Full Length Research Paper

New antimicrobial peptides purified directly from *Bullacta exarata*

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Endogenous antimicrobial peptides are exciting candidates as new antibacterial agents due to their broad antimicrobial spectra, highly selective toxicities and the difficulty for bacteria to develop resistance to these peptides. Marine invertebrates, which rely solely on innate immune system for host defense, are the spectacular resources for new antimicrobial compounds. In order to seek for new effective antibiotics, tissue homogenate of *Bullacta exarata* was treated with trypsin, and was isolated following ultrafiltration, gel chromatography and reverse phase high performance liquid chromatography (reverse-phase HPLC). Antibacterial activities of the peptides purified from *B. exarata* were measured by the agar diffusion test and minimal inhibitory concentration (MIC). Three isolated peptides, BEP-1, BEP-2 and BEP-3, showed activity against *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis*. BEP-1 also showed activity against human pathogen strains (*Staphylococcus epidermidis*, *E. coli* and Methicillin-Resistant *S. aureus*). This research picked out three candidates for new effective antibiotics.

Key words: *Bullacta exarata*, antimicrobial peptides, isolation and purification, antibacterial activity, minimal inhibitory concentration.

INTRODUCTION

Antimicrobial peptides are small peptides existing widely in mammals, amphibians, marine invertebrates and insects, and they appear to be one of the actors in innate immunity. As a component of the innate immune defense system, these peptides are mainly located in epithelia or circulating cells. Antimicrobial peptides are not only the potent, broad spectrum antibiotics, but also an important factor contributing to self-defense system against invading microorganisms, mediating humoral immunity (Bulet et al., 2004). In addition, these peptides have been demonstrated to kill fungi, viruses and cancerous cells (Brogden et al., 2003; Chen et al., 2003). Antimicrobial peptides are also characterized as soluble in water, cationic, amphiphilic, better resistant to heat, etc.

Recently, a number of antimicrobial peptides have been isolated from organisms such as plants, insects, marine invertebrates and vertebrates including human being.

And there have been preliminary works on mechanism of their antimicrobial activities. These peptides, generally act by disrupting membrane integrity and interfering with metabolism (Epand and Epand, 2000; Michael, 2003). The contact between cationic antimicrobial peptides and negatively charged lipid membranes of bacteria transfers cationic charge to the membrane, depolarizing the bacterial membrane and disrupting the normal array of membrane lipid bilayer. In addition, this allows the peptides insert into membrane lipid bilayer to form pores, causing outflow of intracellular potassium ion and finally cell death.

Bullacta exarata is a species of a sea snail, a marine gastropod mollusc in the family Haminoeidae. It is composed of a shell and a soft body. *B. exarata* is abundant in coastlines of the China Seas from Hainan to Yellow Sea in eastern China. The habitat for *B. exarata* includes intertidal flats, including the supratidal zone and subtidal zone.

There is appearance of a growing number of bacteria resistant to conventional antibiotics, forcing the development of antibiotics with novel mechanisms. Endogenous

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Table 1. Diameters of zone of inhibition of extracts from *B. exarata* (mm) ($\bar{x} \pm SD$, $n = 3$).

Type of strain	Part A	Part B	Part C	Part D
<i>S. aureus</i>	1.5 $\pm$ 0.2	5.1 $\pm$ 0.3	0.2 $\pm$ 0.1	4.2 $\pm$ 0.5
<i>E. coli</i>	2.8 $\pm$ 0.5	4.0 $\pm$ 0.4	0	2.0 $\pm$ 0.3
<i>B. subtilis</i>	2.0 $\pm$ 0.4	4.0 $\pm$ 0.3	0	2.2 $\pm$ 0.3

The antimicrobial activity was measured by agar diffusion test. The larger diameter means the more antimicrobial activity.

antimicrobial peptides are considered as exciting candidates due to their broad activity spectrum and the difficulty for bacteria to develop resistance to them (Hancock and Scott, 2000; Lehrer and Ganz, 1999). Marine invertebrates that rely solely on innate immune mechanisms for host defense, as a result, become spectacular resources for the development of new antimicrobial compounds. Here, we purified three novel antimicrobial peptides directly from the body of *B. exarata* for the first time. We also studied their activity spectrum searching for their possible utility as new antimicrobial compounds.

