CHAPTER II

LITERATURE REVIEWS

2.1 Sandworm *Perinereis nuntia* Savigny

The taxonomic definition of the sandworm is as following;

Phylum Annelida

Class Polychaeta

Order Aciculata

Suborder Phyllodocida

Superfamily Nereidoidea

Family Nereidae.

Polychaetes ('Poly' = many; 'chaeta' = hairs) are the largest groups of annelids and majority are marine polychaetes. More than 8,000 species are described in this class. Most of them live in soft or rocky environment on sea floor, abundant from the intertidal zone to depths of over 5,000 meter and distribute around tropical Indo-Pacific. They may be, but rarely, found in freshwater and terrestrial (humid), or parasitic. Most polychaetes fed by sediment swallowing of microphytobenthios, mainly benthic diatoms. Polychaetes in family Nereidae were contained 42 genera and about 500 species (Rouse and Pleijei, 2001).

2.1.1 General morphology of sandworm

The body of sandworm is covered by a flexible external cuticle to make it soft-body. The shape is long flat like and multi segmented. It can be divided into three parts; head, trunk and tail. Head part contains 2 pairs of antennas, 4 tentacular cirri and 1 pair of complex eyes. It has a complex brain which divides into three divisions; a forbrain, midbrain and hindbrain. In trunk part, segment is limited by septa from neighboring segments. Each segment carries parapodia and chaetae and contains digestive, vascular, muscular, nervous system and pair of nephridia for excretion. The parapodia is the flatlike projective on both sides of segment for locomotion and gas exchange. It has a closed circulation system with haemoglobin. The close circulation system consists of medial, dorsal and ventral longitudinal vessels, linked by smaller vessels, capillary beds and gut lacunae. The last part, tail; the posterior section of the body which contains simple terminal anus (Verma, 2005).

2.1.2 Reproductive of sandworm

They are separate sexes, breeding only once in their lifecycle before dying. When male and female sandworms reach maturity, hormonal changes cause their bodies to alter. Their digestive systems break down, to enable large numbers of eggs and sperm to be produced, and most species develop large eyes and swimming legs in preparation for leaving their burrows to spawn at the water surface. Eggs and sperm shed into coelom and leave the body through the nephridia. After the external fertilization, a ciliated free-swimming larval is developed. A combination of a temperature and lunar cycle stimulates the release of pheromones and gametes from all the mature worms in the population. Spawning during spring tides probably ensures the maximum dispersion of fertilized eggs in the water (Pechnik, 2005).

2.1.3 Important of sandworm

Sandworm is an important part of the invertebrate community of intertidal mud flat, providing a source of food for wading birds and fish. They are also used as live bait for anglers and live feed for marine culture especially shrimp farm. At least 6 *Perinereis* species are harvested commercially for these propose, including *P. brevicirris* in Taiwan and *P. nuntia* in Thailand. Sandworm used for shrimp hatcheries in Thailand are collected by digging from sediment shore or from sandworm farm as shown in Figure 2.1. In Japan, *P. nuntia* var. vallata has been investigated as a biological agent for treating sewage waste (Glasby and Hsieh, 2006). Moreover, many bioactive components were identified from sandworm including collagens, glycerophospholipids, eicosapentaenoic acid, fibrinolytic enzyme and antimicrobial peptide (Pan *et al.*, 2004).

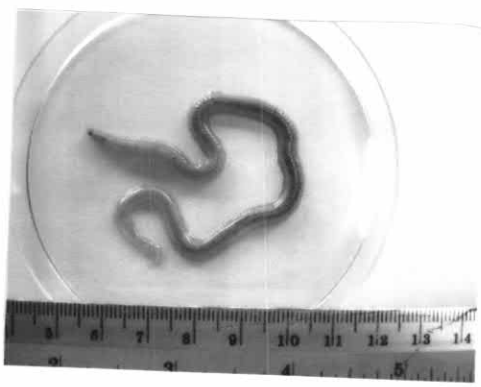
2.2 Proximate composition of sandworm

The marine animals as food for human are the most valuable animals which catch from the sea all over the world. Many researchers have been determined the proximate composition of valuable marine animal. These data are used for improvement of animal's diet formulation. In contrary, little information on the proximate composition of polychaetes is available. Because polychaete is not direct food for human (except some countries) but they are valuable food for marine culture especially for broodstcok shrimp hatcheries.

