African Journal of Microbiology Research Vol. 6(44), pp. 7139-7146, 20 November, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR11.1321
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Isolation and purification of the peptides from *Apostichopus japonicus* and evaluation of its antibacterial and antitumor activities

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Accepted 5 November, 2012

Peptides were isolated and purified from enteron of the *Apostichopus japonicus (A. japonicus)* by methanol and gel filtration chromatography. The antibacterial activity test *in vitro* was carried out by the agar diffusion method. The minimal inhibitory concentration (MIC) of the antimicrobial peptides to *Escherichia coli* (*E. coli)*, *Staphylococcus aureus (Sa), Vibrio harveyi (Vh), Vibrio parahemolyticus (Vp), Bacillus subtilis (Bs), Micrococcus lysodeikticus (Ml), Edwardsiella tarda (Et) and Vibrio anguillarum (Va)* were analyzed by means of an MTT assay. The antimicrobial peptides were also analyzed by SDS-PAGE and high-performance liquid chromatography (HPLC). Antitumor activities were researched by an MTT assay as well. Three compositions are obtained by experimentation that is classified as A1, A2 and A3. A3 inhibits the bacteria and the cancer cells. The MIC to *Ml,* Vp*, E. coli*, *Sa, Vh, Et, Va and Bs* was 0.5, 1.0, 1.0, 1.0, 2.0, 2.0, 2.0 and 4.0 mg/ml, respectively. The proliferation effect of antimicrobial peptides on A549 cell is demonstrated. The SDS-PAGE indicates that the molecular weight of A3 is about 6.5 kD. The HPLC figure illustrates that the composition of A3 is complex with four components shown.

Key words: *Apostichopus japonicas*, antimicrobial peptides, antibacterial activities, sodium dodecyl sulfate polyacrylamide gel electrophoresis, minimal inhibitory concentration.

INTRODUCTION

Antimicrobial peptides (AMPs) are very common in many species and they construct the first line of defense against invading pathogens (Hancock and Diamond, 2000). For this reason, AMPs are critical in the innate immune system, especially in prokaryotes and invertebrate (Boman, 1995). Many peptides frequently display cytolytic activities against a range of pathogenic bacteria and fungi consistent with the idea that they play a role in the host's system of innate immunity. Typically, more than half of AMP's amino acids are hydrophobic. AMPs are active against certain Gram-positive bacteria, Gram-negative bacteria, fungi and viruses (Andreu and Rivas, 1998). A previous study showed that most AMPs have small molecular weight (almost <10kDa). In many species AMPs are evolutionarily conserved (Li et al., 2010). AMPs were first identified in cecropin (Boman and Steiner, 1981).The discovery of new molecules with potential antitumor activity continues to be of great importance in cancer research. A few studies recently showed that peptaibols (a family of antimicrobial peptides) exerted cytotoxicity toward human lung epithelial and breast

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carcinoma cells (Shi et al., 2010). Here, we report the presence of antimicrobial peptides active against cancer cells in the enteron of *A. japonicus*. *A. japonicus* (sea cucumber) has a very high nutritional and pharmaceutical value because it contains many of the beneficial constituents to health like mucopolysaccharide, peptides and collagen (Shen, 2001). Thus, *A. japonicus* is known to be an important food and pharmaceutical resource from the sea. Recently, *A. japonicus* farming has rapidly started to develop (Conand and Byrne, 1993).

Antibiotic resistance in bacteria is a serious medical concern in treating and preventing disease. AMP research may be useful to develop new classes of antibacterial drugs to kill bacteria. For this reason, the antimicrobial peptides were isolated and purified from enteron of *A. japonicus* for the first time. In order to avoid bacterial resistance, we need to produce the antimicrobial peptides to replace antibiotics. At the same time, this study might be helpful in selecting disease resistant *A. japonicus* for aquaculture and prevent outbreaks in crops.