MATERIALS AND METHODS

Peptide extractions

B. exarata were obtained from a commercial shellfish farm (Mamu Farm, Zhoushan in Zhejiang Province). The meat of *B. exarata* was homogenized by high-speed tissue-masher. After being added about 0.5% trypsin (Sinopharm Chemical Reagent Co. Ltd, China) and adjusted to pH 8.7, this tissue homogenate was then incubated for 8 h at 45°C and treated for 30 min at 100°C. Debris were eliminated by centrifugation (11000 g, 20 min, 4°C). The supernatant was separated by ultrafiltration with three types of ultrafiltration membrane (10, 5 and 3 kD) into four parts, which were lyophilized and kept at -20°C until use.

Purification

The active lyophilized supernatant (5 to 10 kD) was reconstituted with 3 ml water, and loaded onto Sephadex G-50 cartridge (2.6 $\times$ 100 cm, Pharmacia). Eluting with water, the effluent was monitored by UV absorption at 280 nm. The fractions obtained were lyophilized, reconstituted and tested for antimicrobial activity as described subsequently. The active lyophilized fraction was reconstituted with water, and submitted to reverse-phase HPLC (P1201, Dalian Elite Analytical Instruments Co., Ltd).

Aliquots of 0.2 ml of the active fraction were subjected to reverse-phase HPLC on a Kromasil100-10 C₁₈ column (10 $\times$ 250 mm, Akzo Nobel) right after equilibrating the column with 0.05% trifluoroacetic acid (TFA) in water. Elution was performed with acetonitrile containing 0.05% TFA, and monitored by UV absorption at 254 nm. All HPLC purification steps were carried out at a flow rate of 1.0 ml/min. The HPLC purification was monitored by UV absorption at 254 nm. The fractions obtained were tested for antimicrobial activity.

Micro-organisms

For experimental use, the bacteria of *Escherichia coli* and

Staphylococcus aureus were offered by NIFDC (National Institutes for Food and Drug Control). *Bacillus subtilis* was offered by Zhejiang Institutes for Food and Drug Control. The human pathogen strains, *Staphylococcus epidermidis*, *E. coli* and Methicillin-Resistant *S. aureus* (MRSA) were isolated from patients by ZhouShan Hospital.

Antimicrobial assays

After incubation on Mueller Hinton agar (Aoboxing Biotech, China) at 37°C for 18 to 20 h, bacteria monoclonal were incubated in Mueller Hinton broth medium (BBI) and quantified by counting. This incubation was not stopped until the concentration was about 10⁸ CFU/ml.

The agar diffusion test (Kirby-Bauer disk-diffusion method) (Wei et al., 2010) was used to distinguish antimicrobial parts in extraction. The culture plate was coated with Mueller Hinton agar, and then swabbed uniformly with broth agar containing tested bacteria. A filter-paper disk, impregnated with tested part of extraction (5 mg/ml), was then placed on the surface of the agar. The size of zone of inhibition was measured after 24 h incubation at 37°C. The diameter of zone of inhibition was expressed as a diameter (average the readings by three separate times) for the area around the filter disk.

The antimicrobial activities of peptides purified by HPLC were determined by measuring the minimal inhibitory concentration (MIC). Serial doubling dilutions of peptides were made in 0.9% NaCl. Two-millilitre aliquots from each dilution were incubated in culture plates with 2 μ l of suspension of bacteria (containing 10³ to 10⁴ CFU) in 18 ml Mueller Hinton broth medium. The ultimate concentrations were 5, 10, 20, 40, 80, 160 μ g ml⁻¹ peptides, with ampicillin as control. After 18 to 24 h incubation at 37°C, the MIC values were expressed as the highest peptide concentration that causes 100% growth inhibition. The MIC₅₀, MIC₉₀ and cumulative inhibitory rate of peptides against human pathogen strains were also given (Wei et al., 2010).

