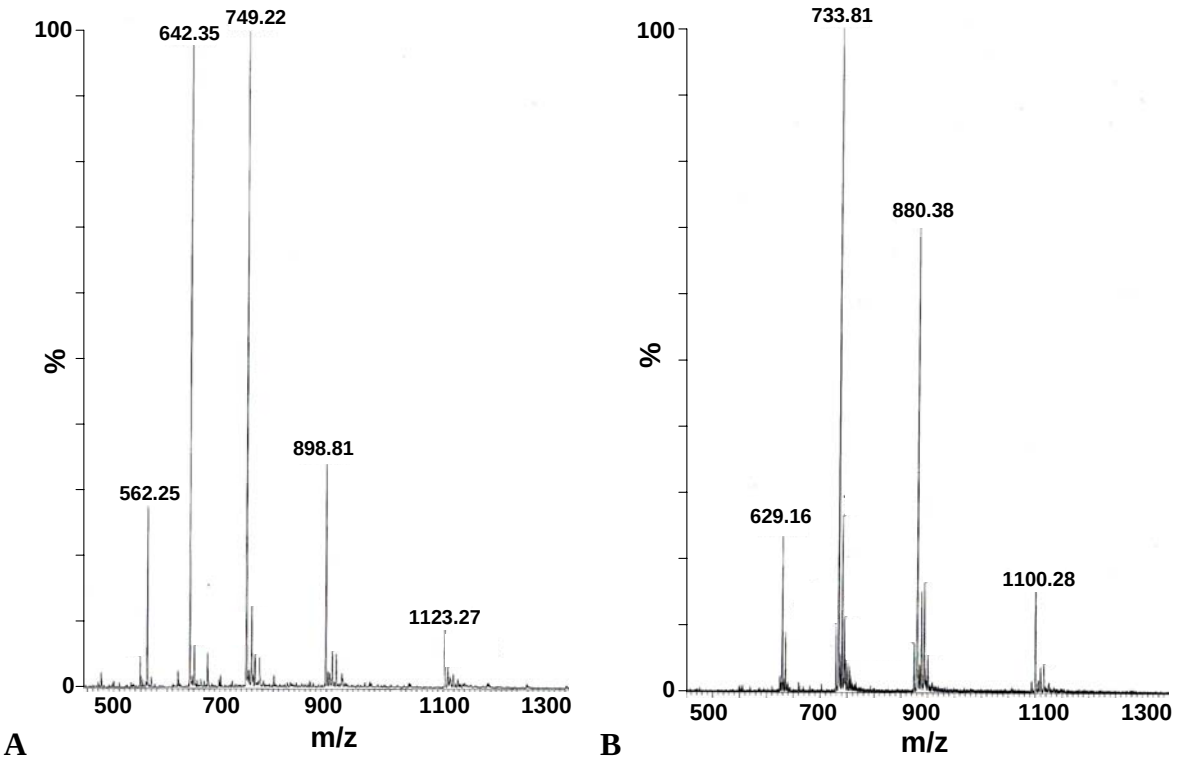
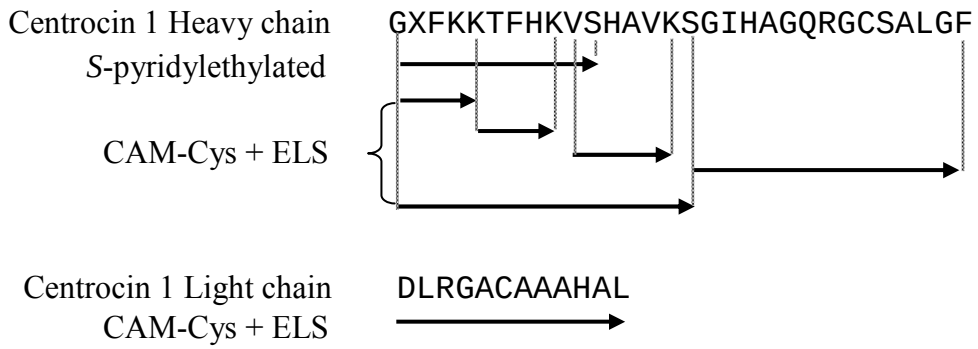


Figure 2 A and B

A



B

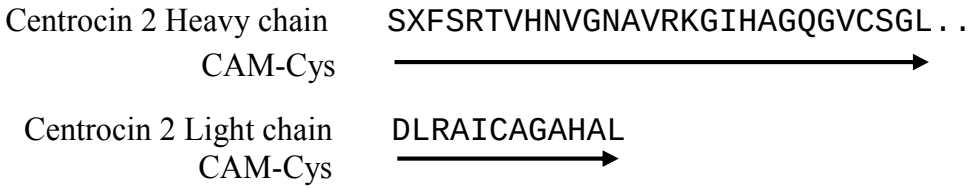
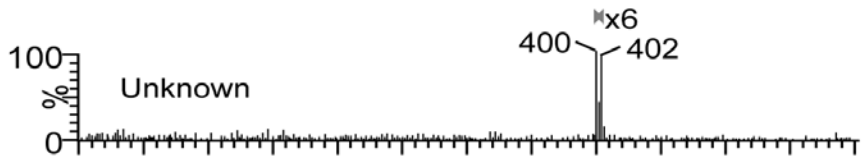
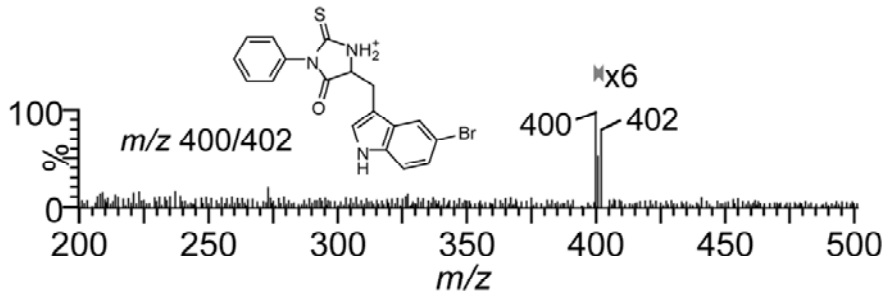