MATERIALS AND METHODS

A. japonicus were collected in 2010 from Dalian, China. They were then given nothing but sea water for the next 48 h to empty the enteron. [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (MTT), Sephedax G-25, dimethylsulfoxide (DMSO), Tris hydroxymethyl aminomethane (Tris), sodium dodecyl sulfate (SDS), Acrylamide , Bis-Acrylamide, RPMI-1640 media, Potassium sorbate, 10% FBS were all from Sigma-Aldrich Chemicals. All other chemicals and reagents were of the highest quality commercially available. The following equipment was utilized: CO_2 incubator (Thermo, USA), centrifuge (Heraeus, Germany), electric heating constant temperature incubator (Donglian, CHINA), Multiskan ascent microplate reader (Thermo, USA) and Shaker (Donglian, China).

Extraction of antimicrobial peptides

Enterons of *A. japonicus* (50 g) were immersed immediately in liquid nitrogen, and then the frozen enterons were ground into powder with a mortar and pestle under liquid nitrogen. Next, the powders were placed into 80% methanol (300 ml) for 24 h (Anastasi et al., 1971). After, the clear supernatant fluid was decanted into another beaker; a second extraction was performed on the remaining precipitate with the same quantity of 80% methanol over a 24 h period. Finally, the supernatant fluid was centrifuged (8000 rpm/min, 20 min, 4°C), and then was lyophilized overnight.

Purification of antimicrobial peptides

The gel filtration chromatography was designed to remove pigments and other macromolecular substances (Zhang et al., 2011). A Sephadex G-25 column (1 × 40 cm) was installed on the purification system and equilibrated by elution buffer (0.5% methanol) for about 2 h. It was eluted at a flow rate of 0.5 ml/min. Crude antimicrobial peptides (1.5 g) were gently loaded onto the column. The flow rate was 0.5 ml/min. The elution was then collected, about 3mL per tube. Lastly, the UV absorbance at 280 nm was monitored (Roberta et al., 2009).

Antimicrobial assays

The following strains of bacteria were used: *E. coli, Sa, Vh, Vp, Bs, Ml, Et, Va.* The bacteria were obtained from Dalian Ocean University. *E. coli, Sa* and *Bs* were inoculated in LB medium (Mark et al., 2006) at 37°C under shaking (120 rpm) for 24 h. *Vh, Vp, Ml, Et* and *Va* were inoculated in 2216E medium (Ballester et al., 1977) at 28°C under shaking (120 rpm) for 24 h. A counting chamber was used to adjust the bacterial concentration to obtain approximately 1×10^6 CFU/ml (Motta and Brandelli，2002). Culture mediums of 2% LB agar and 2216E agar were prepared. About 1mL bacterial suspension was seeded onto a 90 mm diameter Petri dish containing LB agar media or 2216E agar media. A 6 mm diameter filter paper with the samples of interest (5 mg/ml) respectively, was overlaid into the agar surface. Potassium sorbate (positive control groups, 5 mg/ml) and distilled water (negative control groups) were also pipetted on the filter paper disks. Petri dishes were incubated at 37 or 28°C for 24 h and then checked for inhibition halos (Siavash and Fadzilah， 2011).

The minimal inhibitory concentrations (MIC) of the AMPs to bacteria were determined based on methods described by the Clinical and Laboratory Standards Institute (USA). In this experiment, 96 well plates were used. MIC parameters were determined in triplicate by inoculating 100 µL of bacterial suspensions (1×10^6 CFU/ml) into tubes. Antimicrobial peptides were diluted across the rows in twofold serial dilutions from 16 to 0.25 mg/ml. The 96 well plates were incubated at 28°C for 24 h. After incubation, 10 µL of MTT solution (5 mg/ml) was added in each well of the plate. After 4 h, 100 µL of DMSO solution was added to each well of the plate. OD values were tested with a microplate reader. All assays were performed in triplicate (Lauth et al., 2002).