Worm collecting activity

Wild sandworm

Farming scale of sandworm



Farmed sandworm

Figure 2.1 Wild and farmed sandworm

Luis and Passos (1995) reported about proximate of marine polychaete, *Nereis diversicolor* in Portugal. Wild *N. diversicolor* were caught every month from November 1990 till October 1991 and determined for proximate. The results showed that the protein content was maximum 60% (in winter) and minimum of around 47% (in summer). For the fat content, it was maximum 4.4 ± 0.2% wet weight (in winter) and minimum 1.9 ± 0.1% wet weight (in spring). Fatty acid compositions of *N. diversicolor* were determined and found major fatty acid were C16:0 (the usual major fatty acid component in animal tissue), C18:1, C18:2 and C20:5. The fatty acids compositions were 23.37% saturated fatty acids and 65.38% unsaturated fatty acids for all year average. Furthermore, percentage of unsaturated fatty acids was increased during winter. From these results, the report was concluded that polychaetes storage lipid during periods of good sexual immaturity and depleted during maturation and spawning. Moreover, lipid was accumulated during winter and depleted in spring and summer. The degree of unsaturated fatty acids was increased during winter, induced by environmental temperature as an animal to maintain membrane fluidity at low temperature.

Fatty acids compositions in *N. diversicolor* fed with different diet were investigated by Costa (2000). The wild *N. diversicolor* was caught and grew them in laboratory and fed with 6 different experimental diets. The fatty acids compositions of all experiments were determined. The results showed as report from Luis and Passos (1995), even *N. diversicolor* was fed with different diets but the major fatty acids in all tests were C16:0, C18:1 C18:2 and C20:5. It means that the major fatty acids in marine polychaete were not effect by diet.

In Thailand, a few data of proximate composition of polychaete were report. Pimoljinda (1999) reported percentage of protein in marine polychaete, *Diopatra* sp.

and *Perinereis* sp.. The *Diopatra* sp. was contained 58.37% protein and *Perinereis* sp contained 45.32% protein. Meunpol (2005) investigated in wild marine polychaete for broodstock shrimp, sand polychaete; *Perinereis* sp. and mud polychaete; *Marphysa* sp.. Sand polychaete was contained 63.87% protein, 14.19% lipid and 9.26 ± 0.20% moisture while mud polychaete was found 50.90% protein, 5.25% lipid and 8.04 ± 0.16% moisture. However, not available information of proximate composition and nutritional value in wild compared with farmed sandworm (*P. nuntia*). Therefore, the one purpose of this study is to determine the nutritional values of farmed and wild sandworm. The data will be use as a guideline for quality control of farmed sandworm as live feed for aquatic animals.

2.3 The innate immunity of invertebrate animals

There are several barriers which help an organism's defense against surrounding pathogens. The first barrier is the skin or mucosa, which is exposed to the external environment and prevents most of the pathogens. The second barrier is the innate immune system, which is non specific to pathogens. And the third barrier is the acquired immune response system, which adds the features of memory and adaptability. All animal can efficiently prevent infection and protect themselves against surrounding microbes through their innate and adaptive immune system.

