ISJ 7: 132-140, 2010 ISSN 1824-307X

MINIREVIEW

Antimicrobial peptides in Echinoderms

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Accepted May 5, 2010

Abstract

Antimicrobial peptides (AMPs) are important immune effector molecules for invertebrates, including echinoderms, which lack a vertebrate-type adaptive immune system. Here we summarize the knowledge of such peptides in echinoderms. Strongylocins are a novel family of cysteine-rich AMPs, recently identified in the sea urchins, *Strongylocentrotus droebachiensis* and *S. purpuratus*. Although these molecules present diverse amino acid sequences, they share an identical cysteine arrangement pattern, dissimilar to other known AMPs. A family of heterodimeric AMPs, named centrocins, are also present in *S. droebachiensis.* Lysozymes and fragments of larger proteins, such as beta-thymocins, actin, histone 2A and filamin A have also been shown to display antimicrobial activities in echinoderms. Future studies on AMPs should be aimed in revealing how echinoderms use these AMPs in the immune response against microbial pathogens.

Key Words: sea urchin; host defence peptides; celomocyte; innate immunity

Introduction

Antimicrobial peptides (AMPs) are evolutionarily conserved small molecular weight proteins of the innate immune response, with a broad spectrum of antimicrobial activities against bacteria, viruses, and fungi (reviewed by Mookherjee and Hancock, 2007). AMPs appear naturally throughout all three domains of life from unicellular to multicellular organisms (Zasloff, 2002; Riley and Chavan, 2007; Wang *et al*., 2009). By February 2010, more than 1,500 AMPs have been identified (http://aps.unmc.edu/AP/main.php). AMPs are characterized as having less than 100 amino acids and are usually cationic in nature. Nearly all antimicrobial peptides form amphipathic structures which reflect the relative abundance and polarization of hydrophobic and hydrophilic domains within the peptides. The hydrophobicity enables the water-soluble antimicrobial peptides to interact with the hydrophobic lipid bilayer of the microbial membranes.

AMPs are likely to be attracted by and attach to the negatively charged bacterial surfaces because of their cationic nature. The mechanism by which AMPs interact with the cell wall structures of Gram-negative and Gram-positive bacteria has rarely been addressed and is therefore not yet understood (Brogden, 2005). However, once the peptides come into 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 (Yang *et al*., 2001). At sufficiently high concentrations of the peptides orientate perpendicularly and insert into the bilayer, thereby interfering with membrane integrity. However, new paradigms imply that pore-forming is not the only mechanism of antimicrobial activity. 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 (Brogden, 2005).

AMPs are crucial immune effector molecules for invertebrates which lack a vertebrate-type adaptive immune system. They have been identified both in plasma and in various blood cells and their distribution in the host can be site-specific or systemic. They are either expressed constitutively or the expression is induced by exposure to pathogens. They do not only inactivate bacteria *in vitro* and *in vivo* , thereby protecting host organisms against a variety of infections, but they also modulate immunity (Hancock and Diamond, 2000; Zasloff, 2002; Hancock *et al*., 2006). It is worth mentioning that a few AMPs play a role as anti-endotoxins (Scott *et al*., 2002; Bowdish *et al*., 2005) and chemokines in vertebrates (Durr and Peschel, 2002;

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Davidson *et al*., 2004) and might also induce production of cytokines and chemokines (Bals and Wilson, 2003). These immunomodulatory functions do not directly kill microbes, but recruitment and activation of immune cells and signalling molecules improves the host defence system.

In this review we will present an overview of AMPs identified and characterized from echinoderms and present their characteristics. In particularly, we will describe their antimicrobial activities, their primary structure, and posttranslational modifications of these molecules. Finally, we will address some important questions regarding future AMP research on echinoderms.