Antitumor assays

The MTT assay was used for testing the antitumor activities of antimicrobial peptides (Qin et al., 2010). It was determined in triplicate by inoculating 100 µL of lung cancer cells A549 suspensions (1×10^5 CFU/ml) into tubes. Antimicrobial peptides were diluted across the rows in twofold serial dilutions from 10 to 2.5 mg/ml. 5-FU was used as a positive control group (2 mg/ml) and RPMI-1640 media (contain 10% FBS) was used as a negative control group. The total medium volume of each well was 200 µL. The 96-well plates were incubated at 37°C in the CO_2 incubator for 24 h. After incubation, 10 µL of MTT solution (5 mg/ml) was added to each well of the plate. After 4 h, the medium was discarded and 200 µL of DMSO solution was added in each well of the plate. Then the OD value was tested with a microplate reader.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE (Kwabena et al., 2003) was performed in a gel electrophoresis chamber (0.1 × 10 × 10 cm) using a 20% separating gel overlaid with a 5% stacking gel, in the presence of 1% SDS. The migration buffer consisted of 25 mM Tris and 192 mM glycine (pH 8.5). After migration, separating gels were stained with Coomassie brilliant blue G-250, and decolored with destaining solution (45% methanol: 10% iced acetic acid: 45% water).

High-performance liquid chromatography analysis

The purity of the AMPs was measured by analytical reverse-phase HPLC using a C18 column (Waters, USA). The AMPs were

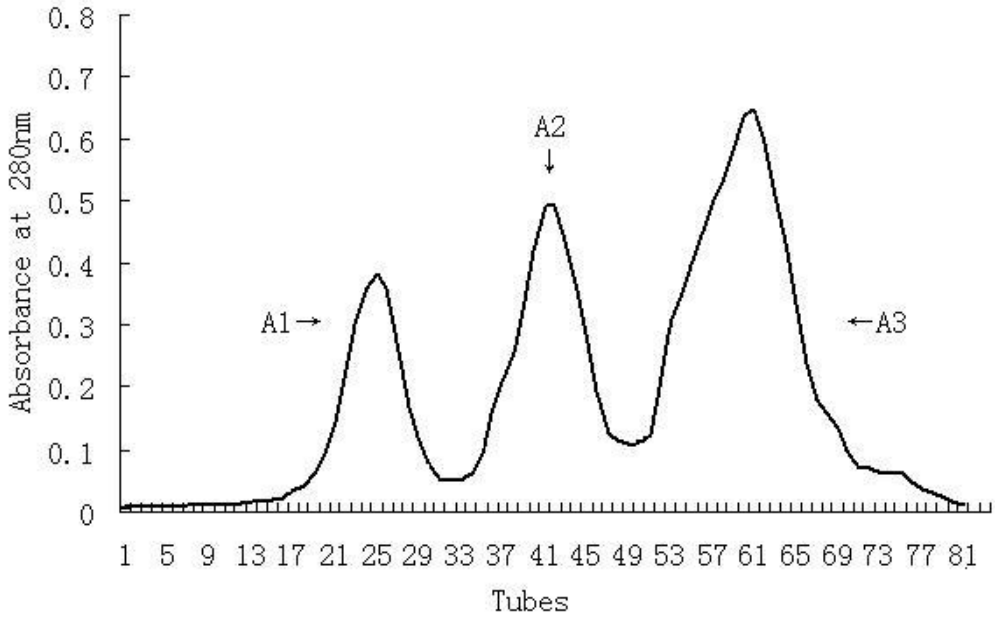

Figure 1. Sephadex G-25 elution profile of antimicrobial peptide purification. Elution was performed with 0.5% methanol about 80 tubes. Three fractions were eluted and names A1, A2 and A3.

solubilized in 80% (v/v) acetonitrile, 0.1% (v/v) TFA and applied to a C18 column in an HPLC. Elution was achieved by an acetonitrile (0~80% v/v) gradient at 1 ml /min. The acetonitrile from 0~30% at 0~15 min，from 30~50% at 15~25 min, from 50~70% at 25~35 min, from 70~80% at 35~40 min. Detection was at 220 nm (Sónia et al., 2004).

Statistical analysis

All the data was analyzed using statistical package for social sciences (SPSS) version 10.0. Each sample was measured in triplicate and the data is presented as means ± standard deviation (SD). The values were obtained from at least three determinants. Data was analyzed using a one way analysis of variance (ANOVA) for multiple comparisons. $P < 0.05$ was considered to be statistically significant.