RESULTS

Antimicrobial extracts isolated directly from the meat of *B. exarata*

The supernatant of the tissue homogenate treated with trypsin was separated by ultrafiltration, and resulted to be Part A (proteins larger than 10 kD), Part B (proteins between 5 to 10 kD), Part C (proteins between 3 to 5 kD) and Part D (proteins smaller than 3 kD).

The antimicrobial activity was measured by agar diffusion test (Table 1). Both Part B and Part D had marked activity against *E. coli*, *S. aureus* and *B. subtilis*.

Figure 1. Five fractions obtained from Part B (5 to 10 kD). The Part B of the extract was loaded onto Sephadex G-50 cartridge. Absorbance peaks were monitored at 280 nm.

Table 2. Diameters of zone of inhibition of five fractions (BEP I to V) separated from active Part B (5 to 10 kD) (mm) ($\bar{x} \pm SD$, $n = 3$).

Type of strain	BEP I	BEP II	BEP III	BEP IV	BEP V
<i>S. aureus</i>	4.0 ± 0.3	1.2 ± 0.1	1.0 ± 0.2	0.2 ± 0.1	0.1 ± 0.2
<i>E. coli</i>	3.1 ± 0.2	2.3 ± 0.2	2.1 ± 0.1	0.4 ± 0.1	0
<i>B. subtilis</i>	2.8 ± 0.2	2.2 ± 0.1	1.7 ± 0.2	0.4 ± 0.2	0

The antimicrobial activity was measured by agar diffusion test. The larger diameter means the more antimicrobial activity.

Part A had moderate activity against *E. coli* and *B. subtilis*, whereas the activity against *Staphylococcus aureus* was mild. Part C had no significant antimicrobial activity.

Purification of antimicrobial peptides from *B. exarata*

The most active Part B (5 to 10 kD) from the supernatant of tissue homogenate was loaded onto Sephadex G-50 cartridge. There were five fractions obtained, which were named BEP I, BEP II, BEP III, BEP IV and BEP V (Figure 1).

The antimicrobial activity was measured by agar diffusion test (Table 2). It showed that BEP I had marked antimicrobial activity against *E. coli*, *S. aureus* and *B. subtilis*. However, BEP II and BEP III had moderate activity against *E. coli* and *B. subtilis*, and had mild activity against *S. aureus*.

The fraction of BEP I, which had marked activity, was submitted to additional reverse-phase HPLC purification step on a Kromasil100-10 C₁₈ column. Finally, three peptides were purified from this fraction, named BEP-1, BEP-2 and BEP-3 (Figure 2).

Antimicrobial activity of peptides from *B. exarata*

Minimal inhibitory concentration (MIC) of the three peptides purified from BEP I with reverse-phase HPLC was measured. All of the three peptides (BEP-1, BEP-2 and BEP-3) had antimicrobial activity against *E. coli*, *S. aureus* and *B. subtilis*, while the activity of BEP-1 was the most marked (Table 3). In addition, the MIC₅₀ values of BEP-1 against three human pathogen strains (*S. epidermidis*, *E. coli* and Methicillin-Resistant *S. aureus*) were 20, 40 and 80 $\mu\text{g ml}^{-1}$, and the MIC₉₀ values were 40, 80 and 160 $\mu\text{g ml}^{-1}$ (Tables 4 and 5).

DISCUSSION

Endogenous antimicrobial peptides in marine invertebrates are exciting candidates for the development of new antimicrobial compounds, due to their broad activity spectrum and the difficulty for bacteria to develop resistance to them (Hancock and Scott, 2000; Lehrer and Ganz, 1999). The results of this study showed three novel antimicrobial peptides in the body of *B. exarata* for the first time and indicated the broad spectrum of their

Figure 2. Reverse-phase HPLC of BEP I. The BEP I, which was isolated from the Part B (5 to 10 kD) of extract, was further purified on Kromasil100-10 C₁₈ column. Absorbance peaks were monitored at 254 nm.

Table 3. Minimal inhibitory concentration (MIC) of BEP-1, BEP-2 and BEP-3 (μg/ml).

Type of strain	BEP-1	BEP-2	BEP-3	Ampicillin
<i>S. aureus</i>	20	80	40	10
<i>E. coli</i>	20	80	80	20
<i>B. subtilis</i>	40	160	80	20

The antimicrobial activity was measured by minimal inhibitory concentration. The lower value of MIC means the more antimicrobial activity.

