Antimicrobial peptides in echinoderm host defense

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Abstract

Antimicrobial peptides (AMPs) are important effector molecules in innate immunity. Here we briefly summarize characteristic traits of AMPs and their mechanisms of antimicrobial activity. Echinoderms live in a microbe-rich marine environment and are known to express a wide range of AMPs. We address two novel AMP families from coelomocytes of sea urchins: cysteine-rich AMPs (strongylocins) and heterodimeric AMPs (centrocins). These peptide families have conserved preprosequences, are present in both adults and pluteus stage larvae, have potent antimicrobial properties, and therefore appear to be important innate immune effectors. Strongylocins have a unique cysteine pattern compared to other cysteine-rich peptides, which suggests a novel AMP folding pattern. Centrocins and SdStrongylocin 2 contain brominated tryptophan residues in their native form. This review also includes AMPs isolated from other echinoderms, such as holothuroidins, fragments of beta-thymosin, and fragments of lectin (CEL-III). Echinoderm AMPs are crucial molecules for the understanding of echinoderm immunity, and their potent antimicrobial activity makes them potential precursors of novel drug leads.

Antimicrobial peptides (AMPs) are widespread molecules found both in prokaryotes and eukaryotes and provide important protection to hosts against pathogens and/or environmental 'invaders'. To date, approximately 2,400 naturally isolated or synthesized AMPs have been listed in the Antimicrobial Peptide Database (APD database, http://aps.unmc.edu/AP/main.php). The majority of these AMPs are from amphibians, insects, mammals, and plants (Wang *et al*., 2009). There are comparatively few research activities throwing light on AMPs in echinoderms. Here, we present an overview of what is currently known about echinoderm AMPs, with a special focus on recently identified AMPs from sea urchins. Characteristics such as antimicrobial activity, peptide structure, expression profile, and biological function in echinoderms are discussed.

1. Echinoderm host defense

Echinoderms are deuterostome invertebrates, with a phylogenetic position closely related to chordates and hemichordates (Kondo and Akasaka, 2012). They are categorized into five classes: Crinoidea (sea lilies and feather stars), Ophiuroidea (brittle stars), Asteroidea (sea stars), Echinoidea (sea urchins, sand dollars, and sea biscuits), and Holothuroidea (sea cucumbers). These animals have a unique morphology that includes a water vascular system and a pentamerously symmetrical body shape in the adults. All living echinoderms are marine species and occupy habitats from the intertidal zone to the deep sea. These environments are rich in bacteria, fungi, viruses, and parasites, and many are potentially pathogenic to echinoderms. Echinoderms have evolved in complex means to overcome these challenges as was first illustrated from the annotation of the purple sea urchin genome (Rast *et al*., 2006).

Echinoderms have an innate immune system, but as all other invertebrates they lack a vertebrate-type adaptive immune system (Smith and Davidson, 1992). However, their ability to reject non-self tissues has been documented for several echinoderm species (reviewed by Smith *et al*. 2010 and Smith *et al*. 2006). Many of their immune activities occur in the

coelomic fluid, which is a complex fluid containing minerals, proteins, and suspended cells called coelomocytes. These coelomocytes are critical immune mediators and are involved in the response against pathogens, injuries, and foreign substances (Smith *et al*., 2010; Smith *et al*., 2006). In general there are four morphologically different types of coelomocytes in echinoderms: phagocytes, vibratile cells, colorless and red spherule cells. However, Asteroidea and Ophiuroidea lack red spherule cells, and Crinoidea lack vibratile cells (reviewed by Ramirez-Gomez and Garcia-Arraras, 2010). Phagocytes constitute the most abundant coelomocyte type in the coelomic fluid (reviewed by Smith *et al*., 2010). Based on morphology, phagocytes can be categorized into three subtype populations. One subset consists of large discoidal phagocytes that have radially oriented actin bundles distributed throughout the cortex from the perinuclear region to the periphery (Henson *et al*., 2003; Henson *et al*., 1999). Another subset consists of large polygonal phagocytes that have actin bundles that are oriented across the cell creating a polygon cell shape (Henson *et al*., 1999). The third subset are smaller cells called small phagocytes (Gross *et al*., 2000) that are always present in filopodial form (Brockton *et al*., 2008). It is known that the sea urchins, *Strongylocentrotus droebachiensis* and *S. purpuratus*, are able to clear injected bacteria quickly from their coelomic fluid (Plytycz and Seljelid, 1993; Yui and Bayne, 1983). In addition, the different types of coelomocytes differ in their activity against microbial intruders. Phagocytic amoebocytes (herein referred to as phagocytes) and red spherule cells from *Paracentrotus lividus* exert a higher antibacterial activity against Gram-negative marine bacteria than the other populations of coelomocytes (Gerardi *et al*., 1990). When erythrocytes are opsonized by molecules in the coelomic fluid, this enhances the capacity of phagocytes from *S. nudus* to take up the erythrocytes (Ito *et al*., 1992). Several humoral factors are involved in defense processes such as clotting, opsonization, encapsulation, cell lysis, and wound healing (Gross *et al*., 1999). For example, the coelomic fluid from four sea urchin