Invertebrates, which lacking adaptive immune system use innate immunity to detect and response to pathogens. The innate immune system is the most primitive first line of inducible host defense. This defense system is essential for the survival and perpetuation of all multicellular organisms. Invertebrates which do not possess immunoglobulins, have developed unique modalities to detect and response to microbial surface antigens like lipopolysaccharide (LPS), lipoteichoic acid,

lipoprotein, peptidiglycan (PGN) and β-D-glucans. Because both invertebrates and vertebrates response to these substances, it is likely that a system recognizing these epitopes emerged at an early stage in the evolution of animal. Moreover, it is well known that various microbial cell wall components elicit a variety of responses that depend on species and cell type. In invertebrates, toll-like receptor mediated antimicrobial peptide production, haemolymph coagulation, melanin formation and lectin-mediated complement activation are prominent immune responses as shown in Table 2.1 (Iwanaga and Lee, 2005).

Table 2.1 Major host defense system in invertebrates animal

1. Haemolymph coagulation system
2. Pro-phenoloxidase (pro-PO) activating system
3. Lectin-complement system
4. Agglutinin-lectin system
5. Antimicrobial peptides
6. Reactive oxygen-producing system
7. Phagocytic system

2.4 Antimicrobial peptides (AMPs)

Antimicrobial peptides (AMPs) are the earliest molecule factors in the evolution of innate immunity and are considered to play a key role in invertebrates host defense. AMPs display broad-spectrum activities against pathogenic bacteria, fungi, envelop virus, parasite and even cancer cell (Cytrynska *et al.*, 2007; Lofgren *et al.*, 2008; Zhao *et al.*, 2007). They have evolved to rapidly neutralize and kill

pathogenic microorganism. Most AMPs are hydrophobic and cationic properties. They have a molecular mass below 10 kDa and take an amphipathic structure (Zhao *et al.*, 2007). Since the original discovery of cecropin in moth *Hyalophora ceropia* (Cheng *et al.*, 2005) and with a development of separation and analytical techniques, a rapid expansion with over 800 AMPs has been discovered and characterized from various species according to the Antimicrobial Sequence Database (Wang and Wang, 2004).

2.4.1 Classification of antimicrobial peptides

As mention above, over 800 AMPs were isolated from many organisms. Therefore, they can be classified into four groups according to their biochemical properties and chemical structures as cationic peptides, anionic peptides, aromatic dipeptides and peptide derived from oxygen-binding proteins (Wang *et al.*, 2007). The cationic peptides are the largest group of AMPs. They were found more than 400 peptides from all AMPs. Member of this groups are broad spectrum with against a large spectrum of microorganisms, including Gram positive bacteria, Gram negative bacteria, fungi, virus and protozoa. Furthermore, antimicrobial activities of anionic peptides, aromatic dipeptides and peptides derived from oxygen-binding proteins are weaker than cationic peptides. The well-know representative of cationic peptides is cecropins which isolate from moth and nematode (Pillai *et al.*, 2005). Cecropins are a family of 3-4 kDa linear amphipatic peptides containing two alpha helix segments linked by a short hinge. One of this groups, maganin from frog skin *Xenopus* sp. was used as a template for pioneering clinical trial for biomedical applications. Moreover, cecropins and maganin are demonstrated anticancer activity (Cytrynska *et al.*, 2007). The more complex structure of cationic AMPs is defensins, a huge group of 4 kDa

open-ended cysteine-rich peptides isolated from vertebrate and invertebrate. They contain alpha helix and link to beta sheet by disulfide bridges, some of this group has 3 or 4 bridges (Vizioli and Salzet, 2002).

Anionic peptides, a novel group of AMPs. They were isolated from human, leech and mussel. These peptides are not broad spectrum, they against only Gram positive bacteria (Tasiemski *et al.*, 2004). However proline – rich anionic peptides killed bacterial cell by causing cytoplasmic protein precipitation and intracellular content flocculation (describe in 2.4.2.2). There are also known peptides affecting important intracellular process, eg. DNA and protein synthesis (Cytrynska *et al.*, 2007).

Aromatic di-peptides and peptides derived from oxygen-binging proteins are smaller group of AMPs than cationic and anionic peptide. Antibacterial peptide which is an aromatic dipeptides structure was isolated from flesh fly. It was very low molecular weight with 573 Daltons and mode of action of this group is not clear. While peptides derived from oxygen-binding protein have been report as bactericide compounds and might be as considered a defense molecule to fight pathogens (Vizioli and Salzet, 2002).