Table 4. Minimal inhibitory concentration (MIC) of BEP-1 against human pathogen strains (μg/ml).

Type of strain (strain number)	MIC _{range}	MIC ₅₀	MIC ₉₀
<i>S. epidermidis</i> (10)	5 - 80	20	40
<i>E. coli</i> (10)	20 - 160	40	80
MRSA (10)	20 - 160	80	160

The antimicrobial activity was measured by minimal inhibitory concentration (MIC). The lower value of MIC means the more antimicrobial activity. MRSA represents Methicillin-Resistant *S. aureus*.

antibiotic activities.

The appearance of a growing number of bacteria resistant to conventional antibiotics has become a serious problem, because of the abuse. The development of new effective antibiotics is a pressing issue. Antimicrobial peptides are not only the potent antibiotics, but also have activity of killing viruses and cancerous cells. Therefore, they may be exciting candidates as new antibacterial agents.

Recently, there are several methods used for purification of antimicrobial peptides, including bacteria inducing,

tissue homogenate, gel chromatography, reverse-phase HPLC, cell culture *in vitro*, etc., (Charlet et al., 1996; Park et al., 1997; Liepke et al., 2001; Tomie et al., 2003; Rajaram et al., 2010). There have been many peptides isolated from marine invertebrates like *Mussel* and *Penaeus* (An et al., 2006). Mitta et al. (1999, 2000) have isolated several antimicrobial peptides from *Mytilus edulis* and *Mytilus galloprovincialis*, including mytilins, myticins and mytimycins. Mytilins have been demonstrated to be essentially active against gram-positive bacteria, and are much less active against gram-negative bacteria.

Table 5. Cumulative inhibitory rate of BEP-1 against human pathogen strains.

Type of strain (total strain number)		Peptide concentration (µg/ml)					
		5	10	20	40	80	160
<i>Staphylococcus epidermidis</i> (10)	Growing strains	9	7	4	1	0	0
	Inhibited strains	1	3	6	9	10	10
	Cumulative inhibitory rate (%)	10	30	60	90	100	100
<i>Escherichia coli</i> (10)	Growing strains	10	10	8	4	1	0
	Inhibitory strains	0	0	2	6	9	10
	Cumulative inhibitory rate (%)	0	0	20	60	90	100
MRSA (10)	Growing strains	10	10	9	7	4	1
	Inhibitory strains	0	0	1	3	6	9
	Cumulative inhibitory rate (%)	0	0	10	30	60	90

According to the research on antibacterial activities of extracts from seven marine organisms from Zhoushan Islands in Zhejiang province (Ma et al., 2010), we here select *B. exarata*, from which the extract has marked activity, as the resource for antimicrobial peptides. Li Y, et al. (2005) induced *B. exarata* with *E. coli*, isolated peptides from its mucus and lymph, and found marked antimicrobial activity against *E. coli* and *S. aureus*. However, the peptides from *B. exarata* are not further purified. In addition, there is still no report on purifying antimicrobial peptides directly from the meat of *B. exarata*.

All these findings confirm the idea that endogenous antimicrobial peptides in marine invertebrates could be good resources for new antimicrobial compounds. However, a more simple way to purify them seems important for the better use of these peptides in future. And it would be necessary to get more purified elements.

Here, we treated tissue homogenate of *B. exarata* with trypsin for the first time. The supernatant of this tissue homogenate was then purified with ultrafiltration, gel chromatography and reverse-phase HPLC. Finally, we got three antimicrobial peptides, named BEP-1, BEP-2 and BEP-3, from *B. exarata*. In addition, we found BEP-1, BEP-2 and BEP-3, especially BEP-1, have activity against *E. coli*, *S. aureus* and *B. subtilis*. BEP-1 was also active against human pathogen strains (*S. epidermidis*, *E. coli* and Methicillin-Resistant *S. aureus*). All these results indicate the broad spectrum of antibiotic activities of BEP-1, BEP-2 and BEP-3. However, the structures and mechanisms of these peptides need further study.

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