·实验研究·

Preliminary Purification and Influencing Factors of Antimicrobial Polypeptides from Porphyra Haitannsis

LIU Lei[1,2], WEI Yu-xi[1△], LIU Qi[2△], ZHAO Ling[1], WANG Ling-yan[1]

(1 Biological Department of Qingdao University, Qingdao 266071, China;

2 Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao 266071)

ABSTRACT Objective: Extracting and purifying the water-soluble protein from Porphyra haitanensis, and to investigate the inhibitory activity on Staphyloccocus aureu. Methods: The water-soluble protein was hydrolysated by pepsin at 37℃ and pH1.8 for 3h. The hydrolysates were purified sequentially by ultrafiltration, Bio-Gel P-10 and DEAE Sephadex A-50 chromatography. The molecular weight of antibacterial polypeptides was detcted by SDS-PAGE. The antimicrobial activity of every fraction obtained during purification procedures was detected by plat stiletto method. The inhibitory effect on Staphyloccocus aureu, and the activity influencing factors of polypeptides were detected. Results: The molecular weight f antibacterial polypeptides was between 43.0KD and 66.2KD. The antibacterial peptides' antimicrobial activity to Staphyloccocus aureus was affected by temperature intensively. On the other hand, the synergistic effects were observed in all combination of DSA-1 + 5%EDTA, DSA-1+ 5% citric acid, DSA-1 + 5%Vc or DSA-1+ 25%DMSO, and antagonistic effects were observed in combination of DSA-1 + 5%VE+ 25%DMSO. Conclusion: The antibacterial polypeptides was purified from water-soluble protein of Porphyra haitanensis, which molecular weight was between 43.0KD and 66.2KD. The antibacterial activity of the antibacterial polypeptides was affected by temperature, some organic acid and some vitamine.

Key words: Porphyra haitanensis; Water-soluble protein; Antimicrobial polypeptide; Purification; Influencing factor

Chinese Library Classification (CLC): 282.71 Document code: A

Article ID:1673-6273(2011)03-476-06

Introduction

Porphyra haitanensis, attached to Rhodophyta, Bangiaceae, Porphyra, is important economic seaweed in our country. Furthermore, traditional Chinese medicine thought that Porphyra has some pharmaceutical functions such as dispelling heat, inducing diuresis for removing edema, reinforcing kidney and so on. However, up to now, the research have showed that the active substances mainly concentrated in laver polysaccharide. For example, sulfated polysaccharide fraction F2 from Porphyra haitanesis (Rhodephyta) showed inhibitory effect on the lipid per-oxidation in vitro [1]. Porphyra yezoensis polysaccharide (PYP) was found to decrease the activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) significantly ($P < 0.05$), lower the liver indexes and malondialdehyde level in hepatical tissues in mice remarkably ($P < 0.05$), and upregulate the total superoxide dismutase (T-SOD) level in liver homogenate ($P<0.01$) [2]. Polysaccharides isolated from Porphyra was degraded into different molecular weight fractions and it was found that fractions with Mr= 49 kDa and 30 kDa can significantly increase the life span of D. melanogaster [3]. Additionally, one antihypertensive peptide from Porphyra yezoensis had a high ACE inhibition rate of 55.0%, a low IC50 value of 1.6 g/L and remained high stability at temperatures of 4℃, 25℃, and 37℃, pH 2.0 and 8.0, and after pepsin and trypsin treatments [4]. This paper was to investigate the extraction, purification and activity affecting factors of antimicrobial polypeptides from Porphyra haitanensis.

1 Materials and Methods

1.1 Materials

Porphyra haitanensis was bought from supermarket in Qingdao; Bacteria: Vibrio anguillarum, Bacillus subtilis, Micrococcus tetragenus, Escherichia coli, Vibrio parahaemolyticus, Staphylococcus aureus were supplied by Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences.

1.2 Methods

1.2.1 Extraction of Water-Soluble Protein from P. haitanensis 300 mL water was added to 15g dry seaweed. The mixture was stirred and then cell was broken by ultrasonic wave to obtain supernatant liquor after freeze centrifugation at 7500 rpm for 30 minutes. 95% ethanol was added to it with the end concentration of ethanol was 89% and stayed at -20℃ for 12 hours. The water-soluble protein was precipitated and the precipitation was further freeze dried and conserved at -20℃ [5-7].