2.4.2 Mechanism of antimicrobial peptides

2.4.2.1 *Mechanism of targets specificity*

Several studies have been carried out to understanding the mode of action of AMPs. The almost common steps to kill microbe are peptide insertion and membrane permeability (describe in 2.4.2.2). However, all biological membranes are composed of a proteins and phospholipids but AMPs display highly abilities to

discriminate between microbial targets and normal host cell. The mechanisms of target specificity of AMPs were proposed by Yeaman and Yount (2003). The mechanisms were membrane composition and charge, membrane asymmetry and microbial ligands.

i) Membrane composition and charge; the phospholipids composition in cell membrane of eukaryotes and prokaryotes cell were different. The phospholipids are divided into several groups based on their net charge. Phosphotidylcholine (PC), phosphotidylethanolamine (PE) and sphingomyelin (SM) are neutral. While phosphotidylglycerol (PG), cardiolipin (CL) phosphotidylserine (PS) are negatively charged. Eukaryotic cell membranes mainly contain neutrally charged phospholipids such as PE, PC or SM. In contrast, prokaryotic cell membranes mainly contain negatively charged phospholipids such as PG, CL or PS. These characteriztic membrane composition and charge properties serve as biomarkers that enable the AMPs to identify and attach microbial cells instead of host cells.

ii) Membrane asymmetry; membranes of both eukaryotes and prokaryotes are not static and not symmetry. The composition and characterization of lipid bilayers of their membranes are different and make them asymmetry. The asymmetry of lipid bilayers is the one mechanism of AMPs for find the invader cell.

iii) Microbial ligands; Microorganism have specific proteins on their cell membrane. Some studies were reported that specific proteins on microorganism surface are important for AMPs binding and start the mechanism.

2.4.2.2 *Mechanism of AMPs action*

The mechanism of AMPs action to kill microorganism is completely different from that of phagocytes and antibodies in adaptive immunity. The

phagocytes kill the invading microorganism by phagocytosis whereas the antibodies target receptors of the invading cells to stop the infection. On the contrary, AMPs most directly interact with cell membrane lipid to form transmembrane pore that cause the target cell to die.

The first step in the mechanism of action occurs when cationic regions of AMPs are attached by negative charged components of the membrane of the pathogens. In Gram negative bacteria, the outer membrane which contain the lipopolysaccharide as well as the outer of the cytoplasmic membrane contain anionic molecules. The peptidoglycan, the component of Gram positive bacteria cell wall, contains negatively charged teichoic and teichuronic acids. These anionic components of bacteria are exterior of the cell, therefore attract antimicrobial peptides to bind to bacteria surface (Hancock and Chapple, 1999). In the solution, AMPs are unstructured peptides. When they attach to microbe membrane, they form secondary structure and insert into membrane bilayers to form pore (Figure 2.2). The pore size causes leakage of cytoplasmic material and hence death of the microbe cell (Figure 2.3). Transmembrane pore-forming mechanisms is not only activity of them, they also have intracellular mechanisms by flocculate of intracellular contents, alter cytoplasmic membrane septum formation, inhibit cell wall synthesis, bind nucleic acid, inhibit nucleic acid synthesis, inhibit protein synthesis and inhibit enzymatic activity (Brogden, 2005).