RESULTS

Purification of antimicrobial peptides with Sephadex G-25

The antimicrobial peptides were purified by gel filtration chromatography and eighty tubes were obtained after this procedure. After the UV absorbance was read at 280 nm, the purification procedure gave three peptide peaks designated as A1, A2 and A3 (Figure 1).The molecular weight was decreased from A1 to A3.

The antimicrobial activities of antimicrobial peptides

The results indicated that the negative control groups, A1 and A2 have no inhibition halos. The positive control groups, A3, have obvious inhibition halos (Figure 2). This means antimicrobial peptides, A3, have antimicrobial activities against Gram-positive and Gram-negative bacteria. The antimicrobial activities of the positive control groups were better than the A3 groups.

The A3 groups mean diameters of inhibition halos to *Vp Sa, Ml, E. coli, Va, Et, Vh, Bs* were: 19.02, 19.00, 18.45, 18.02, 15.51, 13.35, 12.14, 12.00 (Table 1), respectively. The positive control groups mean diameters of inhibition halos to *Ml, Sa, E. coli, Vp, Va, Et, Vh, Bs* were: 20.85, 20.71, 20.71, 20.49, 18.73, 16.95, 16.71, 13.53 (Table 1), respectively. Results are presented more clearly by Figure 3.

The minimal inhibitory concentration of the antimicrobial peptides to bacteria

After incubation with bacteria, the antimicrobial peptides, A3, exhibited obvious growth inhibition on *Vh, E. coli, Ml, Sa, Vh, Et, Va, Bs* with a MIC of 0.5, 1.0, 1.0, 1.0, 2.0, 2.0, 2.0, 4.0mg/ml (Table 2), which implied that there was a different dosage effect between the different Gram-negative and Gram-positive bacteria.

The antitumor activities of antimicrobial peptides

The antimicrobial peptides, A3, were found to present significant antitumor activities on lung cancer cells A549