*Foundation items: Special Fund for Agro-scientific Research in the Public Interest, in ministry of agriculture (200903030)

Auther:Liu Lei, (1985-), female, Master, mainly researching in detection and control to marine microorganism.

Special Fund for Agro-scientific Research in the Public Interest (200903030)

△ Corresponding Author: Wei Yu-xi, E-mail: yuxiw729@163.com; Liu Qi, E-mail: liuqi@ysfri.ac.cn

(Received: 2010-10-07 Accepted: 2010-10-31)

1.2.2 Hydrolysis of the water-soluble protein from P. haitanensis 300 ml water was added to 17g water-soluble protein. The mixture was stirred and then the hydrolysis was started by the addition of 1% pepsin at 37℃, pH1.8 for 1~5h. In order to obtain the optium reaction time, the hydrolysates liquor (60 mL) at different times was removed and centrifuged at 10000 rpm. for 10 minutes. The supernatant liquor of different time was freeze dried and the dry hydrolysate was stored at -20℃ [8-10].

1.2.3 Screening the antimicrobial activity of the hydrolysates at each hour by water-soluble protein enzymolysis from P. haitanensis According to Bluet`s method with some improvements [11, 12], Bcteria (including Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Micrococcus tetragenus, Vibrio parahaemolyticus and Vibrio anguillarum) were activated and bacterial suspension (density: 10^8-10^9 cfu/ml) was prepared. Bacterial suspension (100μ L) was spread onto the Petri dishes. The hydrolysates with the same concentration (120mg/ml) and volume (10μ L) of different time period were injected into the holes. The terrestrial bacteria and marine bacteria were cultivated at 37℃ and 28℃ for 24 h, respectively [11, 12]. Choose one of the 6 bacteria as the indicative bacterium in the followed steps.

1.2.4 Ultrafiltration of hydrolysate solution by LabScale TFF System The pepsin hydrolysate which has the strongest antimicrobial activity was divided into three components by LabScale TFF System: LTS-1 (molecular weight>50,000), LTS-2 (molecular weight among 5,000-50,000), LTS-3 (molecular weight<5,000). The hydrolysate with the strongest antimicrobial activity was freeze dried and conserved at -20℃.

1.2.5 Gel-filtration chromatography by Bio-Gel P-10 The maximum absorption wavelength of component chosen in section 1.2.4 was determined by spectrum scanning [13]. Then, the component was purified by Bio-Gel P-10 chromatography. The elution curve was based on elution volume and absorbance at the maximum absorption wavelength of the component. Fractions which have strong antimicrobial activity among one peak in different tubes were merged, freeze dried and conserved at -20℃ [14-16].

1.2.6 Ion-change chromatography by DEAE Sephadex A-50 The fraction which has the strongest antimicrobial activity is further purified by Bio-Gel P-10 chromatography. And the DEAE Sephadex A-50 chromatography contained 0.1mol/L, 0.2mol/L, 0.3mol/L, 0.4mol/L, 0.5mol/L and 0.6mol/L NaCl as elution solution of discontinuous gradient elution., The elution curve was based on elution volume and absorbance at the maximum absorption wavelength of the component. Fractions which had strong antimicrobial activity among one peak in different tubes are merged, freeze dried and conserved at -20℃ [16-18]. Desalting and molecular weight determination of the fraction (DSA) selected above were carried out by ultrafiltration and SDS-PAGE [19].

1.2.7 Influence factors of DSA′s antimicrobial activity Temperature DSA solution (120mg/mL) was treated at 10℃, 30℃, 50℃, 70℃, 90℃ and 100℃ for 30 min., Respectively. Then the solution was rapidly cooled down by ice water. The diameter of inhibition zone from different treatment groups was determined as above. The control group was treated at 25℃ [20].

Organic acids DSA was added into 5% EDTA solution and 5% citric acid solution (final concentration of DSA was 120mg/mL), respectively. The diameter of inhibition zone of different treatment groups was determined as above. The control group was added 5% EDTA solution, 5% citric acid liquor and 120mg/mL DSA solution, respectively [21].