species, *Anthocidaris crassispina*, *Pseudocentrotus depressus*, *Hemicentrotus pulcherrimus*, and *Holothuria polii*, has hemagglutinating activity against mammalian erythrocytes (Canicattì and Parrinello, 1985; Ryoyama, 1974). An agglutinin from the coelomic fluid of the sea urchin *P. lividus* not only agglutinates animal erythrocytes but also promotes adhesion of the autologous coelomocytes (Canicatti *et al*., 1992). Echinoderm lectins, which are carbohydrate binding proteins, function to bind erythrocytes (Matsui *et al*., 1994), agglutinate bacteria (Kamiya *et al*., 1992), and assist in cell adhesion (Ozeki *et al*., 1991). In addition to AMPs, the innate immune system of echinoderms also has a number of antimicrobial components such as lysozyme, which play an important role in the echinoderm defense system. Generally, lysozymes show antimicrobial functions against Gram-positive bacteria and have been detected in asteroids (Bachali *et al*., 2004; Jollès and Jollès, 1975), echinoids (Shimizu *et al*., 1999), and holothuroids (Cong *et al*., 2009). A larval lysate of *P. lividus* has lysozyme-like and antibacterial activity against marine bacteria (Stabili *et al*., 1994). It is possible that lysozyme, AMPs and other antimicrobial factors act synergistically to provide an effective defense against microbial infections.

The first echinoderm genome to be completely sequenced was the genome of *S. purpuratus* (Sodergren *et al*., 2006). The sequence revealed a complex immune gene repertoire, indicating that sea urchins have an astonishingly high diversity of immune molecules (Hibino *et al*., 2006; Rast *et al*., 2006). Analysis of the *S. purpuratus* genome has uncovered an extreme expansion of several immune recognition receptor families. The genome sequence revealed 253 Toll-like receptor (TLR) genes (Buckley and Rast, 2012), more than 200 NACHT domain-LRR (NLR) genes and 218 scavenger receptor genes (Rast *et al*., 2006). Another large putative immune response gene family encodes the Sp185/333 protein family, which was initially identified by analyzing transcripts from LPS challenged sea urchins (Nair *et al*., 2005; Terwilliger *et al*., 2006). Of 171 genes from the Sp185/333 family that were sequenced, there were 121 unique

gene sequences, and 689 cDNA sequences (Terwilliger et al., 2006; 2007) resulted in a total of 51 different element patterns (Buckley and Smith, 2007); reviewed by Smith (2012). Furthermore, the *S. purpuratus* genome contains homologues of the vertebrate complement protein C3 and factor B, called SpC3 and SpBf, respectively (reviewed by Smith *et al.*, 2001). They were the first complement components to be identified in an invertebrate. The expression of the gene *Sp064* that encodes SpC3 is upregulated after LPS challenge (Clow *et al.*, 2000) and shows typical C3 function by binding to both methylamine and yeast and functioning as an opsonin (Clow *et al.*, 2004). In addition, two transcripts from *S. purpuratus* encode putative complement proteins with domains that are also present in C6 and C7 (Multerer and Smith, 2004). Their deduced proteins might participate in the terminal complement pathway or act as complement regulatory proteins in sea urchins.

Although the recent acquisition of genetic information of echinoderms, such as the *S. purpuratus* genome sequence, allows us a glance at the complexity of the echinoderm immune system, we are far from understanding the immune system of the echinoderms. This is specially true for immune effectors such as antimicrobial compounds, which are understudied and very poorly understood. Considering the intriguing number and diversity of species in the Echinoderm phylum, their microbe-rich habitat, and mostly untapped genetic information, it is not surprising that echinoderms are targeted as a source for discovering novel antimicrobial peptides.