Figure 3 A and B



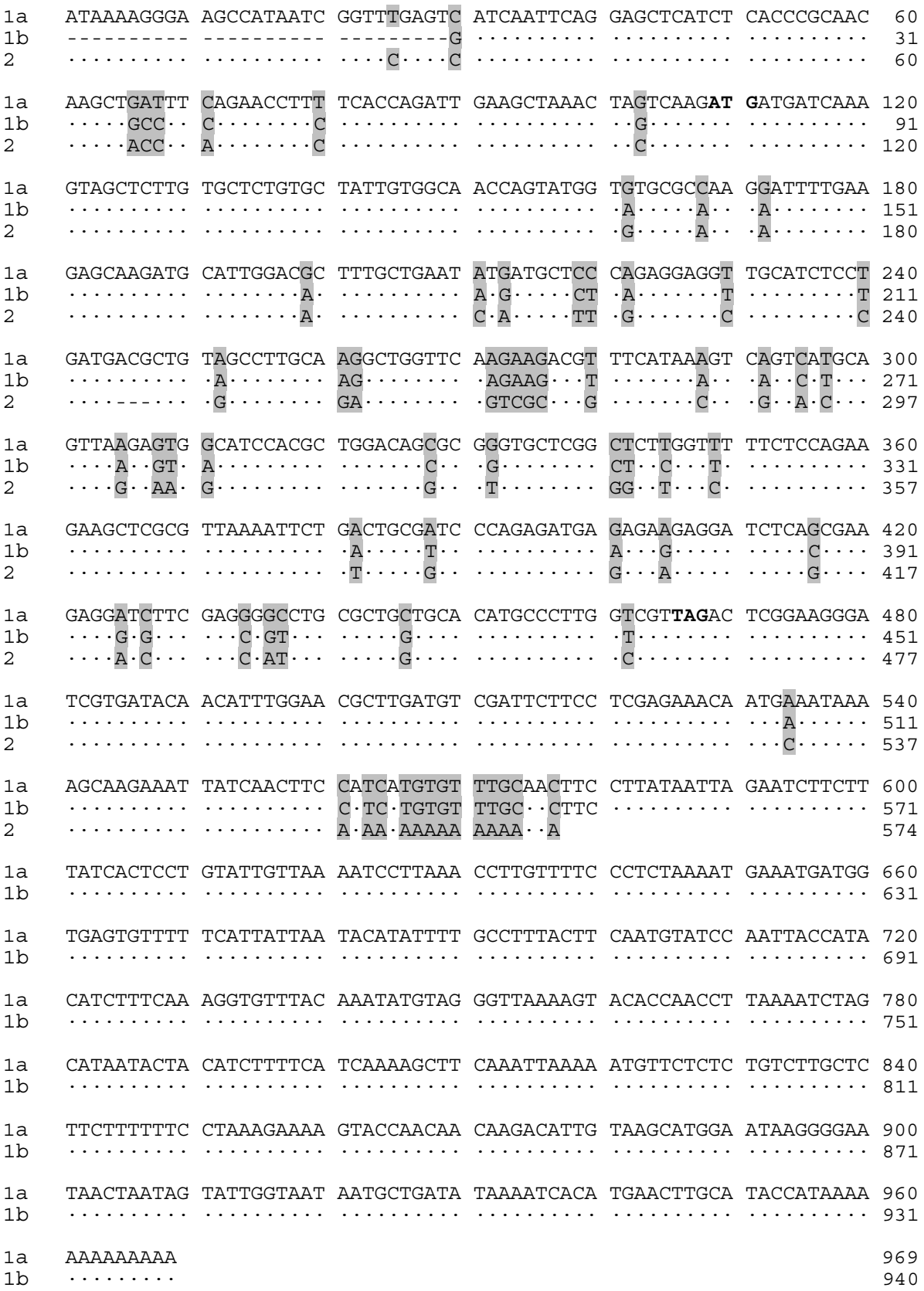
A



B

Figure 4 A and B

A Centrocins 1a/1b/2



B

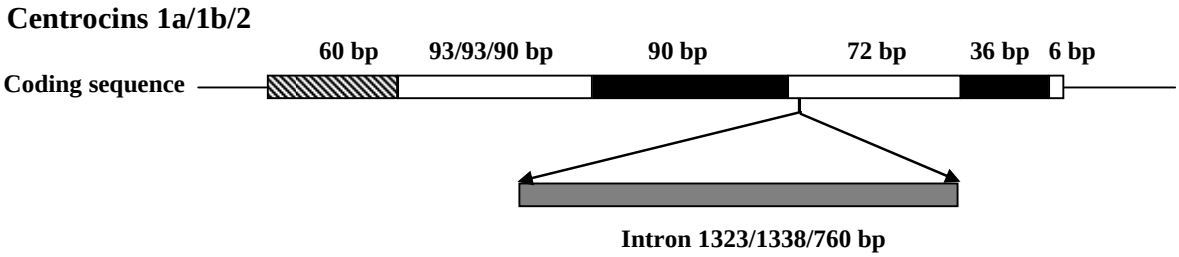
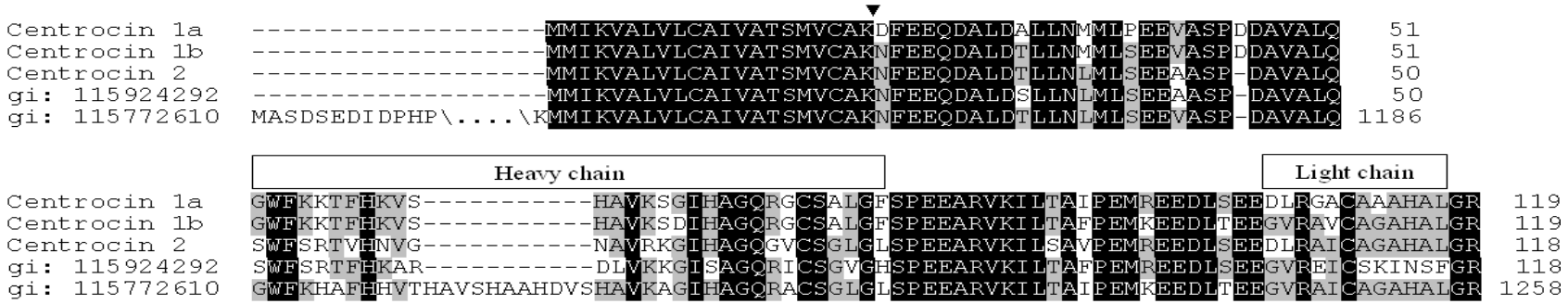


Figure 5 A and B

A



B

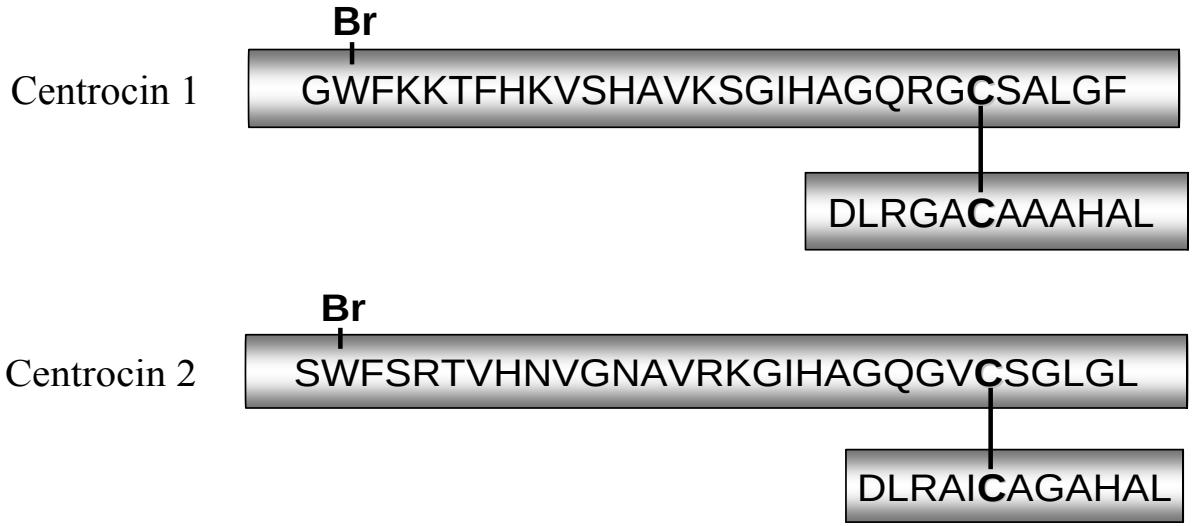
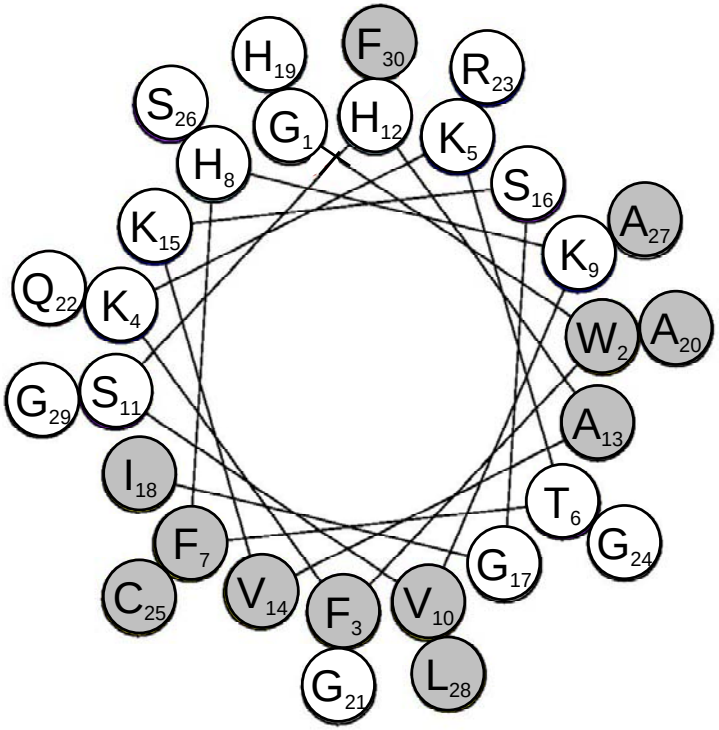
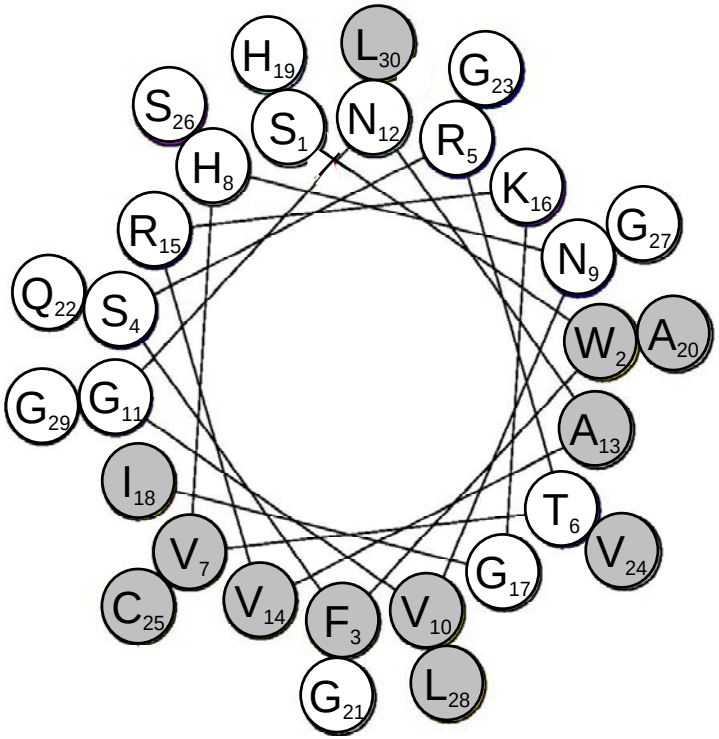