Antimicrobial activities in echinoderms

The phylum echinodermata is a large phylum of deuterostomes containing approximately 7,000 living species divided into the groups Crinoidea (sea lilies), Ophiuroidea (brittle stars), Asteroidea (starfishes), Echinoidea (sea urchins) and Holothuroidea (sea cucumbers). The adult echinoderms have a few major organs such as mouth parts, intestine, nerve ring, and gonad. These organs are located in the cavity, named celomic cavity. The remaining space of the celomic cavity is filled with celomic fluid which contains various "blood cells", the celomocytes. These cells are though to mediate the main immune functions in echinoderms, including antimicrobial activities. Many studies have been conducted to examine the activity of celomocytes lysates and celomic fluid against bacteria, fungi and even tumour cells. Celomic fluid from *Echinus esculentus* possess bactericidal activity against marine *Pseudomonas* sp (Wardlaw and Unkles, 1978). Gerardi *et al.* (1990) found that the highest bacterial growth inhibition against several *Vibrio* sp. is shown by phagocytes (called amoebocytes) and red spherule cells of *Paracentrotus lividus.*. Stabili *et al.* (1996) also discovered antibacterial activity against *V. alginolyticus* in celomocyte lysates and celomic fluid of *P. lividus*. Recently, it was reported that celomocytes of *P. lividus* show a cytotoxic activity against rabbit erythrocytes and the K562 tumour cell line (Arizza *et al*., 2007). Antibacterial activity was detected in extracts of several tissues from the green sea urchin *S. droebachiensis*, the common sea star *Asterias rubens*, and the sea cucumber *Cucumaria frondosa* (Haug *et al*., 2002). Some of the activities detected (in extracts of celomocytes and body walls) were caused by compounds of protein nature.

Several drug discovery projects have screened echinoderms for antibiotic activities. An early study by Rinehart et al (1981) showed that antimicrobial activity was present in 43 % of 83 unidentified species of echinoderms (collected from the west coast of Baja California and the Gulf of California) and 58 % of 36 unidentified Caribbean species displayed antimicrobial activities. In the northern Gulf of Mexico, 80 % of 22 echinoderm species showed antimicrobial activity (Bryan *et al*., 1994). Body wall extracts of echinoderms displayed activity against marine bacteria, but did also inhibit settlement of marine larvae (Bryan et al., 1996). Several antimicrobial molecules (other than AMPs) have been isolated from echinoderms, including echinochrome A (Kuwahara *et al*., 2009; Service and Wardlaw, 1984), steroidal glycosides (Andersson *et al*., 1989; Chludil *et al*., 2002; Levina *et al*., 2009), and polyhydroxylated sterols (Iorizzi *et al*., 1995). Although these results indicate that echinoderms present remarkable activity against microbes, only a few AMPs have been reported.

Antimicrobial peptides in echinoderms

Lysozymes are widely distributed throughout the animal kingdom and catalyze the hydrolysis of peptidoglycans of bacterial cell walls of especially Gram-positive bacteria, and acts as a nonspecific innate immunity molecules against the invasion of bacterial pathogens (Jollès and Jollès, 1984). They are characterized as basic proteins of low molecular weight (15-25 kDa) with an isoelectric point of 10.5–11.0, stable at acidic pH and even at high temperatures. There are several lysozymes or lysozyme-like proteins identified from celomic fluid, celomocytes and other tissues of echinoderms (Jollès and Jollès, 1975; Canicatti and Roch, 1989; Canicatti, 1990; Canicatti and D´Ancona, 1990; Gerardi *et al*., 1990; Stabili *et al*., 1994; Stabili and Canicatti, 1994; Stabili and Pagliara, 1994, 2009; Shimizu *et al*., 1999; Bachali *et al.*, 2004; Cong et al., 2009) (Table 1).

A study by Beauregard and co-workers indicated the presence of AMPs in the celomic fluid of the orange-footed sea cucumber, *C. frondosa* (Beauregard *et al*., 2001). The celomic fluid extract was purified by molecular sieve chromatography and antibacterial activity was detected in separate fractions. A peptide of approximately 6 kDa was identified, but no sequence was reported (Table 1).

In a celomocyte extract from the starfish *A. rubens* showing antimicrobial activity, several protein/peptides with molecular mass around 2 kDa were isolated (Maltseva *et al*., 2004; Maltseva *et al*., 2007) (Table 1). Two peptides were identified as part of the histone molecule, H2A. Two other peptides were identified as fragments of actin, while one peptide was a fragment of filamin A. In addition, four other AMPs were detected, but not characterized. It had been known for long time that histones have antibacterial properties (Hirsch 1958). Histone-derived fragments (H1, from H2A and H2B) have shown to possess antimicrobial activity (From *et al*., 1996; Robinette *et al*., 1998; Patrzykat *et al*., 2001; Richards *et al*., 2001; Fernandes *et al*., 2002). Although Maltseva *et al.* (2004, 2007) also identified fragments of actin and filamin A in the active extract, the antimicrobial activity of these fragments need to be further investigated.