Vitamins DSA was added into 5% Vc solution and 5% VE solution (including 25% DMSO as solvent), respectively, and the final concentration of DSA was 120mg/mL. The diameter of inhibition zone of different treatment groups was determined as above. The control group was 5% Vc solution, 5% VE solution (including 25%DMSO) and 120mg/mL DSA-1 solution [21].

2 Results

2.1 Antimicrobial activity screening result of the hydrolysates by water-soluble protein enzymolysis from P. haitanensis

The hydrolysates by water-soluble protein enzymolysis from P. haitanensis only had strong antimicrobial activity to Staphyloccocus aureus, and hydrolysates obtained in different enzymolysis time presented different antimicrobial activity. Among these, hydrolysate (H-3) in 3 hours posessed the largest inhibition zone (diameter: 2.8cm) (Fig.1). Therefore, Staphyloccocus aureus was selected as the indicative bacterium for antibacterial activity test.

Fig.1 Effects of different enzymolysis time on the antimicrobial activity of the hydrolasate to Staphyloccocus aureus

2.2 Antimicrobial Activity of varied molecular-weight fractions of H-3 by ultrafiltration

H-3 could be divided into three components by LabScale TFF System: LTS-1(molecular weight range: >50,000), LTS-2(molecular weight range: 5,000-50,000) and LTS-3 (molecular weight

range: <5,000). Although all the three components had antimicrobial activity to Staphyloccocus aureus, LTS-1 has the largest inhibition zone (diameter: 3.2cm) (Fig.2). So LTS-1 was chosen to be further purified by chromatography.

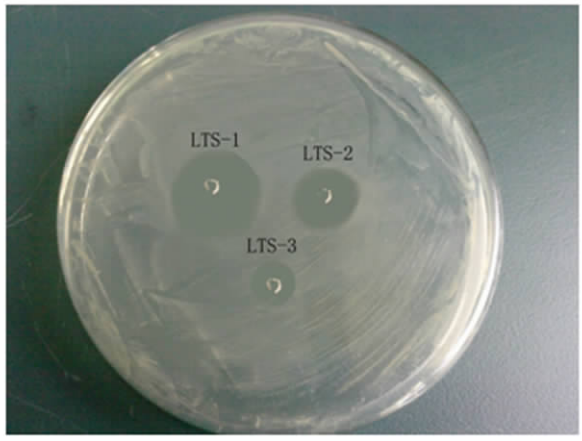

Fig.2 The antimicrobial activity of LTS-1, LTS-2 and LTS-3 to Staphyloccocus aureus

2.3 Purification result of LTS-1 by Bio-Gel P-10

LTS-1 solution was scanning under 200nm~600nm by UV-vis spectrophotometer. The result showed that 245nm is the maximum absorption wavelength of LTS-1 (Fig.3) , which can be the UV detecting wavelength when LTS-1 was purified by chromatography.

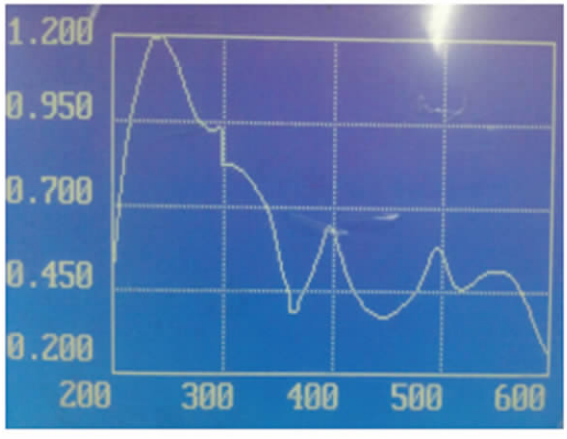

Fig.3 The maximum absorption wavelength Scanning result of LTS-1 by UV-vis spectrophotometer

There were three clear absorption peaks in the elution curve of LTS-1 by Bio-Gel P-10 chromatography (Fig.4). Peak 1 was much wider than the other two peaks. The result of antimicrobial activity determination showed that only fraction 1 (BGP-1) in peak1 presented antimicrobial activity to Staphyloccocus aureus. Therefore, BGP-1 still needed further purification.