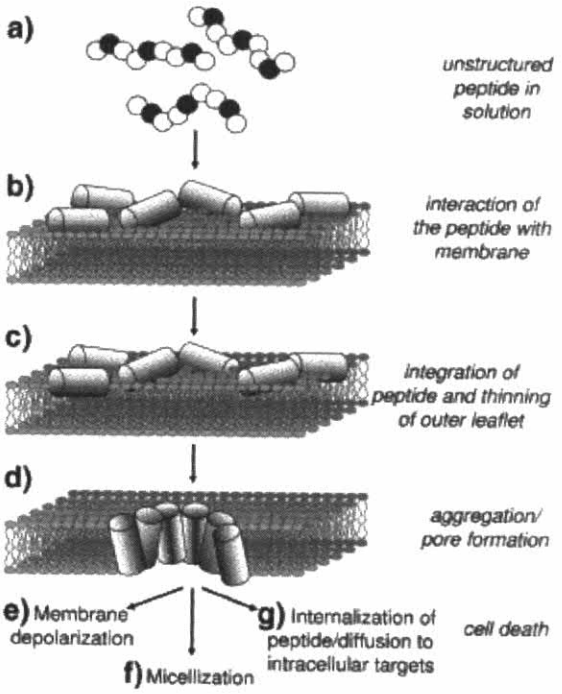


Figure 2.2 Model for the mechanism of action of AMPs (Straus and Hancock, 2006).

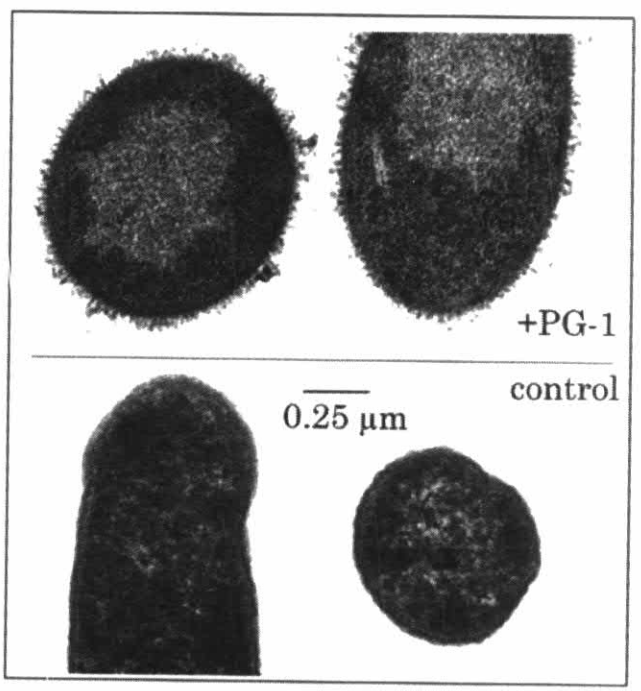


Figure 2.3 Effect of AMPs (portegrin, PG-1) on *E. coli* cell. (Upper) Bacteria were exposed to 50 μg/ml for 15 min and shown leakage of cytoplasmic material compare with control normal cell (lower) (Gidalevitz *et al.*, 2003).

These mechanisms of AMPs are also effective against resistance pathogenic microorganism. Moreover, the development of resistance of microorganism to AMPs appears to be difficult because change of phospholipids composition of cell membrane may effect on transport and enzymatic system which impaction to cell survival (Lofgren *et al.*, 2007). Therefore, AMPs are candidates as new antimicrobial agents due to their broad antimicrobial spectra, highly selection and the difficulty for microbe to develop resistance to these peptides (Tincu and Taylor, 2004).

2.4.3 Antimicrobial peptides from marine invertebrates

Marine invertebrates also have innate immune mechanisms that include both humoral and cellular response. Humoral immunity in them is antimicrobial agents present in the blood cell and plasma. Cellular immunity of them is based on cell defense reactions including encapsulation, module formation and phagocytosis (Iwanaga and Lee, 2005). The circulating haemolymph in marine invertebrates contain biologically active substance such as lectins, clotting factor and antimicrobial peptide defense system. All of these systems contribute to a self defense system in marine invertebrates against invading microorganism which high number in sea water. Moreover, the tissue such as the gut and respiratory organs are direct exposed to pathogenic microorganism in environment. The survival of marine invertebrates in this environment suggests that their innate immune system is effective and robust (Tincu and Taylor, 2004).