Recently, Schillaci *et al*. (2009) reported that a 5 kDa peptide fraction from the celomocytes of *P. lividus* presents activity against both Gram-positive and Gram-negative bacteria and fungi (Table 1). The peptide fraction also showed the ability to inhibit the formation of *Staphylococcus* biofilms. The authors suggested that the antimicrobial and antistaphylococcal biofilm activity of this peptide

Table 1 Antimicrobial peptides and proteins characterized in echinoderms

Class and genus	Origin (tissue/cell type)	Compound	Mw (kDa)	Reference
Asteroidea				
Asterias forbesi	Cell-free celomic fluid	Complement-like		Leonard et al., 1990
A. rubens	Body wall	Peptides/proteins	<20	Haug *et al*., 2002
	Celomocytes	Fragments of actin, histone H2A, and filamin A	1.8-2.4	Maltseva *et al*., 2007; Maltseva *et al*., 2004
	Celomocytes	Peptides	2.0-4.7	Maltseva *et al*., 2007; Maltseva *et al*., 2004
		Lysozyme	15.5	Jollès and Jollès 1975; Bachali *et al*., 2004
Marthasterias glacialis	Eggs	Lysozyme-like		Stabili and Pagliara, 1994, 2009
	Body wall mucus	Lysozyme-like		Canicatti and D´Ancona, 1990
Echinoidea				
Holothuria scabra	Celomic fluid	Lectin	>10	Gowda *et al*., 2008
H. polii	Celomocytes	Lysozyme-like		Canicatti and Roch, 1989
	Various tissues	Lysozyme-like		Canicatti, 1990
Paracentrotus lividus	Celomocytes	Lysozyme-like		Gerardi *et al*., 1990
	Celomocytes	Protein	~ 60	Stabili *et al*., 1996
	Celomocytes	Peptide	~ 5	Schillaci *et al*., 2009
	Larvae	Lysozyme-like		Stabili *et al*., 1994
	Seminal plasma	Lysozyme-like		Stabili and Canicatti, 1994
	Various tissues	Lysozyme-like		Canicatti, 1990
Strongylocentrotus droebachiensis	Celomocytes	Stongylocins	5.6-5.8	Li *et al*., 2008
	Celomocytes	Centrocins	4.4-4.5	Li *et al*., 2010b
S. intermedius	Celomic fluid	Lysozyme	14	Shimizu *et al*., 1999
S. purpuratus	Celomocyte cDNA	SpStrongylocins[a]	5.6-6.1	Li *et al*., 2010a
Holothuroidea				
Cucumaria echinata	Whole body	Fragments of lectin CEL-III	2.0-4.2	Hatakeyama *et al*., 2004; Hisamatsu *et al*., 2008
C. frondosa	Celomic fluid	Peptides	~ 6	Beauregard *et al*., 2001
	Various tissues	Peptides/proteins	<20	Haug *et al*., 2002
Parastichopus californicus	Celomic cavity	LPS-binding compound		Dybas and Fankboner, 1986
Stichopus japonicus	Intestine cDNA	Lysozyme[a]	14	Cong *et al*., 2009

[a] Recombinantly produced and tested for activity.

fraction is associated with beta-thymosin like fragments. Beta thymosins have been reported to induce the production of metalloproteinases, display chemotactic and anti-inflammatory activities (Huff *et al*., 2001) and even function as AMPs released by human platelets (Tang *et al*., 2002).

Several AMPs have been isolated and characterized from the green sea urchin, *S. droebachiensis*. One group is the cysteine rich peptides named strongylocins (Li *et al*., 2008). Homologous genes, named SpStrongylocins, were also identified in the sister species *S. purpuratus* (Li *et al*., 2010a). All these native peptides or recombinant equivalents show antibacterial activity against both Gram positive and Gram negative bacteria (Table 2). In addition, there are several heterodimer structured peptides, named centrocins, identified from *S. droebachiensis* (Li *et al*, 2010b). They show strong potent activity, not only against Gram-positive and Gram-negative bacteria, but the longest peptide chain of the dimers also show strong activity against fungi and yeasts.

In silico analysis of strongylocins and comparison of the native peptide sequences and the ones deduced from cDNA sequences, show that these peptides contain three regions: a signal peptide, a prosequence and a native sequence (Fig. 1). Analysis , using both the neutral network model and the hidden Markov model (Bendtsen et al., 2004; http://www.cbs.dtu.dk/services/SignalP/), indicates that a signal peptide cleavage site is located between the amino acid number 22 and 23 for all these peptides.