2. AMPs

While the amino acid composition which defines the structure of AMPs is diverse, some basic features seem to be common. AMPs are relatively short amino acid sequences, usually less than 100 amino acids (Ganz, 2003; Maroti *et al.*, 2011), and according to the APD database, the average length of all registered peptides is around 32 residues with an average net positive charge of 3.18 (Wang *et al.*, 2009). AMPs commonly contain many positively charged residues

such as arginine and lysine, which result in the net positive charge (Hancock and Sahl, 2006; Zasloff, 2002). Most antimicrobial peptides fold into amphipathic structures, which reflect the relative abundance and polarization of hydrophobic and hydrophilic domains in their structural conformation. These fundamental features of AMPs also determine their antimicrobial properties such that the hydrophobic regions enable water-soluble AMPs to interact with the hydrophobic lipid bilayer of bacterial cell membranes (Brogden, 2005; Matsuzaki, 1999), which are known to be predominantly composed of phosphatidylglycerol, cardiolipin, or phosphatidylserine, resulting in a negative net charge on their outer leaflet (Yeaman and Yount, 2003). Studies on both artificial model membranes and bacterial membranes have shown that AMP-membrane interaction is initiated by electrostatic attraction (Scott *et al.*, 1999; Silvestro *et al.*, 1997; Zhao *et al.*, 2001), followed by attachment (Heller *et al.*, 1999; Huang, 2000; Wu *et al.*, 1995), insertion (Lee *et al.*, 2004; Yang *et al.*, 2001), which leads to the loss of membrane integrity (Lee *et al.*, 2004) and ion leakage through transmembrane pores or detergent-like solubilization of the cell membrane. Several models have been proposed to describe the mechanisms of action involved in AMP-dependent membrane damage that occurs when the AMP concentration reaches a certain threshold of a peptide/membrane lipid ratio. The AMPs form peptide helices that insert into the membrane either as a toroidal pore, a barrel stave, or a carpet model. The 'toroidal pore' model suggests that the lipid layer bends continuously and associates with the hydrophobic region of peptide forming the pore (Matsuzaki *et al.*, 1996; Matsuzaki *et al.*, 1998). According to the 'barrel-stave model', peptide helices insert into the membrane as parallel staves and form barrel-like pores (Cantor, 2002; He *et al.*, 1995; Lee *et al.*, 2004; Yang *et al.*, 2001). In the 'carpet model', peptides show 'detergent-like' activity and disrupt the bilayer by forming micelles (Bolintineanu and Kaznessis, 2011; Ladokhin and White, 2001; Shai, 1999). In addition, the pores formed by AMPs in supported lipid bilayers can expand in size from the nano to micrometer scale resulting in a point of complete membrane

disintegration (Rakowska *et al*., 2013). Some AMPs are able to translocate across the membrane and interact with intracellular targets. The mammalian proline-rich AMPs PR-39 and indolicidin inhibit protein and/or DNA synthesis in *Escherichia coli* (Boman *et al*., 1993; Subbalakshmi and Sitaram, 1998). In addition, buforin II and tachyplesin can bind nucleic acids (Park *et al*., 1998; Yonezawa *et al*., 1992); histatins reduce enzyme activity (Kavanagh and Dowd, 2004); and mersacidin inhibits cell wall synthesis (Brotz *et al*., 1998). Furthermore, AMPs are typically active against microbes in a dose-dependent manner, whether they act on the membrane or intracellular targets (Jang *et al*., 2010; Mochon and Liu, 2008; Muñoz *et al*., 2012; Romani *et al*., 2013; Vasudevan *et al*., 2008).

As a group of crucial immune-related molecules in multicellular organisms, it is not surprising that some AMPs have multiple roles, which not only have a direct antimicrobial function but also act indirectly as modulators of innate immunity, such as inducing chemokine production and influencing the processes of apoptosis, angiogenesis, and wound healing (Guilhelmelli *et al*., 2013; Hancock and Sahl, 2006). For example, magainin and gomesin induce apoptosis by increasing the activity of caspase-3, which is a frequently activated death protease (Cruz-Chamorro *et al*., 2006; Paredes-Gamero *et al*., 2012). PR-39 is involved in the regulation of angiogenesis (Li *et al*., 2000), promotion of wound repair (Gallo *et al*., 1994; Shi *et al*., 1996), and modulation of cytokine production (Veldhuizen *et al*., 2014). Therefore, such moonlighting functions should always be considered when AMPs are discovered or used as drug leads.

AMPs play an important role in host defense and might therefore serve as models for pharmaceutical drug leads. The pharmaceutical industry has shown a growing interest in AMPs, due to their attractive properties, such as high target specificity, high potency, and minimal toxicity/side-effects to the patient (Góngora-Benítez *et al*., 2014; Northfield *et al*., 2014). Examples of AMPs that have entered clinical trials are the lactoferricin derivative LTX-109

and the indolicidin variant CP-226 called omiganan (Hancock and Sahl, 2006). Historically, AMPs have mainly been isolated from mammals, insects and plants. In recent years, however, the focus of bioprospecting activities has been shifting towards marine resources. Echinoderms, which have approximately 7,000 extant species and contribute significantly to marine biodiversity, are therefore expected to be a valuable source of bioactive and therapeutic peptides. What we currently know about some of the AMPs in echinoderms and their activities is the focus of the remainder of this review.