Figure 6 A and B



A



B

Figure 7 A and B

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Expression of antimicrobial peptides in coelomocytes and embryos of the green sea urchin (*Strongylocentrotus droebachiensis*)

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Abstract

Antimicrobial peptides (AMPs) play a crucial role in innate immunity. We have previously reported the isolation and characterization of the AMPs, strongylocins 1 and 2, and centrocin 1, from extracts of coelomocytes of *Strongylocentrotus droebachiensis*. Here we show that these AMPs are expressed in phagocytes. In addition, the transcripts of strongylocin 1 were detected in vibratile cells and/or colorless spherule cells, while the transcripts of strongylocin 2 were found in red spherule cells. Results from immunoblotting and immunocytochemistry studies showed that centrocin 1 was produced by phagocytes and stored in granular vesicles. Co-localization of centrocin 1 and phagocytosed bacteria suggests that the granular vesicles containing centrocin 1 may be involved in the formation of phagolysosomes. We analyzed the temporal and spatial expression of AMPs throughout larval development. Strongylocins were expressed in the early pluteus stage, while centrocin 1 was expressed in the mid pluteus stage. The spatial expression pattern showed that centrocin 1 was mainly located in the secondary mesenchyme cells (SMCs) forming the coelomic pounches around the stomach and the esophagus. In addition, a few patrolling SMCs were detected in some larval arms. Together, these results suggest that AMPs are expressed in different types of coelomocytes and that centrocin 1 is involved in response against bacteria. Furthermore, the expression of AMPs in larval pluteus stage, especially in SMCs, indicates that AMPs and SMCs are engaged in the larval immune system.

Keywords

Antibacterial peptide; strongylocin; centrocin; sea urchin; coelomocytes; embryo; larva; secondary mesenchyme cells; innate immunity

1. Introduction

Antimicrobial peptides are commonly found in the animal and plant kingdoms. They serve as the first line of active host defense against pathogens. So far there are more than 1200 AMPs recorded in a database for AMPs [1]. AMPs are commonly characterized by a short amino acid sequence (< 100 aa), positive net charge and an amphipathic structure [2]. AMPs are active against a wide range of pathogenic organisms like bacteria, fungi and viruses [3]. The elimination and inhibition of invading pathogens by AMPs is very important for invertebrates which depend on the innate immune system alone [4, 5].

Sea urchins have a simple anatomic structure. The organs, like intestine, gonads, nerve ring etc., are protected by a hard skeleton forming the coelomic cavity. Coelomocytes are circulating in the liquid (coelomic fluid) which is filling the coelomic cavity. They are considered to play an important role in immune responses like allograft rejection [6], bacterial clearance [7, 8], encapsulation and opsonisation [9]. Coelomocytes upregulate the transcription of *profilin* in response to injury and lipopolysaccharide (LPS). This results in cytoskeletal modifications or changes in cell shape following immune activation [10, 11]. There are four main subpopulations of coelomocytes: phagocytes, vibratile cells, colorless and red spherule cells (reviewed by [12]). Phagocytes carry out many immune related activities, such as encapsulation, opsonisation, graft rejection and antibacterial activity [9, 13-15]. Vibratile cells are associated with clotting and movement or agitation of coelomic fluid [16]. Red spherule cells contain echinochrome A which shows antibacterial activity [17]. The immune function of colorless spherule cells has not been identified yet. In our previous studies, coelomocyte extracts from the green sea urchin (*S. droebachiensis*) showed antibacterial activity against both Gram-positive and Gram-negative bacteria [18]. Four of the

active compounds were identified and characterized as antimicrobial peptides called strongylocins and centrocins [19, 20].

The genome of *S. purpuratus* has been sequenced [21] and shows that the complexity of immune-related genes in this organism is far beyond our anticipation, this applies for recognition receptors in particular [22]. However, very few immune effector molecules have been identified. Known immune effectors are the putative protein family 185/333 [23], echinochrome A [17] and antimicrobial peptides [19, 20]. Immune staining experiments showed that expression of 185/333 proteins is localized in phagocytes [24]. On the other hand, echinochrome A was identified as a pigment of red spherule cells [17]. Although both centrocins and strongylocins were originally isolated from coelomocytes, it was unknown whether these peptides are co-localized in phagocytes or not.

Moreover, only few studies have thrown light on the immunity of sea urchin larvae. It has been reported that larvae of the sea urchin (*Lytechinus pictus*) are able to pinocytize ferritin from the gut luminal cells [25]. Mid-gastrula stage embryos of *L. variegatus* were observed to phagocytose microinjected yeast [26]. In embryos of *S. purpuratus*, transcription of a homologue of the complement component C3 was upregulated after continuous exposure to heat killed pathogenic bacteria [27]. These results suggest that embryos also have a defense system that responds to stimuli. Although the AMPs have been isolated from coelomocytes of adult animals, the question is whether these molecules are produced in embryos and also involved in embryonic immunity.

In this paper we show that the different types of coelomocytes express AMPs differently. Transcription of strongylocin 1 was found in phagocytes, vibratile cells and/or colorless spherule cells, while strongylocin 2 was shown to be transcribed in phagocytes and red spherule cells. However, transcripts of centrocin 1 were only found in phagocytes. Our results from immunostaining experiments illustrate that centrocin 1 is located in the

cytoplasmic granules which are likely associated with phagocytolysis of bacteria. We also show that the transcripts of these AMPs were present from the pluteus stages and that the expression increased during the later developmental stages. In addition to that, we also show that centrocin 1 is localized in SMCs around the digestive tract and some SMCs are able to migrate to the arms as well.

2. Materials and methods

2.1. Animals and Bacterial strains

Green sea urchins *(S. droebachiensis)* were collected off the coast of Tromsø, Norway, and kept in fresh flowing seawater at 12 °C.

Escherichia coli strain DH10B containing the plasmid pBAD*RFP_{EC2} for expression of DsRed [28] was grown in LB broth or on LB plates containing 1.5% agar both with 100 μg/ml ampicillin at 37 °C. DsRed fluorescence was observed 4 h after inducing DsRed expression with 0.2% arabinose (at an OD_{600} of 0.6). These bacteria (10^4 per ml) were employed to challenge coelomocytes for 20 minutes *in vitro*.

Embryos and larvae were collected from a local sea urchin hatchery (Troms Kråkebolle AS, Tromsø, Norway). Batches of eggs for experiments were more than 98% fertilized. The larvae were maintained in fresh flowing sea water at 8 °C.

2.2. Coelomocyte preparation

Whole coelomic fluid (WCF) was withdrawn and mixed with an equal volume of ice-cold calcium and magnesium free anti-coagulating buffer containing 70 mM EDTA and 50 mM imidazole as described in [15].

In order to obtain different types of coelomocytes, WCF was separated by discontinuous gradient centrifugation (modified from Gross et al. [15]). Briefly, the iodixinol gradient (Optiprep, Oslo, Norway) was made by underlayering 5 ml of successively denser solutions (from bottom to top: 60, 30, 20, 10, and 5% of Optiprep) into a 50-ml centrifuge tube at 4 °C. Five ml of WCF in anti-coagulating buffer were added on top of the discontinuous gradient and centrifuged at 1500 g for 30 min at 4 °C. Four layers of coelomocytes were observed after centrifugation. From the bottom to the top, the layers contained red spherule cells, colorless spherule cells, vibratile cells and phagocytes. Although discontinuous density gradient centrifugation could separate coelomocytes into several layers, it was difficult to get a pure population of each cell type. The dominant coelomocyte populations were phagocytes. However, the distance between the phagocyte layer and the layer containing the vibratile cells, and the distance between the layer near the bottom containing the red spherule cells and the next layer containing the colorless spherule cells were sufficient for the isolation of relatively pure population of phagocytes and red spherule cells, respectively. However, the vibratile cell layer and the colorless cell layer were too close to each other after centrifugation to allow for successful separation. Thus, the vibratile and colorless spherule cells were pooled.

WCF in anti-coagulating buffer with 5% L-15 medium (total 200ul) was loaded into poly-L-lysine coated 8-well plates and incubated for 20 min at 12 °C. The cells were incubated with 10^4 live *E. coli* expressing DsRed for 20 min at 12 °C.