Table 2 Susceptibility of bacterial strains to the antibacterial peptides, strongylocins and centrocins, isolated from *S. droebachiensis* celomocytes, and recombinant SpStrongylocins (Li *et al*., 2008; Li *et al*. 2010a, b)

Peptide	Minimal inhibitory concentration (μM)			
	Listonella anguillarum	*Escherichia coli*	*Corynebacterium glutamicum*	*Staphylococcus aureus*
Strongylocin 1 [a]	2.5	5.0	2.5	2.5
Strongylocin 2 [a]	1.3	5.0	2.5	2.5
Recombinant SpStrongylocin 1 [b]	15.0	7.5	7.5	15.0
Recombinant SpStrongylocin 2 [b]	15.0	7.5	3.8	15.0
Centrocin 1 [a]	2.5	1.3	1.3	2.5
Centrocin 2 [a]	2.5	2.5	1.3	5.0

[a] Minimal inhibitory concentration (MIC) was determined as the lowest concentration of peptide causing an optical density less than 50 % of the growth control without any peptide present.
[b] 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.

It is not surprising that strongylocin 1 and SpStrongylocin 1 contain an identical signal peptide sequence since they share high amino acid sequence similarity. However, SpStrongylocin 2 does not share an identical signal peptide with strongylocin 2, but with strongylocin 1. Although no experimental data show which mechanism assists the migration of these precursors of strongylocins and SpStrongylocins within the cells, the diversity of the signal peptides suggests that strongylocin 2 likely employs a divergent intracellular trafficking pathway than the other sea urchin AMPs.

Analysis of strongylocins and SpStrongylocins show that their prosequences are located at the N-terminal region, following the signal peptide. It is common that prosequences are located at the N-terminal or the C-terminal region, or even in between parts of the mature sequences. The prosequence is considered to act as an intracellular steric chaperone during the folding process (Inouye, 1991), and it has been shown that prosequence-supported protein folding is crucial for processing of the proper protein having antifungal activity (Reichhart and Achstetter, 1990). In addition, it has been shown that the prosequences in some proteolytic enzyme precursors inhibit the activity of the mature proteins (Neurath, 1989). The prosequences of strongylocins and SpStrongylocins are negatively charged sequences which may neutralize the positive charges of the mature sequences (Li *et al*., 2008; Li *et al*., 2010a, b). Therefore, the precursor molecules are likely less toxic to the host cells than the mature peptides. The peptides will obtain their activity during their maturation once the prosequences are removed.

Strongylocins contain six cysteine residues, which form three disulfide bridges (Li *et al*., 2008; Li *et al*., 2010a). Cysteine-rich AMPs are found in various phyla, such as the defensin families in both vertebrates and invertebrates, tachystatins in horse shoe crabs and circulin A in plants (Daly *et al*., 1999; Wang *et al*., 2009). Table 3 shows that strongylocins and SpStrongylocins display a novel cysteine location pattern compared to other known cysteine-rich peptides containing 6 cysteines. This indicates that these AMPs may not form the same disulfide bridge linkages as the other peptides containing six cysteines, since proteins that have the same cysteine residue pattern tend to have identical disulfide connections (Bania *et al*., 1999; Bontems *et al*., 1991; Pallaghy *et al*., 1994). Disulfide bridges are crucial for the antimicrobial activity of most cysteine-containing AMPs (Daher *et al*., 1986; Mandal and Nagaraj, 2002), and they may also stabilize the conformation of the molecules (Selsted and Ouellette, 2005). Therefore, the disulfide linkages may aid strongylocins and SpStrongylocins to resist proteolysis within the celomocytes, and/or after their release into protease-containing environments.