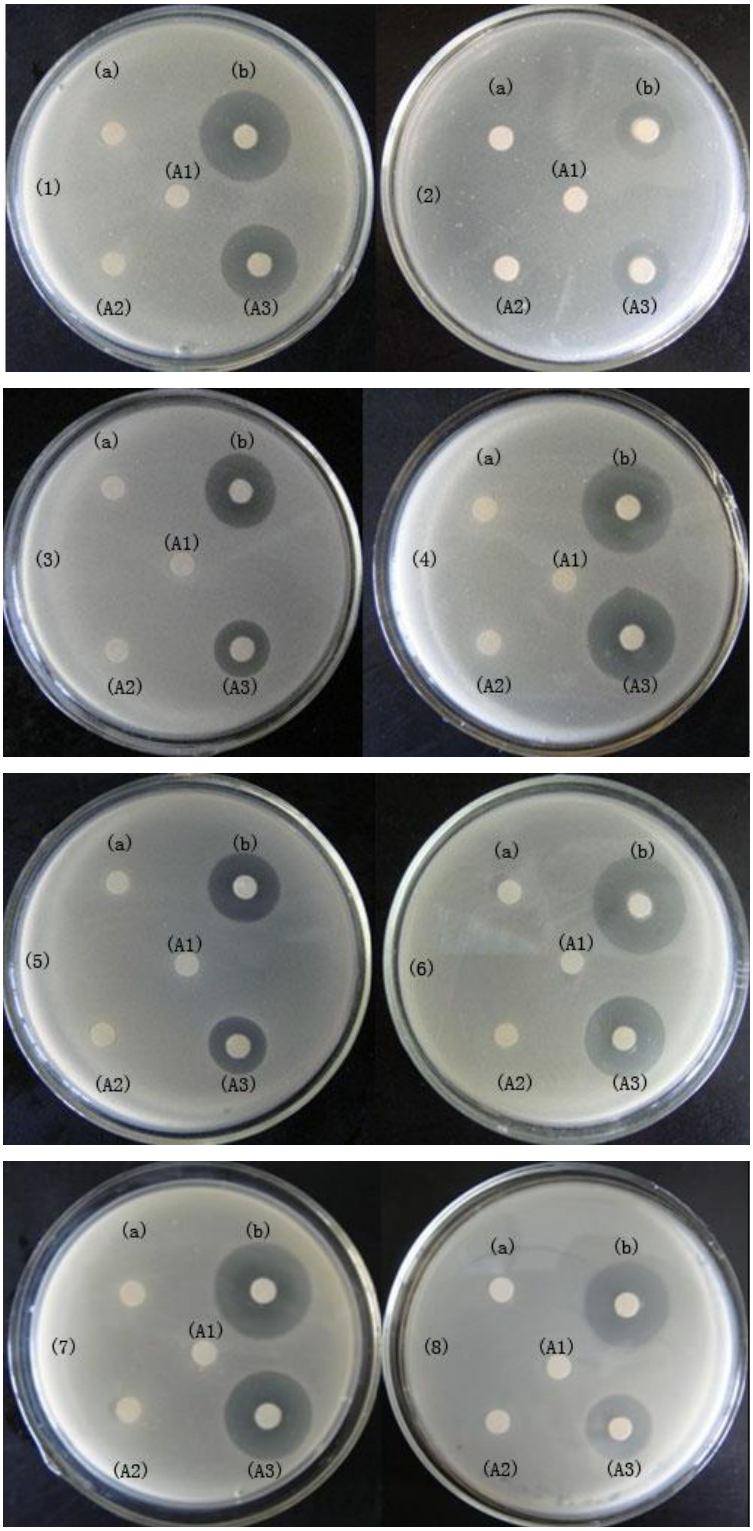

Figure 2. Inhibition halos of compounds determined by the disk paper method (NCCLS, 2002). Letters represent the substances used. (a) Distilled water (negative control groups); (b) 5 mg/ml Potassium sorbate (positive control groups); (A1) 5 mg/ml antimicrobial peptides A1; (A2) 5 mg/ml antimicrobial peptides A2; (A3) 5 mg/ml antimicrobial peptides A3.Numbers represent the bacteria. (1) *E. coli*; (2) *Bs*; (3) *Vh*; (4) Vp; (5) *Et*; (6) *Ml*; (7) *Sa;* (8) *Va*.

Table 1. The mean diameter values (mm) of inhibition haloes.

Bacteria	*E. coli*	*Bs*	*Vh*	Vp	*Et*	*Ml*	*Sa*	*Va*
Distilled water [a]	5	5	5	5	5	5	5	5
Potassium sorbate (5 mg/ml)[b]	20.71 ± 0.26	13.53 ± 0.15	16.71 ± 0.85	20.49 ± 0.28	16.95 ± 0.04	20.85 ± 0.19	20.71 ± 0.15	18.73 ± 0.13
A1(5 mg/ml)[a]	5	5	5	5	5	5	5	5
A2(5 mg/ml)[a]	5	5	5	5	5	5	5	5
A3(5 mg/ml)[b]	18.02 ± 0.05	12.00 ± 0.56	12.14 ± 0.08	19.02 ± 0.10	13.35 ± 0.16	18.45 ± 0.30	19.00 ± 0.21	15.51 ± 0.16

([a, b]), Means with different letters in the same column. Significant differences with the negative control are presented by [b] (P <0.05).