3. AMPs in Echinoderms

The first completely sequenced genes encoding AMPs in echinoderms were the strongylocins, which are expressed in the coelomocytes of the green sea urchin, *S. droebachiensis* (Li *et al*., 2008). This family of peptides includes two native members, named SdStrongylocins 1 and 2, and each has putative isoforms (SdStrongylocins1b and 2b). Native SdStrongylocin 1 is a peptide of 48 amino acids of 5.6 kDa with an isoelectric point (*pI*) of 9.34, while native SdStrongylocin 2 has 51 amino acids, is 5.8 kDa with a *pI* of 9.65. The SdStrongylocins display potent activity against both Gram-positive and Gram-negative bacteria at minimal inhibitory concentrations (MIC) of 1.3 – 2.5 μM. *Listonella anguillarum*, a marine fish pathogen, is especially susceptible to SdStrongylocin 2. The peptides are cysteine rich, with six cysteines distributed throughout the sequence (Fig. 1A). Cysteine-rich AMPs have been discovered in many species from both the animal and plant kingdoms. The number of cysteines and their location pattern in the sequence determines the formation of disulfide bridges and the conformational structure of the peptides, which influences the antimicrobial activity of the AMPs (Selsted and Ouellette, 2005). The human native neutrophil α-defensin, which has six cysteines, is active against viral or bacterial targets but loses this activity when linearized (Daher *et al*., 1986; Mandal and Nagaraj, 2002). A cysteine-rich AMP from a legume that is correctly folded with regard to the disulfide bonds is

not only dramatically more active against bacteria but also more stable than peptides that are alternatively folded with incorrect disulfide bonds (Haag *et al*., 2012). Strongylocins have a unique cysteine arrangement pattern compared to cysteine location pattern of other cysteine rich AMPs (Table 1). The cysteines form three disulfide bonds, which may influence the antimicrobial activity of the peptide and protect the molecule from proteolytic degradation during biosynthesis in the coelomocyte and subsequent function upon secretion in the presence of microbes (Li *et al*., 2008; Selsted and Ouellette, 2005). Although the disulfide bonds in the stronglocins are important for the peptide activity, the exact orientation of the disulfide bonds that function in the native peptides is, however, currently unknown.

Homologous peptides of SdStrongylocin are also expressed in the purple sea urchin, *S. purpuratus*, and named SpStrongylocins 1 and 2 (Li *et al*., 2010a). Recombinant SpStrongylocins (*r*SpStrongylocins) are active against both Gram-positive and Gram-negative bacteria; however, membrane integrity studies showed that *r*SpStrongylocins 1 and 2 do not enhance the permeability of the bacterial membrane (Li *et al*., 2010a). This suggests that SpStrongylocins may have intracellular targets or affect microbial killing in other ways. Based on the identical cysteine location pattern and high sequence similarity among the SdStrongylocins and SpStrongylocins, especially between SdStrongylocin 1 and SpStrongylocin 1, it is likely that the strongylocins employ a similar mode of antimicrobial action.

Another set of AMPs identified from the coelomocytes of *S. droebachiensis* are centrocins 1 and 2 (Li *et al*., 2010b). The native forms of two similar peptides isolated from *S. droebachiensis* were named Centrocin 1 (4.5 kDa) and Centrocin 2 (4.4 kDa), respectively. Subsequent cDNA analyses revealed an isoform, which is closely related to Centrocin 1, therefore named Centrocin 1b. Centrocins 1 and 2 are cationic peptides with *pI*s of 10.06 and 9.69, respectively, and are active against both Gram-positive and Gram-negative bacteria at a

MIC range of 1.3 – 2.5 µM. The centrocins are heterodimeric peptides which consist of a heavy chain (30 aa) and a light chain (12 aa) that are linked by an internal disulfide bond (Fig. 1B and 1C). The heavy chain of the centrocins contributes to the antimicrobial activity of the heterodimer, while the light chain does not have any detectible activity or influence the activity of the heavy chain against microbes (Li *et al*., 2010b). Although the light chain of centrocins alone is inactive against microbes, the formation of a heterodimeric structure in the native centrocins infers that the light chain may aid in stabilization of the molecules and make the peptides more resistant to attack by proteases (Li *et al*., 2010b). Other dimeric AMPs that have been identified from other animals include distinctin from tree frog, *Phyllomedusa distincta* (Batista *et al*., 2001), cryptdin-related sequence (CRS) peptides from mouse, *Mus musculus* (Hornef *et al*., 2004), cathelicidin CAP11 from neutrophils of guinea pig, *Cavia porcellus* (Yomogida *et al*., 1996), PMAP36 from pig, *Sus scrofa* (Scocchi *et al*., 2005), dicynthaurin and halocidin from the tunicate, *Halocynthia aurantium* (Jang et al., 2002; Lee et al., 2001). The dimeric structure of these types of AMPs influences antimicrobial activity, solubility, and protease resistance (Dempsey *et al*., 2003; Dewan *et al*., 2009; Hara *et al*., 2001; Lee *et al*., 2008; Liu *et al*., 2010; Shin *et al*., 2010).