Strongylocin 2 contains a tryptophan in the mature sequence which is likely brominated (Li *et al*., 2008). Several AMPs with bromotryptophan have also been isolated from other marine organisms, for example styelin D from the tunicate, *Styela clavata* (Taylor *et al*., 2000), cathelicidin from the hagfish, *Myxine glutinosa* (Shinnar *et al*., 2003), and hedistin from the annelid, *Nereis diversicolor* (Tasiemski *et*

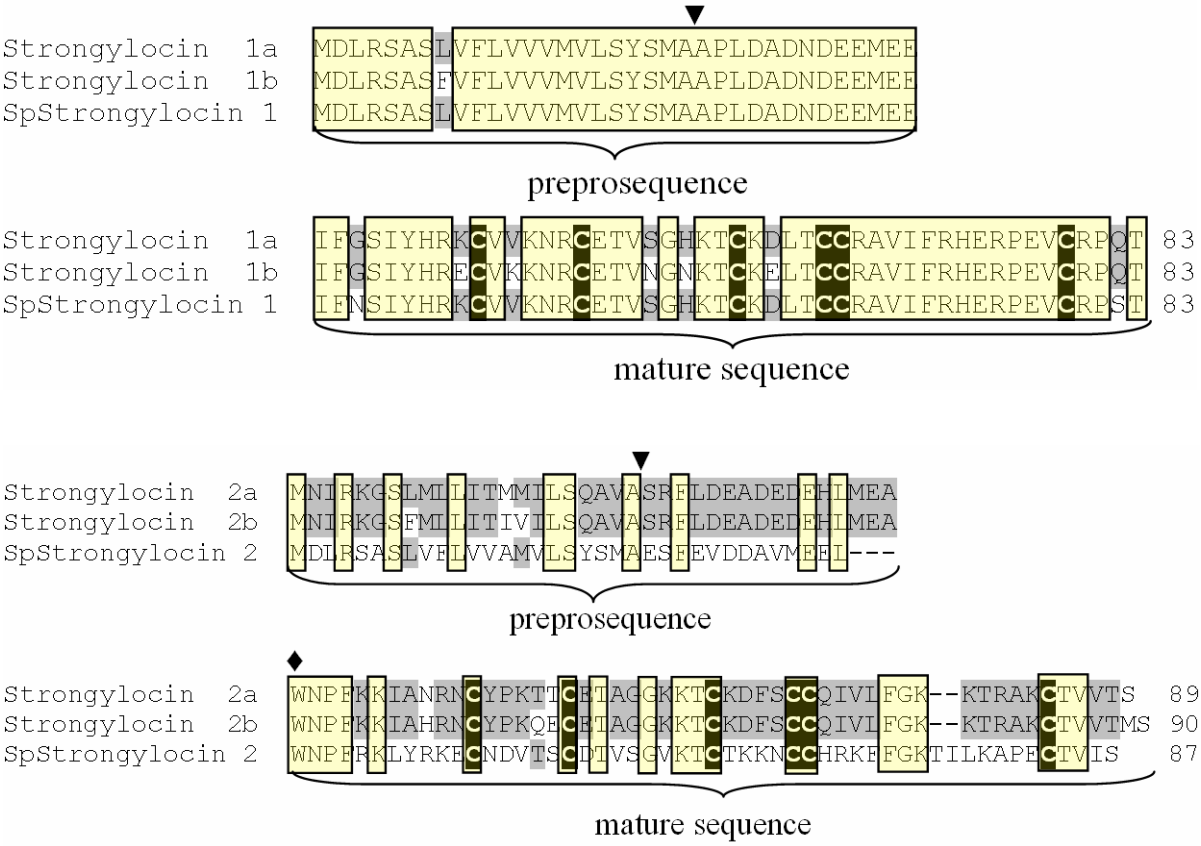

Fig. 1 Alignment of strongylocins from *S. droebachiensis* and SpStrongylocins from *S. purpuratus*. The predicted cleavage site between the signal peptide and the proregion is shown (▼). The first amino acid in active strongylocin 2 and SpStrongylocin 2 are likely a modified tryptophan (♦). Identical amino acids are shown in boxes, similar amino acids are shaded in grey, and cysteines are highlighted in black.

al., 2007). Brominated amino acids may protect the peptides from proteolysis, and/or increase the biological activity of peptides (Bittner *et al*., 2007). Although SpStrongylocin 2 also contains a tryptophan residue in the same position as strongylocin 2, the recombinantly produced SpStrongylocins, having a non-brominated Trp residue, are still antimicrobial active (Li *et al*., 2010a). Therefore, we speculate that the brominated tryptophan affects the peptides by enhancing their stability, but does not affect their antimicrobial activity (Li *et al*., 2010a). This suggestion is supported by results showing no difference in the antimicrobial activity between the heavy chain of the dimeric peptide centrocin 1, with or without the brominated modification (Stensvåg, unpublished).