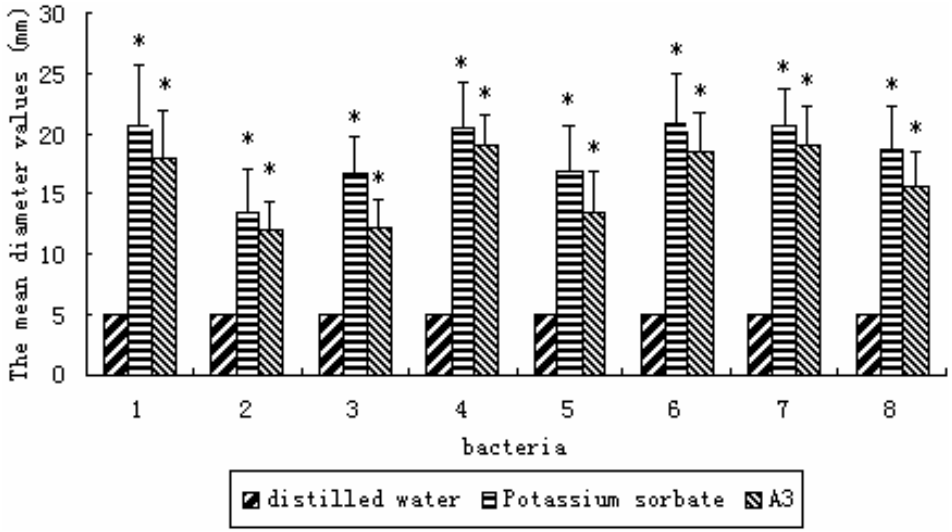

Figure 3. The mean diameter values (mm) of inhibition halos. Numbers represent the bacteria. (1) *E. coli*; (2) *Bs*; (3) *Vh*; (4) Vp; (5) *Et*; (6) *Ml*; (7) *Sa*; (8) *Va*. Distilled water was treated as negative control group. *Denotes significant difference compared with negative control group (* P<0.05).

Table 2. The MIC values of the antimicrobial peptides A3.

Bacteria	*E. coli*	*Bs*	*Vh*	Vp	*Et*	*Ml*	*Sa*	*Va*
MIC (mg/ml)	1.0	4.0	2.0	0.5	2.0	1.0	1.0	2.0

The minimal inhibitory concentration (MIC) of the antimicrobial peptides to *E. coli*, *Sa, Vh,* Vp, *Bs, Ml, Et, Va* by MTT method was analyze.

Table 3. Growth inhibition of antimicrobial peptides at different concentration.

Samples	Concentration (mg/ml)	Absorbance (492 nm)	Inhibition rate (%)
Antimicrobial peptides A3 (mg/ml)	2.5	1.63 ± 0.06	2.51
	5.0 [a]	1.29 ± 0.07	22.93
	10.0[a]	1.00 ± 0.05	40.22
5-Fu	1.0 [a]	0.48 ± 0.03	71.20
RPMI-1640 media[b]	----	1.67 ± 0.14	----

([a, b]), Means with different letters in the same column. Significant differences with the negative control are presented by [a] (P <0.05).