2.3. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the pellets of coelomocytes or larvae using the QIAZol™ reagent according to the manufacturer's instruction (QIAGEN, Gaithersburg, MD). The

concentration and quality of total RNA were measured using the Nano-drop ND-1000 spectrophotometer (Nano Drop Technologies, Wilmington, DE).

The RT-PCR was conducted in a thermocycler (Model 2720, Applied Biosystems, Foster City, CA) with the following steps. Total RNA (1 μg) was added for a 10 μl-reaction with 1 μl dNTP (10 mM) and 1μl random hexamer. The mixture was incubated for 10 min at 70 °C followed by an ice chill. M-MLV reverse transcriptase (0.5 μl; Sigma-Aldrich, St Louis, MO), 1 μl RNase inhibitor (20 units/μl) and 1 μl 10 × reaction buffer were added and the reaction was conducted at 25 °C for 10 min, 37 °C for 50 min and 94 °C for 10 min.

Expression of transcripts of strongylocin 1, strongylocin 2 and centrocin 1 was analyzed with primers: 5' ATCAACCCAACTTCAAGATG and 5' ATGGTGAATCCTGTCTAGGT (for strongylocin 1); 5' CAGTGTTGTGTTCCTCGATCA and 5' CTTGCCGAAGAGGACGAT CT (for strongylocin 2); 5' GTCAGTCATGCAGTTAAGAGT and 5' CTAACGACCAAGGGCATGTG (for centrocin 1); 5' GCGACGGATCCTTAGAATGTCT and 5' ACCCGTGACGACCATGGT (for 18S rRNA). Amplification of different transcripts was performed on a thermocycler using 1 μl cDNA as a template, 2.5 μl 10 × Optimized DyNAzyme™ Buffer, 1 μM the forward and reverse primers, 0.5 μl dNTP 10 mM, 0.2 μl (2 U/μl) DyNAzyme™ II DNA polymerase (Finnzymes, Espoo, Finland) and water to bring the reaction volume up to 25 μl. PCR was carried out using 94 °C for 5 min, 35 cycles of 94 °C for 30 sec, 55 °C (strongylocin 1)/60 °C (strongylocin 2)/57 °C (centrocin 1) for 30 sec and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. Amplification of 18S rRNA transcripts was done with the annealing temperature at 60 °C and only by 28 cycles. The PCR products (5 μl) were analyzed by electrophoresis on a 1.2% agarose gel and documented with the Bioimaging system, Syngene (Syngene, Cambridge, UK).

2.4. Antisera

The heavy chain of centrocin 1 with bromine in tryptophan as previously described [20] was covalently linked to thyreoglobulin. Polyclonal rabbit antisera were prepared against the heavy chain (MedProbe, Oslo, Norway).

2.5. Immunoblotting

Protein samples were lysed in sample buffer (0.1M Tris-HCl pH6.8, 24% Glycerol, 1% SDS, 2% β-mercaptoethanol, 0.2% (w/v) Coomassie blue G-250). After 5 min incubation at 95 °C, the samples were analyzed by 15% SDS-polyacrylamide gel electrophoresis (SDA-PAGE) [29]. The proteins were transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, Billeria, MA) in 1 × NuPAGE transfer buffer (Invitrogen, Carlsbad, CA) containing 10% methanol for 50 min at 100 Volt (XCell Blot module, Invitrogen, Carlsbad, CA). Membranes were blocked in TBST buffer (200 mM Tris pH 7.4, 140 mM NaCl, 0.1% Tween 20) with 5% non-fat dried milk for 1 h, followed by incubation with anti-centrocin 1 heavy chain antiserum, named anti-centrocin-H (1:4000 dilution in TBST with 2% dried milk) for 2 h at room temperature. After washing three times with TBST buffer, membranes were incubated with goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (1:5000 dilution in TBST with 2% dried milk; GαRIg-HRP; Sigma-Aldrich, St Louis, MO) for 1 h at room temperature. Membranes were rinsed three times with TBST buffer and then incubated with an enhanced chemiluminescent substrate (Pierce, Rockford, IL) followed by exposure with chemilumiscent detection film (Roche Diagnostics, Indianapolis, IN).

2.6. Immunofluorescence staining of phagocytes

After settling of the phagocytes onto the poly-L-lysine coated surface of cover slips or 8-well plates, they were fixed with 4% paraformaldehyde in anticoagulation buffer for 15 min, rinsed with anticoagulation buffer three times and then incubated with methanol for 10 min at -20 °C. Subsequently the samples were washed three times with PBS (0.15 M phosphate buffer pH 7.4, 0.2 M NaCl) and incubated with blocking solution (1% bovine serum albumin, BSA in PBS) for 30 min at room temperature (RT). Then the cells were incubated with anti-centrocin-H antisera (1:400 dilution in blocking solution) for 1 h followed by three washing steps with PBS. The samples were incubated with 4', 6-diamidino-2-phenylindole (DAPI; 1:1000 dilution; Invitrogen, Carlsbad, CA) and goat anti-rabbit immunogloblin conjugated to Alexa Fluor 488 (1:400 dilution; GαRIg-AF488; Invitrogen, Carlsbad CA) in blocking solution for 30 min at RT. Cells treated in the plates were rinsed as described above and mounted with ProLong® Gold Antifade solution (Invitrogen, Carlsbad, CA), followed by inspection with microscope.

2.7. Immunofluorescent staining of larvae

Pluteus larvae were incubated with methanol for 20 min at -20 °C. The samples were then incubated with TPBS buffer (0.5% Triton X-100 in PBS) for 1 h at -4 °C. Subsequently the samples were incubated with the blocking solution (2% BSA and 0.1% Tween-20 in PBS, PBST) for 1 h at RT followed by three washing steps with PBST. Then the larvae were labeled with anti-centrocin-H antisera (1:200 dilution in blocking solution) for 1 h at RT followed by three washing steps with PBST. The samples were then incubated with GαRIg-AF488 (1:400 dilution; Invitrogen, Carlsbad CA) in blocking solution for 1 h at RT. The larvae were washed three times by PBST and then mounted with ProLong® Gold Antifade

solution (Invitrogen, Carlsbad, CA). Negative control was conducted as described above, exception of using pre-immune sera or omission of the anti-centrocin-H antisera.

2.8. Microscopy

Cells and larvae were documented with an Axioplan fluorescence microscope (Zeiss, Oberkochen, Germany) or a TCS-SP5 (AOBS) confocal microscope (Leica Microsystems CMS GmbH, Mannheim, Germany).

3. Results

3.1. Expression patterns of strongylocins and centrocin 1 in coelomocytes

Transcripts of strongylocins and centrocin 1 were detected in the the separated fractions of coelomocytes mentioned above (Fig. 1). The data showed that strongylocin 1 was detected in phagocytes and the mixture of vibratile and colorless spherule cells. The transcripts of strongylocin 2 on the other hand were found in phagocytes and red spherule cells. Furthermore, the transcripts of centrocin 1 were only detected in phagocytes.

The presence of a messenger RNA within a cell does, however, not always indicate that this transcript is translated. To know whether the centrocin 1 peptide is actually expressed and stored in coelomocytes, immunoblotting was conducted using the antisera with specificity against the heavy chain of centrocin 1. The results of the Western blot indicated that centrocin 1 is expressed in phagocytes and is not present in the other cell types (Fig. 2). The specificity of the antisera was verified, showing no cross reaction with proteins from *S. droebachiensis* coelomocytes.

3.2. Distribution of centrocin 1 in phagocytes

The distribution of centrocin 1 in phagocytes was detected by fixing phagocytes on cover slips. Subsequently immunostaining was performed. The phagocytes spread in all directions when added to the cover slips (Fig. 3A and Fig. 4A). Immunoflourescent labeling of centrocin 1 showed that the peptide was located in cytoplasmic granular vesicles, mainly around the perinuclear region (Fig. 3B).

3.3. Response of phagocytes to bacterial challenge *in vitro*

Phagocytosis is one of the most important immune responses to invading particles in sea urchins. To determine whether centrocin 1, a phagocyte-specific AMP, plays a role in the immune activity during the phagocytic reaction, the phagocytes were added live *E. coli* expressing DsRed. After 20 min incubation, bacteria had been phagocytosed and were located inside the cells around nucleus (Fig. 4B). Immunostaining using the anti-centrocin-H antisera confirmed the location of the phagocytosed bacteria (Fig. 4C). The bacterial cells showing a yellow color suggested that centrocin 1 peptides were attached to these phagocytosed cells. However, two bacterial cells were still red, indicating they were localized outside the phagocytes and thus not phagocytosed (arrows Fig. 4C).