Future studies on AMPs in echinoderms

AMPs are important immune defence molecules for echinoderms that lack a vertebrate-type adaptive immune system (reviewed by Smith *et al*., 2010). It is therefore interesting to know whether these AMPs are secreted into the celomic fluid or stored in the granula of certain cell types. It has been documented that neutrophilic granules packed with human cathelicidin and defensins are fused with phagocytic vacuoles, where they contain certain concentrations of AMPs to eliminate phagocytosed pathogens (reviewed by Ganz, 2003; Lehrer, 2004; Brogden, 2005). For penaeidins in shrimp, it seems that haemocytes migrate towards the infection sites and then release the AMPs presumably by lysis of the cells (Munoz *et al*., 2002). Echinoderms have a large celomic cavity filled with circulating fluid which could dilute AMPs if they were secreted or released by the cells. Therefore, further investigation on the distribution of AMPs in echinoderms might uncover whether the peptides are involved in 1) intracellular elimination of pathogens; 2) local release of AMPs at the infection sites; or 3) massive release of AMPs to regulate other immune activities. Although several studies have documented mammalian AMPs as immune regulatory molecules (Reviewed by Hancock *et al*., 2006 and Diamond *et al*., 2009), unfortunately, there are not many investigations on invertebrate AMPs. It has been reported that *Tachypleus tridentatus* haemocyte granules can release tachyplesin after

Table 3 Comparison of cysteine location patterns in AMPs containing six cysteines

Peptide family	Cysteine location patterns [1]	Animals
Strongylocins	C – C – C – **CC** – C	*S. droebachiensis*
SpStrongylocins	C – C – C – **CC** – C	*S. purpuratus*
Beta-defensins	C – C – C – C – **CC**	*Bos taurus*
Alpha-defensins	C – C – C – C – **CC**	*Homo sapiens*
Tachystatins	C – C – **CC** – C – C	*T. tridentatus*
Knottin-type AMPs	C – C – **CC** – C – C	*Phytolacca americana*
Thionins type III and IV AMPs	**CC** – C – C – C – C	*Sorghum bicolor*
Insect defensins	C – C – C – C – C – C	*Rhodnius prolixus*

[1] Adjacent double cysteine residues are highlighted in yellow. Information regarding cysteine arrangements in the different peptides was obtained from the *Antimicrobial Peptide Database* (Wang *et al*., 2009).

encountering microbial endotoxins and form a complex (Iwanaga *et al*., 1998; Hirakura *et al*., 2002). This complex will then block the activation of factor C, which is crucial for haemolymph coagulation (Nakamura *et al*., 1988). Pancer *et al*. (1999) identified an *NF-κB* homologue, transcription factor in *S. purpuratus* celomocytes According to analysis of the immune related gene repertoire of *S. purpuratus* (Hibino *et al*., 2006; Rast *et al*., 2006), Toll-like receptor (TLR) genes, interleukin (IL)-17 genes, IL receptors, and tumor necrosis factor (TNF) family members are present in the sea urchin genome. It is possible that AMPs are involved in the endotoxin-induced signalling pathway from the TLR to NF-κB, and/or suppress inflammation. Therefore, it is important to keep in mind that AMPs may play multiple immune roles in the echinoderm´s immunity as in other species (Hancock and Diamond, 2000; Lehrer, 2004; Durr *et al*., 2006; Hancock *et al*., 2006; Mookherjee and Hancock, 2007; Cuthbertson *et al*., 2008; Smith *et al*., 2008).

The discovery of AMPs in echinoderms has attracted attention from both pharmaceutical and academic sectors. However, there should be more focus on searching for new AMPs in Crinoidea and Ophiuroidea by newly developed high-throughput screening systems or other proteomic techniques since there is limited information about antimicrobial compounds from these echinoderm classes.

In summary, AMPs are important host defence molecules in invertebrates. Investigations of antimicrobial peptides/proteins in echinoderms have revealed two novel families of AMPs in Strongylocentrotus, lysozymes in various species and fragments of larger proteins having antibacterial activity. These AMPs do not only attract interest as potent drugs or drug leads, but may also become useful in studying the echinoderm immune system.

Acknowledgements

Writing this review was supported by grants from the University of Tromsø, The Marine Biotechnology in Tromsø (MABIT) research program (No. BS0034) and the Norwegian Research Council (Nos. 178214/S40 and 184688/S40). Oleg Sokolov is appreciated for translating the Russian papers.

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