Analysis of the deduced sequences of strongylocins and centrocins (Fig. 1) shows that the putative precursor molecules have preprosequences and that the centrocins have an interchain region between the heavy chain and the light chain (Li *et al*., 2010a; Li *et al*., 2010b; Li *et al*., 2008). AMPs are often derived from larger precursor molecules (Zasloff, 2002), and hence, the AMPs become biologically active mature molecules after one or more post-translational modification steps that include the removal of a presequence (also called a signal sequence) and/or prosequences, hydroxylation, halogenation (usually bromination), and glycosylation (Wang, 2012). Presequences, functioning as signal sequences, are likely important for targeting the ribosome to the rough endoplasmic reticulum (ER) and for guiding protein

translocation across the ER membrane (Coleman *et al*., 1985; Reddy *et al*., 2004). The presequences are highly conserved in SdStrongylocin 1, SpStrongylocins 1 and 2, but have a different sequence in SdStrongylocin 2; however, all are hydrophobic (Fig. 1). In addition, the centrocins (including the putative form from *S. purpuratus*) share a conserved presequence (Li *et al*., 2010b), which differs from the strongylocin presequences. Three sorts of presequences in sea urchin AMPs are conserved within each family, but divergent among them. This indicates that these presequences might have different evolutionary origins and exert various pathways for intracellular translocation. Prosequences of strongylocins and centrocins contain several negatively charged amino acids, which make these AMP precursors less cationic. Many prosequences are known to inhibit the activity of the precursor until they are cleaved (Neurath, 1989); however, prosequences may also act as intramolecular chaperones, which aid in proper folding of the mature AMP. Prosequences with a net negative charge, such as in strongylocin and centrocin prosequences, likely silence the activity of the AMPs in the precursor form and prior to activation in the coelomocyte. Therefore, it is not surprising that preprosequences are crucial for translocation and inactivation of precursor molecules until AMPs acquire mature functions by post-translational modifications.

Brominated natural products are broadly distributed in marine organisms, but few are peptides. The aromatic amino acid tryptophan contains a heterocyclic indole group with 5 possible carbon positions (carbon number 2, 4, 5, 6, and 7) where a hydrogen can be substituted with a bromine atom. Most natural brominated peptides isolated contain either 2-, 5-, or 6-bromothryptophan residues (Bittner *et al*., 2007). Protein containing brominated tryptophan is resistant to degradation by proteases (Shinnar *et al*., 2003). One example is stylein D, a 32-residue long AMP isolated from the tunicate *Styela clavata*, which contains a 6-bromotryptophan residue in its sequence. The native peptide exerts antibacterial activity

against *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) at a range of pH and salinity. However, a synthesized version of styelin D with a non-brominated tryptophan shows decreased activity at low pH and/or high salinity (Taylor *et al*., 2000). This suggests that the post-translational modification of styelin D may preserve the antimicrobial activity at low pH and/or high salinity. Similarly, the bromotryptophan-containing cathelicidins, isolated from the Atlantic hagfish (*Myxine glutinosa*), are likely to be less susceptible to proteolysis (Uzzell *et al*., 2003). Hedistin is another brominated AMP, isolated from the coelomocytes of the marine annelid *Nereis diversicolor* (Tasiemski *et al*., 2007), and here the non-halogenated synthetic peptide is equally active as the native peptide. This suggests that bromination is the basis for making the peptide more resistant to proteases rather than being required for the antimicrobial activity. Protease resistance would extend the activity *in vivo* and thereby sustain the antimicrobial activity. SdStrongylocin 2 also contains a bromotryptophan residue and shows potent activity against bacteria (Li *et al*., 2008); however, the recombinant SpStrongylocin 2 also shows antibacterial activity, even though the tryptophan residue is not brominated (Li *et al*., 2010a). Likewise, the centrocins have a bromotryptophan residue in their heavy chain, which, if not brominated, does not alter the microbicidal activity of these peptides (Bjorn *et al*., 2012). Although we cannot exclude the possibility that the bromination of the native dimeric centrocins and SdStrongylocin 2 may have some influence on their antimicrobial activity, this post-translational modification may have effects similar to that suggested for hedistin and the hagfish cathelicidins, which is to protect the peptides from protease degradation.