Although the images from normal fluorescence microscopy indicated that granular vesicles containing centrocin 1 were able to translocate within the cytoplasm towards invading bacteria, three-dimensional resolution was needed to verify an exact co-localization. Confocal microscope images confirmed that granular vesicles were concentrated around the bacteria (Fig. 4D). Furthermore, phagocytes containing many intracellular bacteria tend to contain less free granular vesicles. This observation suggested that granular vesicles were

likely fused with phagosomes and therefore released centrocin 1 which targeted and subsequently eliminated the bacteria.

3.4. Expression patterns of strongylocins and centrocin 1 in larvae

The transcripts of strongylocins and centrocin 1 were examined in embryos and larvae in different developmental stages. Transcripts of strongylocins and centrocin 1 were not detected in blastula and gastrula stages (Fig. 5). Transcripts of strongylocins 1 and 2 were detectable in the beginning of the pluteus stage. The expression level of both these genes increased during the mid pluteus and the late pluteus stages. Transcripts of centrocin 1 were detected at the mid pluteus stage. During the larval development, the expression level of centrocin 1 was higher at the late pluteus stage than at the earlier stages. The transcripts of strongylocin 2 were the highest expressed among three AMPs transcripts.

3.5. Distribution of centrocin 1 in larvae

To detect the localization of centrocin 1, mid-pluteus larvae were immuno-labeled with antisera. The peptide was found mainly in the SMCs at coelomic vesicles and sacs around the stomach and the esophagus showing a strong signal (Fig. 6A and B). In addition, several centrocin 1 containing SMCs were detected close to the ectodermal walls in some arms (Fig. 6C). Although the negative controls, with the presera and without primary antisera, showed a faint green color spreading in the whole larva (Fig. 6D and E), it is evident that the centrocin 1 positive signals were intense green spots presumably being located in cells.

Discussion

We have previously isolated two novel families of AMPs, the cysteine-rich strongylocins and the heterodimeric centrocins, from the coelomocytes of the green sea urchin, *S. droebachiensis* [18-20]. Coelomocytes are considered to mediate defense functions in sea urchins [12]. Their predominant cell type is phagocytes that are involved in phagocytosis, graft rejection, encapsulation and clotting reactions (reviewed by [12, 30]). Although Service and Wardlaw reported echinochrome A from red spherule cells having antibacterial activity [17], there is very little information about non-phagocyte coelomocytes. In the present study, it is the first time that transcripts of strongylocins are detected in red spherule cells, and vibratile and/or colorless cells. Strongylocin 1 was detected in red spherule cells. However, it is yet unknown whether strongylocin 2 expresses exclusively in vibratile cells or in colorless spherule cells or in both cell types as the discontinuous gradient centrifugation can not provide a complete separation between these cell types. This problem is common to most gene expression profiling studies in coelomocytes [15]. Phagocytes on the other hand, express both strongylocins and centrocin 1. Altogether this suggests that other types of coelomocytes than phagocytes are likely involved in the host defense system.

AMPs have been identified in the haemolymph, both in the plasma and haemocyte cells of various invertebrates [31]. The putative immune proteins 185/333 have been shown to be localized on the outer cell membranes of phagocytes and are likely secreted into the coelomic fluid [24]. In the present study, centrocin 1 was only found in phagocytes (Fig 1 and 2) and located in the granular vesicles (Fig 3). Two intact red colored bacteria present in Fig 4C, indicate that the centrocin 1 peptide likely performs its function inside phagocytes instead of being secreted into the coelomic fluid. In addition, centrocin 1 likely carries out its function inside phagocytes during the fusion of the granular vesicles with phagosomes. Phagocytosis

of foreign material has been reported for *S. purpuratus* [7] and *S. droebachiensis* [8, 32, 33]. We know that centrocin 1 effectively kills bacteria *in vitro* [20]. The results in this study reveal that centrocin 1 attaches to phagocytosed bacteria (Fig. 4C and D). Therefore, the recruitment of granules containing centrocin 1 to form phagolysosomes can influence or accelerate the degradation of bacteria.

AMPs are in general either expressed constitutively or the expression is induced by exposure to pathogens [5]. Strongylocins and centrocins were isolated from healthy adult individuals, which indicate that these peptides are constitutively expressed in coelomocytes [18-20]. The number of granular vesicles tends to decrease during the *in vitro* challenge (Fig. 4) presumably because of the formation of phagolysosome. It is also shown that the number of phagocytes can drop significantly during the clearance of bacteria [8]. Although we have no direct evidence that the amount AMPs first decreases and then stabilizes to a normal level, it is tempting to assume that this is the case following the recovery of the number of phagocytes.

Sea urchin eggs are surrounded by a jelly coat that consists of polysaccharides and glycoproteins [34]. Such a jelly coat provides protection from bacterial infection before fertilization and throughout the subsequent embryogenesis [35]. Later, throughout the blastula and the gastrula stages, physical separation and protection from the environment is provided by the ectodermal body wall. The transcript of the gene *Sp064*, which encodes a homologue of complement C3, is detectable in unfertilized eggs and throughout embryogenesis peaking just prior to and during gastrulation [27]. Significantly increased *Sp064* transcripts in plutei are found after incubation with heat killed *Vibrio diazatrophicus* introduced at the blastula stage. Pinocytosis was firstly detected in developing *L. pictus* in pluteus larvae, especially in the stomach and the intestine [25]. SMCs have been observed phagocytosing microinjected yeast cells throughout the mid gastrula stage of *L. variegatus*

[26]. Although we now know that the expression of strongylocins and centrocin 1 is detected at the early and mid pluteus stage, respectively, it is yet unknown which mechanisms are employed to modulate their gene expression. The digestive tract, however, is completed during the early pluteus stage [36], which is believed to increase the risk to encounter pathogens. Therefore, the simultaneous occurrence of the expression of AMPs suggests that strongylocins and centrocin 1 are most likely involved in immune activity from the pluteus stage. Since the centrocin 1 labeled SMCs are mainly located around the stomach and esophagus, this may indicate that these cells are able to take part in immune responses against pathogens entering through the digestive tract.

One important factor influencing the larval development is the temperature [37], and warmer temperatures usually accelerate the growth of larva. In our study, one batch of larvae was grown in sea water at 5.4 °C. As expected, the expression of AMPs and the respective developmental stages characteristic for their expression were approximately delayed 7 days (23rd day, data not shown), when compared to growth at 8.0 °C (16th day; data presented in this paper). Thus, the development of larvae can be described by a factor, the water temperature multiplying time [38]. Taken together, this implies that the expression of AMPs is most likely related to larval development and is detectable at approximately 124 day·°C .

SMCs in the gastrula of *L. variegatus*, are able to phagocytose microinjected yeasts [26]. In the late gastrula of *Asterina pectinifera*, mesenchyme cells show extreme fusogeneic activity amongst themselves when inoculated on a culture dish [39]. Recently, it has been shown that most mesenchyme cells construct a dynamic network structure beneath the body wall in bipinnaria larvae of *A. pectinifera*. These mesenchyme cells phagocytically respond to a variety of foreign material [40]. Mesenchyme cells share amoeboid, phagocytic behavior and have chemotactic properties resembling phagocytic coelomocytes of adult animals [41]. In our study, many centrocin 1 labeled SMCs are located in the coelomic vesicles which

likely take part in the formation of the coelomic cavity in the adult animals. Considering that centrocin 1 only is expressed in phagocytes of adult animals, it is tempting to speculate that these SMCs develop specific tissues where phagocytes are later matured. Furthermore, detection of centrocin 1 labeled SMCs in only some of the arms of the larvae suggests that these cells or some of them are able to patrol beneath the body wall and thereby assist in immune defense. This would be in agreement with a finding in larvae of the starfish *A. pectinifera* that has SMCs which are highly motile and seem to patrol in the blastocoel in response to pathological situations and/or penetration by foreign materials [40].

AMPs are regarded as immune effector molecules which play an important role as a first line of host defense. Although we do not have antibodies against strongylocins 1 and 2 to detect these peptides inside cells, this work clearly proves that AMPs from *S. droebachiensis* are expressed in different types of coelomocytes. These results suggest that not only phagocytes are involved in the immune response of sea urchins, but also other types of cell most likely contribute to the host defense. Although the results of the *in vitro* bacterial challenge illustrate that centrocin 1 participates in the clearance of bacteria in the phagolysosome, it is unknown which signaling cascade might be involved in activating this process. According to our results, the expression of AMPs starts in the pluteus stage of larvae, suggesting that AMPs also are important in the developmental stages of the sea urchin.