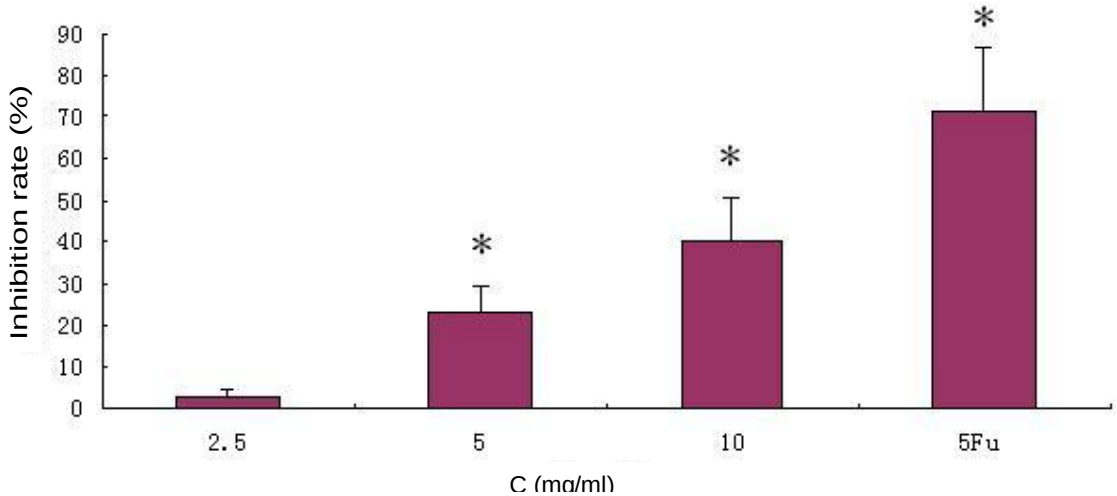

Figure 4. Proliferation effect of antimicrobial peptides on A549 cell line. The antimicrobial peptide, A3, showed significant inhibition of cell proliferation on lung cancer cells A549 in a dose-dependent manner. The 5-Fu (positive control groups) had highest antitumor activities. RPMI-1640 media was treated as negative control group. * Denotes significant difference compared with negative control group (* P<0.05).

when the concentration was above 5.0 mg/ml and the weaker antitumor activities (2.51% inhibition rate) was observed at the concentration of 2.5 mg/ml (Table 3). The RPMI-1640 media (negative control groups) have no antitumor activities, and the 5-Fu (positive control groups) and A3 have obvious antitumor activities. The proliferation effect of antimicrobial peptides on A549 cell is presented by Figure 4.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis

The antimicrobial peptide, A3, molecular weight determined by SDS-PAGE was 6500 Da (Figure 5). Only one main peptide band was visualized by SDS-PAGE, which demonstrated that the crude peptides had been purified with gel filtration chromatography.

High-performance liquid chromatography analysis

The antimicrobial peptides A3 extracts of *Apostichopus Japonicus* were subjected to RP-HPLC fractionation, resulting in the elution of four fractions which were named B1, B2, B3 and B4 (Figure 6). The peak time for each was 16.62, 17.80，19.22 and 21.76min, respectively.

DISCUSSION

The antimicrobial peptides were separated by Sephadex G-25 gel filtration chromatography, and three parts of the peptides (A1, A2 and A3) were obtained. This applied methodology selects for hydrophobic peptides and has often been used for the isolation of small, hydrophobic peptides. The molecular weight of A3 is 6.5KDa by SDS-PAGE, so A3 is considered to be an antimicrobial peptide according to its molecular weight, isolation and purification methods. Since the fraction contained several peptides, it was further separated by RP-HPLC steps. The B4 fractionation presented itself with the most antimicrobial and antitumor activities (Lester et al., 1999). We are aware of the fact that the peptide extracts used in

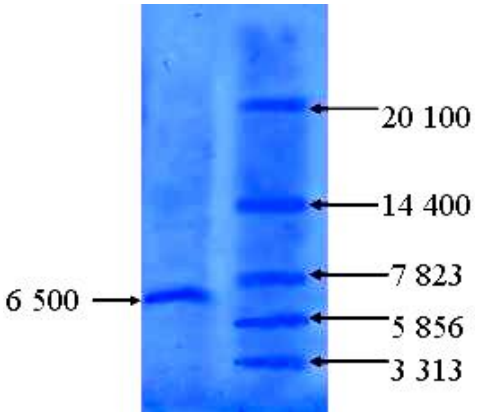

Figure 5. SDS–PAGE of the antimicrobial peptide extracts from the enteron of *Apostichopus Japonicus.* A molecular mass marker was run simultaneously and stained with Coomassie brilliant blue G250. Letters represent the substances used. A3: antimicrobial peptide A3; M: maker.