Sea urchins have four major populations of coelomocytes based on morphological differences (reviewed in Smith *et al*., 2010). Phagocytes constitute the majority of coelomocytes and are divided into three subtypes; however, cell separation techniques limit purification of the subtypes. Recent results of AMP gene expression from *S. droebachiensis*

show that transcripts of SdStrongylocin 1 are present in the phagocyte class of coelomocytes and in a fraction that is composed of a mixture of vibratile and colorless spherule cells. Furthermore, transcripts of SdStrongylocin 2 are present in both phagocytes and red spherule cells, while transcripts of centrocin 1 are only present in phagocytes in cytoplasmic vesicles (Li *et al*., 2014). Phagocytes challenged with co-cultured bacteria *in vitro*, resulted in centrocin 1 attaching to these phagocytosed bacteria inside the phagolysosomes. These studies suggest that sea urchin AMPs exert their antibacterial activities inside of coelomocytes and not in the coelomic fluid. Therefore, presequences direct the precursors of centrocin to the lysosomes. This is in agreement with previous observations where antibacterial activity was detected in extracts of coelomocytes and not in extracts of cell-free coelomic fluid (Haug *et al*., 2002).

Sea urchin eggs are embedded in a jelly coat composed of polysaccharides and glycoproteins (Jondeung and Czihak, 1982), which, in combination with maternal molecules, provides immune protection to the embryos (Kitazume *et al*., 1994). During development, sea urchin embryos and larvae face pathogenic challenges in the marine environment once they emerge from the fertilization envelope as multi-cellular organisms with an ectodermal body wall that quickly develop the digestive tract. Transcripts of the gene *Sp064*, which encodes a homologue of complement C3 that functions as an opsonin (Clow *et al*., 2004), is present in unfertilized eggs and throughout embryogenesis (Shah *et al*., 2003). Phagocytosis of injected yeast cells has been observed for the secondary mesenchyme cells at the mid gastrula stage of *Lytechinus variegatus* (Silva, 2000), and pinocytosis occurs in the cells lining the stomach and intestine of the pluteus larvae of *L. pictus* (Huvard and Holland, 1986). The question is: when do AMPs contribute to sea urchin larval innate immunity? In *S. droebachiensis*, transcripts of SdStrongylocins are present in the early pluteus stage whereas transcripts of centrocin 1 are detected in the mid pluteus stage (Li *et al*., 2014). Moreover, centrocin 1 is

mainly present in blastocoelar cells at coelomic vesicles and sacs around the stomach and esophagus, and also in a few blastocoelar cells that patrol close to the ectodermal walls in some arms. Thus, AMPs have a crucial role in immunity, not only in adult sea urchins but also in larvae.

There are several other AMPs that have been identified in echinoderms (Table 2). A number of partial peptide sequences that show antimicrobial activity were identified from a coelomocyte extract from the sea star *Asterias rubens*, including fragments of histone H2A, actin and filamin A (Maltseva *et al*., 2007). These peptides, which have a molecular mass of 1.8 - 2.4 kDa, are highly cationic with a predicted *pI* ranging from 11.3 to 12.3. Four other AMPs were also detected in *A. rubens* coelomocytes, but the sequences were not identified. A coelomic fluid extract of the sea cucumber *Cucumaria frondosa* was purified by molecular sieve chromatography, and an unsequenced AMP of about 6 kDa was detected (Beauregard *et al*., 2001). Two peptide fragments, called holothuroidins 1 and 2, from the sea cucumber *H. tubulosa* have activity against human pathogens, with MICs in the mM range (Schillaci *et al*., 2013). Helical wheel analysis indicated that the holothuroidins probably adapt to an α-helical structure with considerable amphipathic character. Synthetic holothuroidins also inhibit biofilm formation of three bacterial strains, *S aureus*, *S. epidermidis*, and *Pseudomonas aeruginosa*. Another synthetic peptide, corresponding to the α-helical region of CEL-III (a fragment of a lectin isolated from the sea cucumber *C. echinata*) shows strong antibacterial activity against *S. aureus* and *Bacillus subtilis* through a marked permeabilization of the bacterial cell membrane (Hatakeyama *et al*., 2004; Hisamatsu *et al*., 2008). Recently, peptides of <5 kDa, which might be fragments of beta-thymosin, were detected in coelomocyte extracts of the sea urchin *P. lividus*, and show activity against Gram-positive and Gram-negative bacteria and inhibited the formation of biofilms (Schillaci *et al*., 2010; Schillaci *et al*., 2012). Although these preliminary investigations only cover very few echinoderm

species, the exciting findings infer that echinoderms deserve more efforts to discover new active compounds.