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Fig. 1. Expression of strongylocins and centrocin 1 in different coelomocyte fractions. Three fractions of coelomocytes were separated by the discontinuous density gradient centrifugation. Total RNA was isolated from phagocytes, the mixture of vibratile and colorless spherule cells, and red spherule cells. The RNA was analyzed for expression of strongylocins 1 and 2, and centrocin 1. The 18S rRNA was employed as the constitutive control. *P* phagocytes, *M* the mixture of vibratile and colorless spherule cells, *R* red spherule cells.

Fig. 2. Immunoblotting showing that centrocin 1 is produced by phagocytes. The blotting membrane was incubated with anti-centrocin H antisera and subsequently with GαRIg-HRP and substrate. *S*, the synthetic heavy chain of centrocin 1; an amount of 0.5 mg of protein from cell lysate was added per lane. *P*, phagocytes; *M*, the mixture of vibratile and colorless spherule cells; *R*, red spherule cell. The detected peptide from the cells includes both the heavy chain and light chain and is indicated by an arrow.

Fig. 3. Expression of centrocin 1 in phagocytes. Centrocin 1 is labeled by the anti-centrocin H antisera and GαRIg-AF488 (green) while DNA is labeled by DAPI stain (blue). Phagocytes spread on the cover slips and were imaged with a light field microscope (A). The location of centrocin 1 was imaged in cytoplasmic granular vesicles with a fluorescence microscope (B). Bar, 20 μm.

Fig. 4. Localization of centrocin 1 in phagocytes after *in vitro* challenge with an *E. coli* strain expressing fluorescent. The challenged phagocytes were observed through a light field microscope (A), a fluorescence microscope (B, C) and a confocal microscope (D). Fluorescent detection of *E. coli* (red, recombinantly expressed DsRed), centrocin 1 (green,

described in the legend for Fig. 3) and DNA (blue, DAPI) are shown. There are two intact *E. coli* cells (arrows in B and C). Co-localization of *E. coli* and centrocin 1 appears as yellow in the images (C and D). It can be seen that phagocytosed bacteria are located in cytoplasma close to nucleus. In addition, the phagocyte labeled 2, has less granular vesicles and more phagocytosed bacterial cells than the phagocyte 1 (D). Bar, 20 μm in A-C; 2 μm in D.

Fig. 5. Expression of strongylocins and centrocin 1 in different developmental stages of embryos and larvae. Total RNA was isolated and analyzed for the expression of AMPs in embryos and larvae. The expression of the 18S rRNA was used as the control. *B* blastula, *G* gastrula, *EP* early-pluteus, *MP* mid-pluteus, *LP* late-pluteus.

Fig. 6. Confocal images of the centrocin 1 peptide in sea urchin larvae. Immuno-labeled centrocin 1 was visualized in the mid-pluteus larval stage. The micrographs reveal the presence of centrocin 1 in the mesenchyme cells. In the whole animal, centrocin 1 is mainly located in mesenchyme cells around the stomach region with a strong green signal (A). A higher magnification of the image shows centrocin 1 positive SMCs around the digestive tract (B). In addition, centrocin 1 is found in some of the larval arms showing dense green signal (arrow heads in C). The negative controls with the pre-immune sera (D) and omission of the primary antisera (E) show an evenly faint green color as background. Bar, 100 μm in A, D and E; 50 μm in B and C.

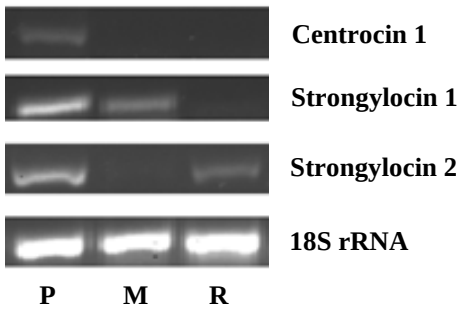

Centrocin 1
Strongylocin 1
Strongylocin 2
18S rRNA
P
M
R

Fig. 1

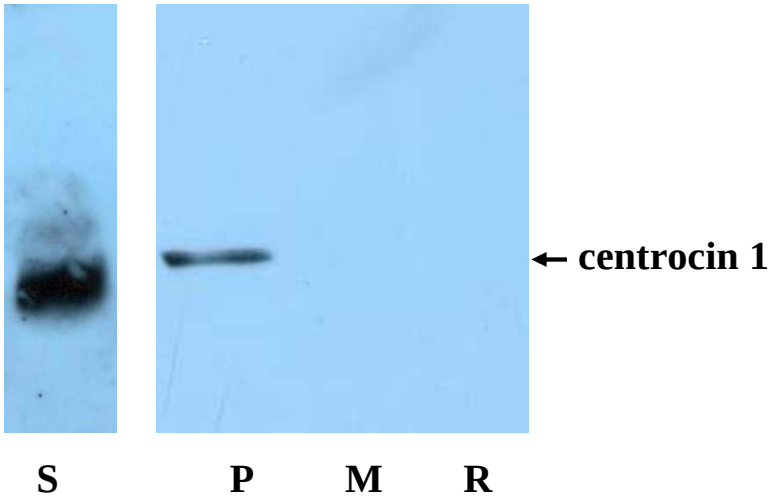
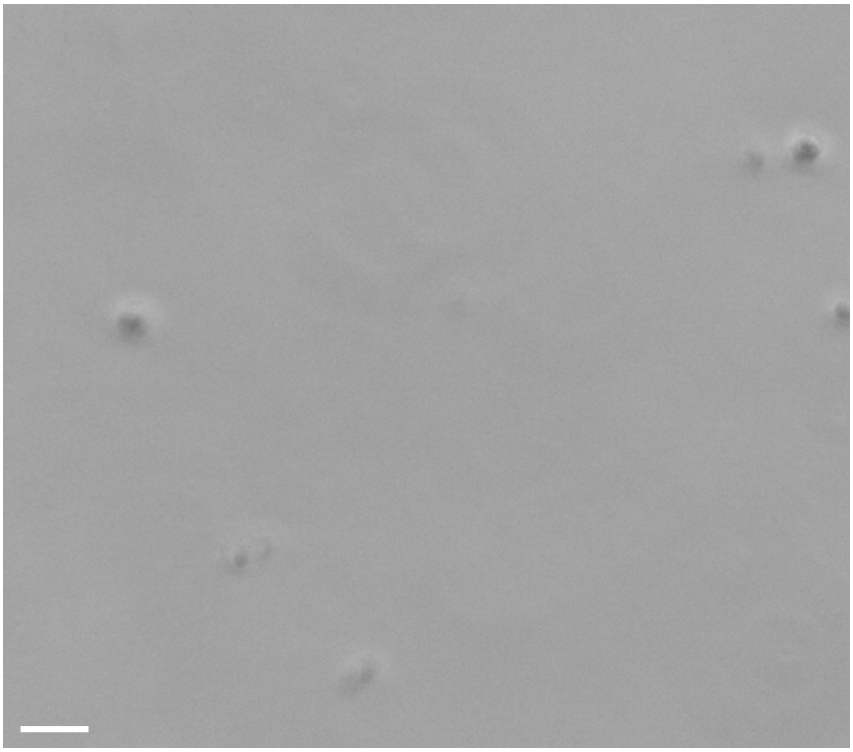
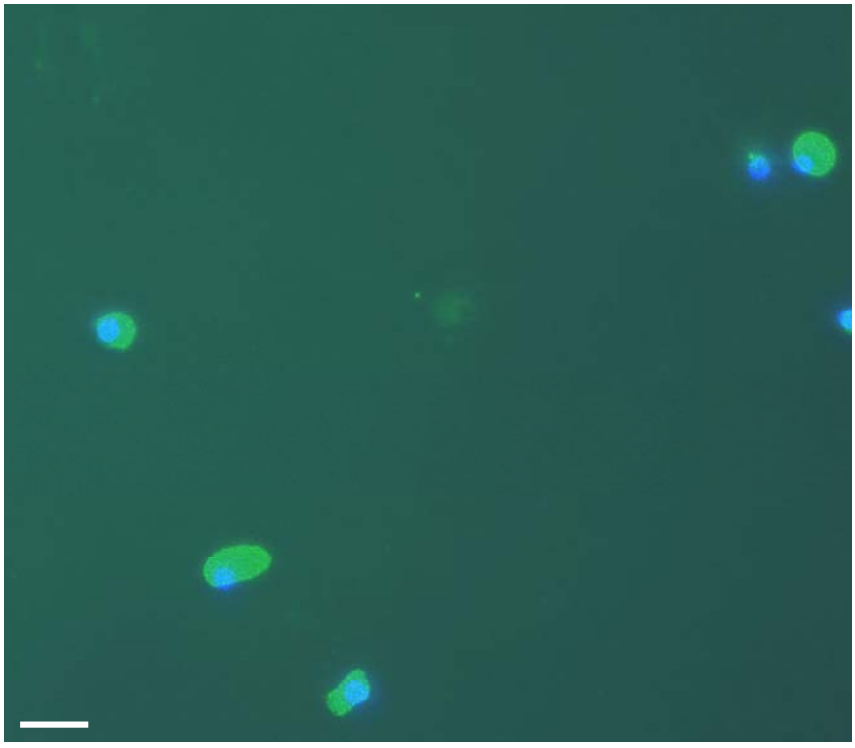