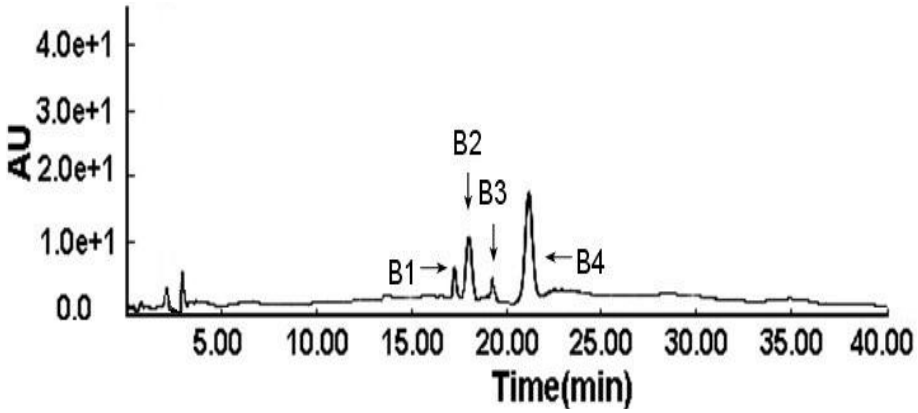

Figure 6. The result of RP-HPLC chromatography of A3 fractions. Chromatogram profile of fractionation of antimicrobial peptide A3 extract of the *Apostichopus Japonicus* under RP-HPLC with a C-18 column, under linear gradient from 0 to 80% (v/v) acetonitrile (containing 0.1% TFA), at a flow rate of 1.0 ml/min by monitoring at UV 220 nm.

this study were not purified to homogeneity. The future construct will be obtained by MALDI-TOF-MS.

It was observed that antimicrobial peptides A1 and A2 did not have antibacterial activities against *E. coli*, *Sa, Vh, Vp, Bs, Ml, Et* and *Va* as the antimicrobial peptide, A3, showed antibacterial activities against these bacteria. The highest activities of the antimicrobial peptide, A3, were observed against *Vp.* The mean diameters of inhibition halos varied from 12.14 to 19.02mm against marine important pathogens such as *Vh* (12.14 mm), *Et* (13.35 mm), *Va* (15.51 mm), *Ml* (18.45 mm), and Vp (19.02 mm). Also, the antimicrobial peptide, A3, was tested against the familiar pathogens such as *Bs* (12.00 mm), *E. coli* (18.02 mm) and *Sa* (19.00 mm). From this experiment, it is true that the antimicrobial peptide, A3, has a broad spectrum of antimicrobial activities both against Gram-positive and Gram-negative bacteria, as the MIC values were in the range 0.5~4 mg/mL. The MIC of the antimicrobial peptide, A3, towards *Vp* was only 0.5 mg/ml; it is likely to *Vp* belongs to intestinal bacteria, at the same time, the antimicrobial peptide, A3, was separated from the enteron of the *A. Japonicus*, so antimicrobial peptide, A3, is more sensitive to *Vp*. By the same token, it is clear that the antimicrobial peptide, A3, have higher antimicrobial activities against marine bacteria. In conclusion, the results of this study indicated that the extracts of

antimicrobial peptide have a capacity to inhibit the growth of pathogenic microorganisms. Therefore, they could be used as antimicrobial in the medical industry.

In this study, the antimicrobial peptide, A3, showed significant inhibition of cell proliferation on lung cancer cells A549 in a dose-dependent manner. The high concentration of antimicrobial peptide, A3, (above 5.0 mg/ml) had induced tumor cell necrosis. The antimicrobial peptide, A3, at a concentration of 10 mg/ml exerted proliferation effects on lung cancer cells A549 with an inhibition rate of 40.22%.

In conclusion, the antimicrobial peptide, A3, has the antimicrobial and antitumor activities in a dose-dependent manner, and the mechanism of the biological activities was presumably subject to interaction with alien cell membrane (Rogan et al., 2006). It is obvious that the antimicrobial peptides serve as a component of the *A. Japonicus*' defense system, and future research will pay attention to the antimicrobial peptides structure and its biological role in vivo. Further work should be carried out on genomic studies and reduced toxicity to normal cells. It may be a vital peptide, not only because of the fundamental research, but also because of the potential pharmaceutical application as an antimicrobial or antitumor medicine. This study might be helpful in selecting disease resistant *A. Japonicus* for aquaculture and enhance research to protect *A. Japonicus* from important microbial infections.

ACKNOWLEDGEMENTS

We thank Prof. Shengtao Hou and Mark Perfetto for their critical reading of this manuscript. This work was supported by the project Innovative Team of Education Department of Liaoning Province: Marine functional genomics and proteomics research and its application (No. 2009T054), Project of Administration of Ocean and Fisheries of Liaoning Province (2011007) and Dalian Science and technology research (No. 2007B11NC066).

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