4. Summary

In the last few decades, thousands of AMPs have been characterized, and some of their killing mechanisms and other functions have been addressed. In order to identify potent antimicrobial agents, exploration has shifted from terrestrial species towards marine species that inhabit regions from the intertidal zone to the deep sea. Echinoderms, being the second largest group of deuterostomes, constitute a huge potential source for new bioactive peptides that may lead to new drugs. In this review, we have provided an overview of antimicrobial peptides found in echinoderms. A broad range of bioactivities have been attributed to AMPs, including bactericidal, fungicidal, anti-inflammatory activity, wound healing, and inhibition of biofilm formation. In addition, AMPs from echinoderms have provided insights into the functional importance of some post-translational modifications, and unique functional domains and conformational structures, which may benefit the design of new peptide drugs, such as proteolytic resistant AMPs, AMP mimetics, chimeric AMPs, AMP congeners, AMP conjugates, and immobilized AMPs (Brogden and Brogden, 2011).

AMPs are crucial effector molecules that are essential for effective echinoderm innate immunity. Li *et al.* (2014) demonstrated that AMPs appear and function from the early developmental stages throughout the remainder of the sea urchin lifespan. Although AMPs act as downstream effector molecules in animal immunity, mammalian AMPs are also considered as immunoregulatory molecules (Hancock and Sahl, 2006; Hilchie *et al.*, 2013). However, there is currently no evidence to suggest that echinoderm AMPs function to modulate the expression of chemokines/cytokines during echinoderm immune defense. The complexity of immune receptors in echinoderms has been unveiled by the analysis of the genome of *S. purpuratus* (Rast *et al.*, 2006). This complexity raises many questions such as how immune

receptors can recognize and distinguish various pathogens, trigger signaling pathways, and activate effector molecules such as AMPs. Future work will help us to address questions of AMP activities and to better understand the role of AMPs in echinoderm immunity.

Acknowledgements

Table 1. Cysteine location patterns in AMPs containing 6 cysteine residues.

Peptide family	Cysteine location patterns [a]	Animals
Buthinin, Cycloviolacins, Circulins	C – C – C – C – C – C	Insects, plants
Thionins, Phoratoxin,	CC – C – C – C – C	Plants
Knottin-type AMPs, PaAMPs	C – C – CC – C – C	Plants
Tachystatins	C – C – CC – C – C	Horseshoe crab
Strongylocins	C – C – C – CC – C	Echinoderms
Alpha-defensins	C – C – C – C – CC	Mammals
Beta-defensins	C – C – C – C – CC	Mammals, birds

[a] Adjacent double cysteine residues are boxed. Information regarding cysteine arrangements in the different peptides was obtained from the *Antimicrobial Peptide Database* (Wang *et al*., 2009).
"–" one or more non-cysteine amino acids.

Table 2. Antimicrobial peptides and proteins characterized in echinoderms.

Class and genus	Origin	Peptides	MW (kDa)	Reference
Asteroidea				
Asterias rubens	Coelomocytes	Fragments of Actin, Histone H2A, and filamin A [a]	1.8-2.4	Maltseva *et al*., 2007
	Coelomocytes	Peptides	2.6-4.7	Maltseva *et al*., 2007
Echinoidea				
Paracentrotus lividus	Coelomocytes	Fragments of ß-thymosin [a]	1.3-2.3	Schillaci *et al*., 2010
Strongylocentrotus droebachiensis	Coelomocytes	SdStrongylocins	5.6-5.8	Li *et al*., 2008
	Coelomocytes	Centrocins	4.4-4.5	Li *et al*., 2010b
S. purpuratus	Coelomocyte cDNA	SpStrongylocins [b]	5.6-6.1	Li *et al*., 2010a
Holothuroidea				
Cucumaria echinata	Whole body	Fragments of CEL-III protein [a, c]	2.0-4.2	Hatakeyama *et al*., 2004
C. frondosa	Coelomic fluid	Sequence unknown	~ 6	Beauregard *et al*., 2001
Holothuria tubulosa	Coelomocytes	Holothuroidins [a, c]	1.4-1.5	Schillaci *et al*., 2013

[a] Putative AMPs, derived from larger precursor molecules.
[b] Recombinantly produced peptides.
[c] Synthetic peptides.

A.