← centrocin 1
S
P
M
R

Fig. 2



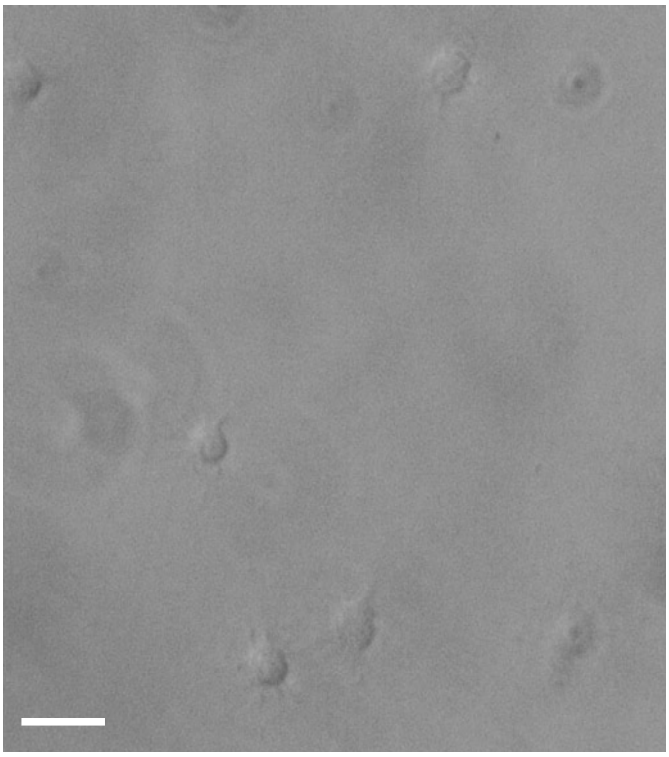
A



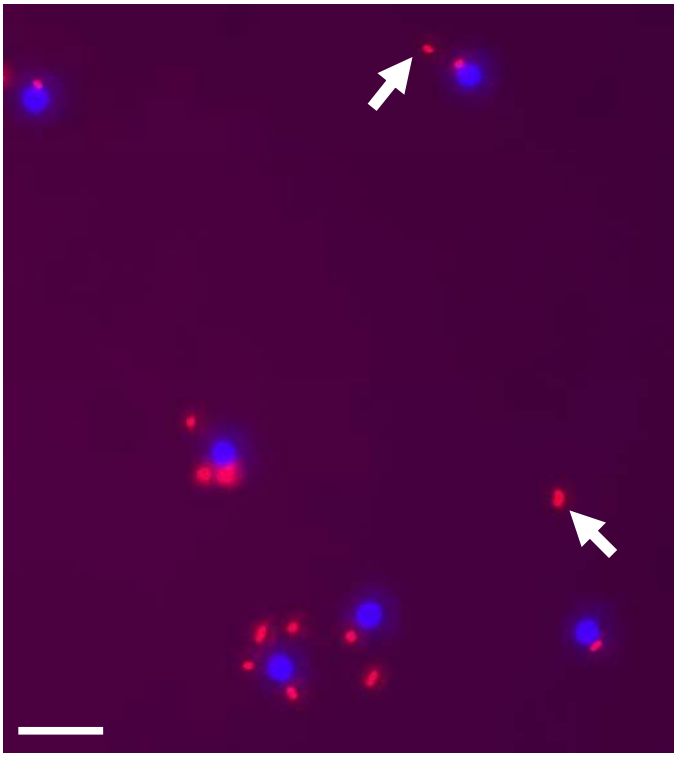
B

Fig. 3

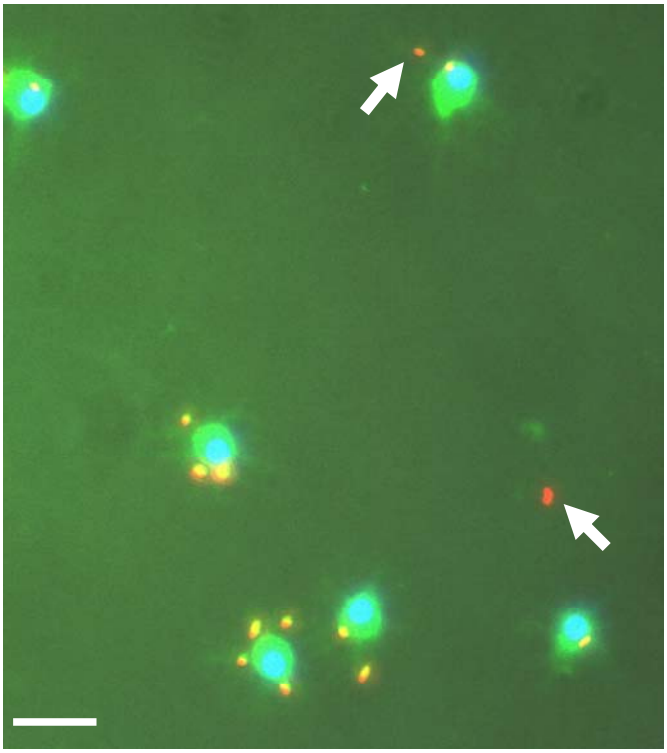
A



B



C



D

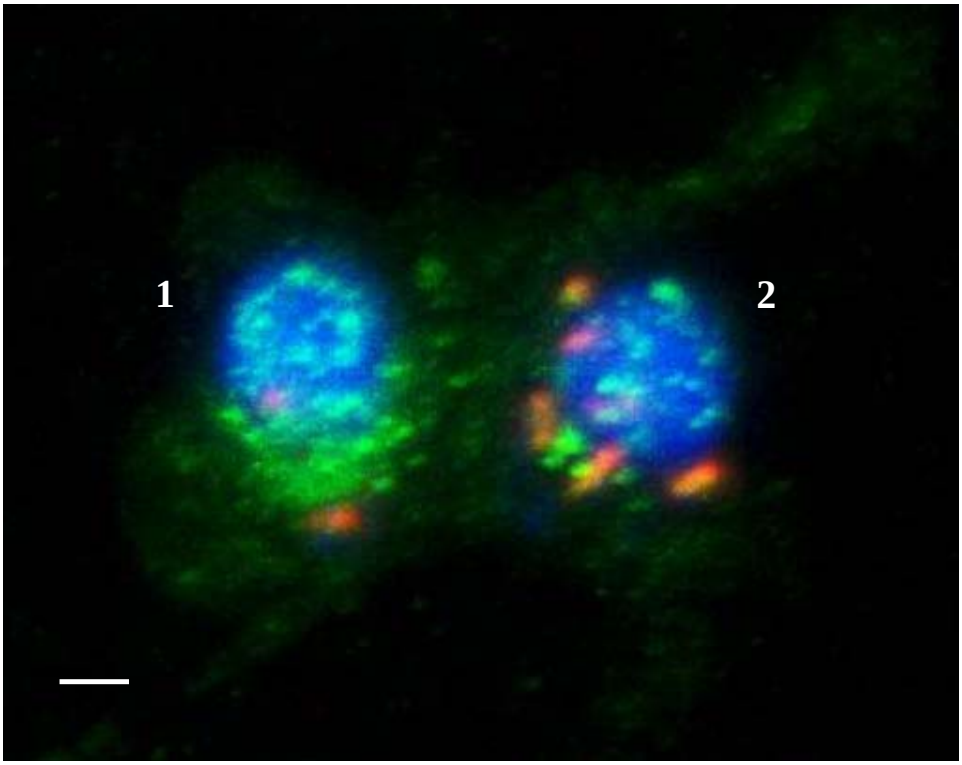


Fig. 4.