The ocean covers 71% of the surface of the earth and contains approximately half of the total global biodiversity, with estimates ranging between 3 and 500 x 10^6 different species. Therefore, the marine invertebrates that rely on innate immune

mechanisms for host defense is a spectacular resource for the development of new AMPs (Tincu and Taylor, 2004).

A variety of AMPs were discovered and characterized from representatives of marine invertebrates, including, mollusks, chelicerates, crustaceans, ascidians and polychaetes. A numerous AMPs from them have been characterized not only to increase knowledge on innate immunity but also for the development of possible treatment of bacteria infection affecting aquaculture (Tasiemski *et al.*, 2007). Among them are from the haemocytes and plasma of mussle *Mylilus galloprovincialis* and *M. edulis* (Mitta *et al.*, 1999), from skin and mucus of the sea hare *Dolabella auricularis* (Iijima *et al.*, 2003), from the haemocytes of the Japanese horseshoe crab *Tachylecus tridentatus* (Osaki *et al.*, 1999), from the haemocyte of black tiger shrimp *Penaeus monodon* (Hu *et al.*, 2006), from whole body of tunicate *Halocynthia aurantium* (Jang *et al.*, 2002; Lee *et al.*, 2001) and from mesoglea of jelly fish *Aurelia aurita* (Ovchinnikova *et al.*, 2005) etc. Some AMPs from marine invertebrates were shown in Table 2.2

Marine polychaetes are also interested for characterization of AMPs. They live in estuary sediments and sandy beaches rich in microorganisms and toxic agents resulting from pollution. Their abundance in this type of environment suggests these worms have developed efficient immunodefense and detoxification strategies (Tasiemski *et al.*, 2007). Moreover, only few data described the participation of AMPs in marine annelids.

Table 2.2 AMPs from marine invertebrates

Group	Species	AMPs	MW and amino acid residue	Amino acid sequence	Reference
Chelicerates	*Tachypleus tridentatus* (horseshoe crab)	Tachystatin	5.0 kDa, 41 residues	20 residues N-terminal; DYDWSLKGPPKCATYG QKCR	Osaki *et al.*, 1999
Ascidians	*Halocynthia auranthium* (solitary sonicate)	Dicynthaurin	6.2 kDa, 30 residues	ILQKAVLDCLKAAGSSLSKAAITAIYNKIT	Lee *et al.*, 2001
		Halocidin	3.4 kDa, 18 and 15 residues	WLNALLHHGLNCAKGVLA and ALLHHGLNCAKGVLA	Jang *et al.*, 2002
Mollusks	*Dolabella auricularia* (sea hare)	Dolabellanin	3.8 kDa, 33 residues	SHQDCYEALHKCMASHSKPFSCSMKFHM CLQQQ	Iijima *et al.*, 2003
	Crassostrea virginica (American oyster)	Arthopod defensin	4.2 kDa, 38 residues	GFGXPWNRYQXHSHXRSIGRLGGYXAGSLRL TXXXYR *	Seo *et al.*, 2005
	Argopecten irradians (bay scallop)	Big defensin	9.2 kDa, 84 residues	20 residues N-terminal; AIPIAYVGMAVAPQVFR WLV	Zhao *et al.*, 2007
Coelenterate	*Aurelia aurita* (jelly fish)	Aurelin	4.3 kDa, 38 residues	AACSDRAHGHIESFKSFCKDSGRNGVKLRAN CKKTCGLC	Ovchinnikava *et al.*, 2006

* X = undetermined amino acid residues

Table 2.2 AMPs from marine invertebrates (continue)