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SdStrongylocin  1a  MDLRSASLVFLVVVMVLSYSMAAPLDADNDEEMEE
SdStrongylocin  1b  MDLRSASFVFLVVVMVLSYSMAAPLDADNDEEMEE
SpStrongylocin   1  MDLRSASLVFLVVVMVLSYSMAAPLDADNDEEMEE
                              preprosequence

SdStrongylocin  1a  IFGSIYHRKCVVKNRCETVSGHKTCKDLTCCRAVIFRHERPEVCRPQT
SdStrongylocin  1b  IFGSIYHRECVKKNRCETVNGNKTCKELTCCRAVIFRHERPEVCRPQT
SpStrongylocin   1  IFNSIYHRKCVVKNRCETVSGHKTCKDLTCCRAVIFRHERPEVCRPST
                              mature sequence


SdStrongylocin  2a  MNIRKGSLMLLITMMILSQAVASRFLDEADEDEHLMEA
SdStrongylocin  2b  MNIRKGSFMLLITIVILSQAVASRFLDEADEDEHLMEA
SpStrongylocin   2  MDLRSASLVFLVVAMVLSYSMAESFEVDDAVMEEL---
                              preprosequence
                    *
SdStrongylocin  2a  WNPFKKIANRNCYPKTTCETAGGKKTCKDFSCCQIVLFGK--KTRAKCTVVTS
SdStrongylocin  2b  WNPFKKIAHRNCYPKQECETAGGKKTCKDFSCCQIVLFGK--KTRAKCTVVTMS
SpStrongylocin   2  WNPFRKLYRKECNDVTSCDTVSGVKTCTKKNCCHRKFFGKTILKAPECTVIS
                              mature sequence
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B.

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Centrocin 1a  MMIKVALVLCAIVATSMVCAKDFEEQDALDALLNMMLPEEVASPDDAVALQ
Centrocin 1b  MMIKVALVLCAIVATSMVCAKNFEEQDALDTLLNMMLSEEVASPDDAVALQ
Centrocin 2   MMIKVALVLCAIVATSMVCAKNFEEQDALDTLLNLMLSEEAASP-DAVALQ
                              preprosequence
              *
Centrocin 1a  GWFKKTFHKVSHAVKSGIHAGQRGCSALGFSPEEARVKILTAIPEMREEDLSEEDLRGACAAAHALGR
Centrocin 1b  GWFKKTFHKVSHAVKSDIHAGQRGCSALGFSPEEARVKILTAFPEMKEEDLTEEGVRAVCAGAHALGR
Centrocin 2   SWFSRTVHNVGNAVRKGIHAGQGVCSGLGLSPEEARVKILSAVPEMREEDLSEEDLRAICAGAHALGR
              heavy chain        prosequence (interchain)   light chain
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C.

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              *
Centrocin 1a  GWFKKTFHKVSHAVKSGIHAGQRGCSALGF
                                      |
                           DLRGACAAAHAL


              *
Centrocin 2   SWFSRTVHNVGNAVRKGIHAGQGVCSGLGL
                                      |
                           DLRAICAGAHAL
```
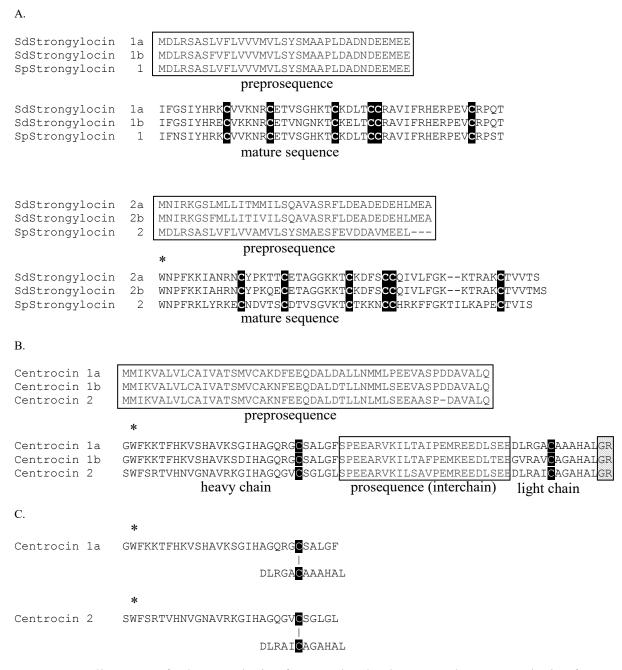
Figure 1. Alignment of SdStrongylocins from *S. droebachiensis* and SpStrongylocins from *S. purpuratus* (A), centrocins from *S. droebachiensis* (B), and the structure of centrocins (C). The preprosequences (or prosequence) are boxed. The first amino acid in the mature SdStrongylocin 2, SpStrongylocin 2 and the second amino acid in the mature centrocins are brominated tryptophans (*). Cysteines are shaded in black. The gray shaded and boxed C-terminal dipeptides in the precursors of centrocins, are cleaved off in the mature peptides.

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