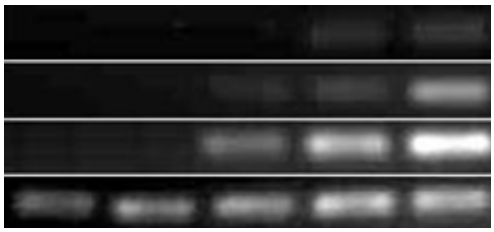
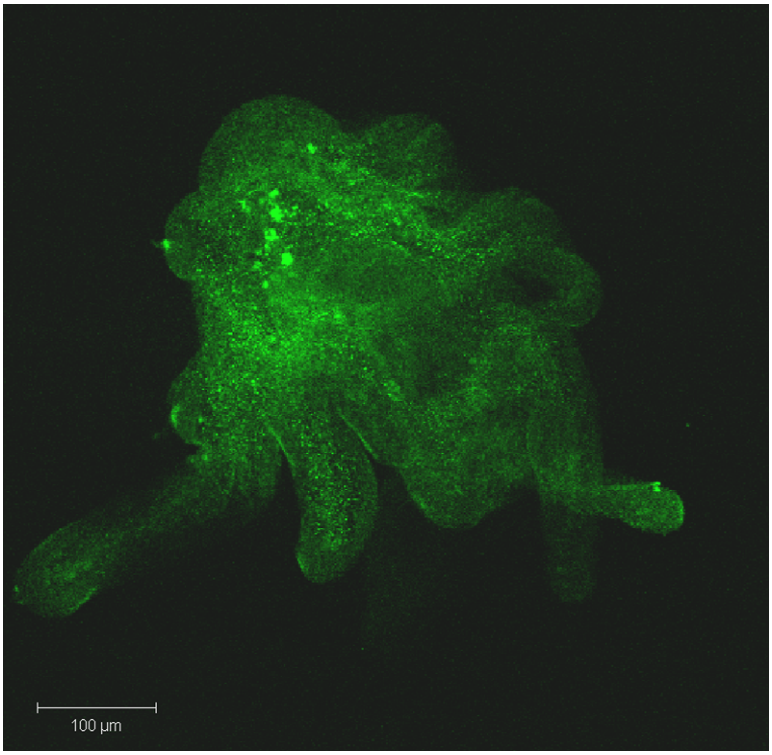
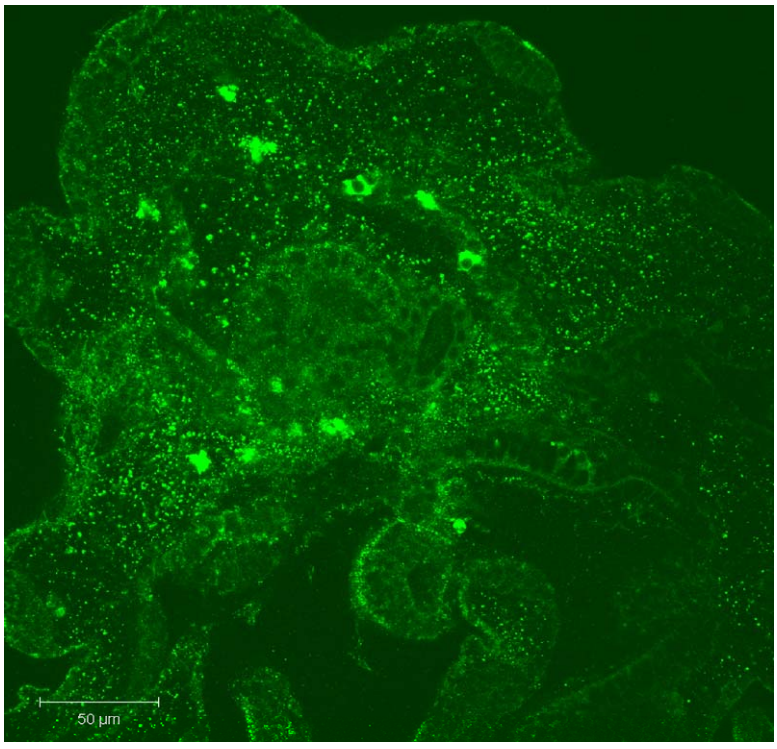

Centrocin 1
Strongylocin 1
Strongylocin 2
18S rRNA
B
G
EP
MP
LP

Fig. 5.

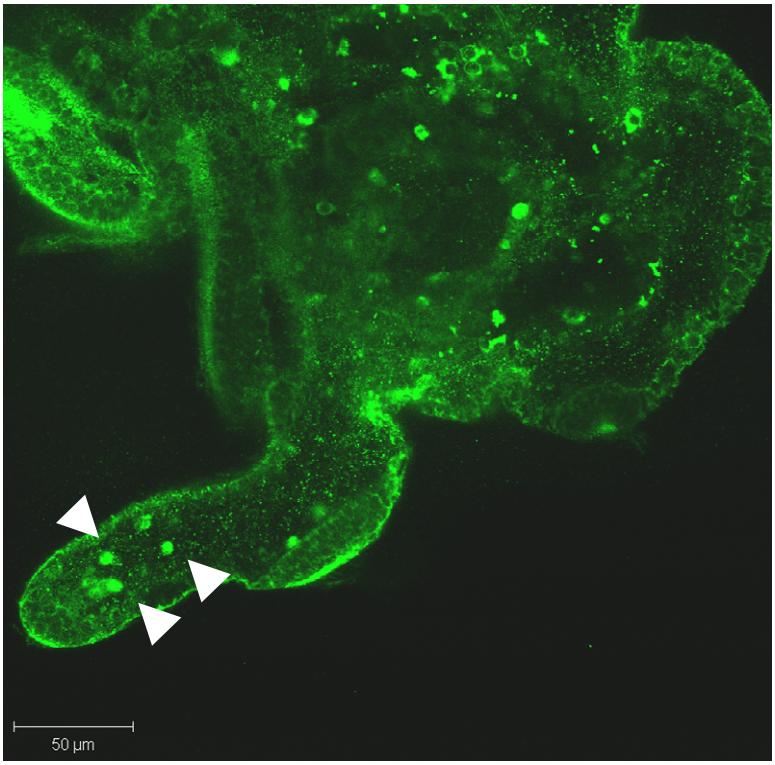


A

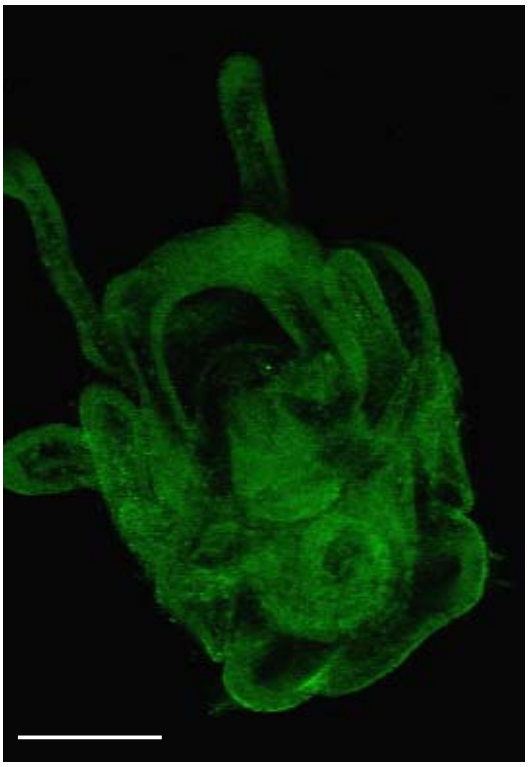


B

C



D



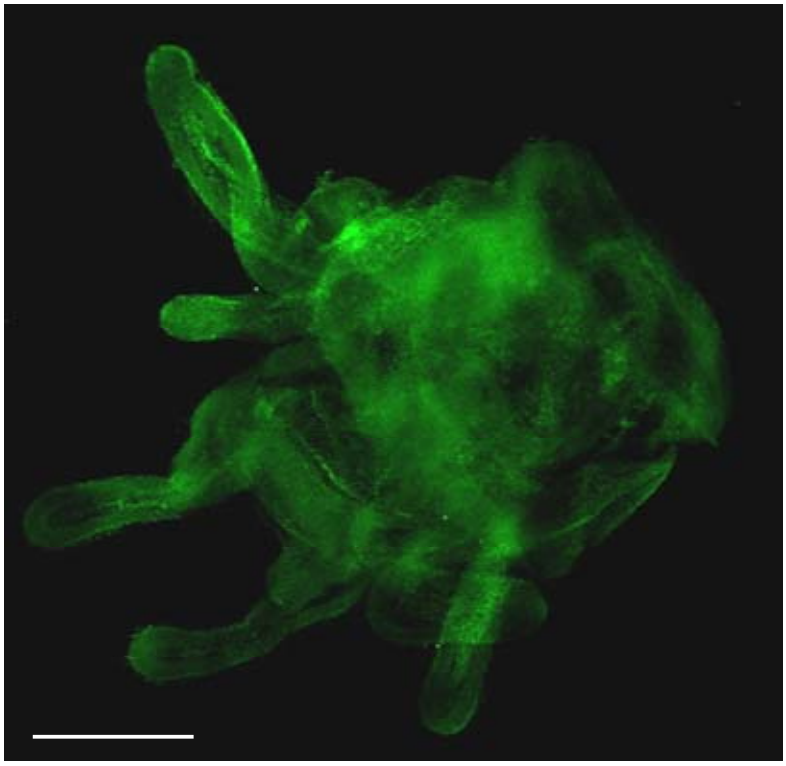
E 

Fig. 6.

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