Group	Species	AMPs	MW and amino acid residue	Amino acid sequence	Reference
Crustaceans	*Penaeus monodon* (black tiger shrimp)	Penaeidin-5	5.8 kDa, 55 residues	20 residues N-terminal; QGYQGGYTRPFPRPPYG GGY	Hu *et al.*, 2006
	Penaeus monodon (black tiger shrimp)	Crus-like*Pm*	124 residues	20 residues N-terminal; QDKGNADTRFLGGVGV PGGG	Amparyup *et al.*, 2007
	Scylla serrata (mud crab)	Scygonadin	102 residues	20 residues N-terminal; GGALALLMPLIVSAIIT MVG	Wang *et al.*, 2007
	Hyas araneus (Spider crab)	Arasin	4.3 kDa, 37 residues	SRWPSPGRPRPFPGRPKPIFRPRPCNCYAPPCP CDRW	Stensvag *et al.*, 2008
Polychaete	*Perinereis aibuhitensis* (sandworm)	Perinerin	5.9 kDa, 51 residues	FNKLKQGSSKRTCAKCFRKIMPSVHELDERRR GANRWAAGFRKCVSSICRY	Pan *et al.*, 2004
	Arenicola marina (lugworms)	Arenicin 1 Arenicin 2	2.7 kDa, 21 residues 2.7 kDa, 21 residues	RWCVYAYVRVRGVLVRYRRCW RWCVYAYVRIRGVLVRYRRCW	Ovchinnikava *et al.*, 2004
	Nereis diversicolor (clamworm)	Hedistin	2.5 kDa, 20 residues	LGAW$_{Br}$rLAGKVAGKVAGTVATYAW$_{Br}$NR*	Tasiemski *et al.*, 2007

* W_{Br} = Bromotryptophan

The isolation of the AMPs from marine polychaete was first reported from *Perinereis aibuhitensis* Grube (sandworm). This novel peptide, name perinerin was extracted from homogenated by acidic extraction. It was purified by heparin-affinity column and reverse-phase HPLC. Perinerin consists of 51 amino acid residues, 5.9 kDa and found 2 intramolecular disulfide bridges. About 0.3 µg of the pure perinerin was recovered from 1 g of worm. It was shown broad spectrum activities and activities against *Pseudomonas aeruginosa*, *Bacillus megaterium* and *Aerococcus viridans*; which were higher than other microorganisms. The synthetic perinerin showed activities similar to that of the native peptide (Pan *et al*., 2004).

Furthermore, mode of action of perinerin against *B. megaterium* and *P. aeruginsa* were determined. The results showed that less than 3 min is sufficient to kill Gram positive bacteria and required 1 hr to kill Gram negative bacteria. Further research of perinerin was cloned coding sequence into pET 32 a (+) vector and expression as a Trx fusion protein in *E. coli*. The recombinant perinerin exhibited a similar antimicrobial activity to the native peptide (Zhou *et al*., 2007).

Ovchinnikova (2004) discovered AMPs; arenicin 1 and 2 from lugworm *Arenicola marina*. These AMPs were isolated from coelomocytes of the lugworm in acidic condition. The coelomocyte were purified by ultrafiltration, preparative continuous acid-urea polyacrylamine gel filtration electrophoresis and reverse phase HPLC. Both arenicins were shown to be active against Gram positive bacteria *Listerine monocytogenes*, Gram negative bacteria E. *coli* and fungi *Candida albicans*. Molecular masses of them were 2.7 kDa and each arenicin has one disulfide bond. In 2007, Ovchinnikova was reported the over expression of arenicin 2 as a fused form in *E. coli* and synthesized of both arenicins. The results showed that recombinant and

synthetic arenicins were identical to natural peptides in respect of their molecular masses, amino acid sequence and antimicrobial activity.

AMP named hedistin was identified from coelomocytes of clamworm *Nereis diversicolor*. The coelomocytes were extracted by acidic solution, purified by solid-phase extraction and reverse phase HPLC. The molecular mass of hedistin was 2.5 kDa and found bromotryptophan residues in its molecule. The native peptide showed activity against only Gram positive bacteria *Micrococcus luteus* and *M. nishinomiyaensis* and marine bacteria *Vibrio alginolyticus*. Then synthetic hedistin was performed and tested activities against several bacteria. The results of synthetic peptide still were not against Gram negative bacteria (except *V. alginolyticus*) and showed low activity against other Gram positive bacteria *Staphylococcus* sp. (Tasiemski *et al.*, 2007).