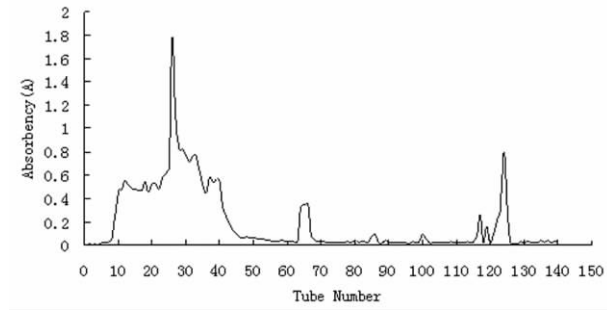

Fig.4 Elution Curve of LTS-1 by Bio-Gel P-10 chromatography

2.4 Purification result of BGP-1 by DEAE Sephadex A-50 chromatography and SDS-PAGE analysis

There were five absorption peaks in the elution curve of BGP-1 by DEAE Sephadex A-50 chromatography (Fig.5). The determination result of antimicrobial activity to Staphyloccocus aureus indicated that only fractions (DSA-1 and DSA-2) in peak 1 and peak 2 showed antimicrobial activity (Fig.6). Moreover, the diameter of inhibition zone (3.9cm) of DSA-1 is bigger than that of DSA-2 (0.8cm). So DSA-1 as the optimum fraction was further studied in the follows.

DSA-1 was still polypeptide mixture, and its molecular weight range was between 66.2KD and 43.0KD. This result also indicated that DSA-1 needed further purification(Fig.7).

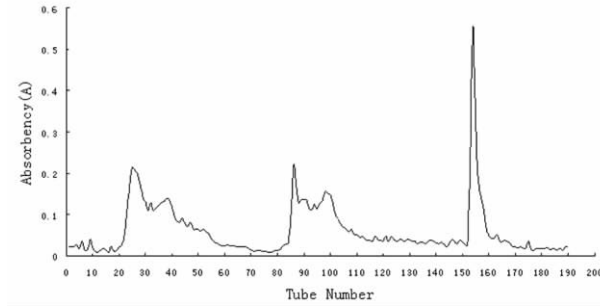

Fig.5 Elution Curve of BGP-1 by DEAE Sephadex A-50 chromatography

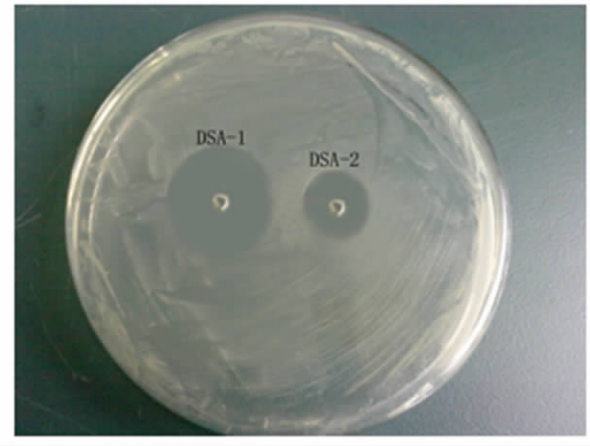

Fig.6 DSA-1 and DSA-2's antimicrobial activity to Staphyloccocus aureus

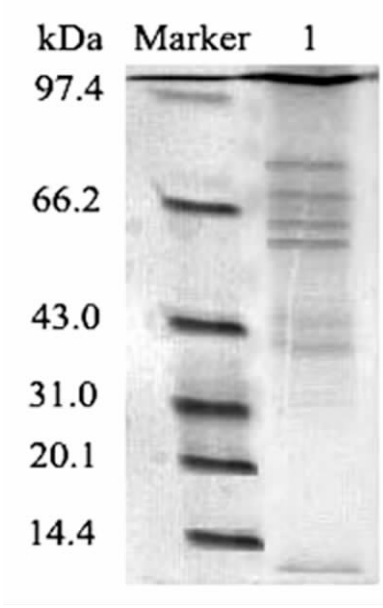

Fig.7 Result of molecular weight determination of DSA-1 by SDS-PAGE

2.5 Influence factors of DSA−1′s antimicrobial activity

Influence of temperature on antibacterial activity of DSA-1 was shown in Fig. 8. It could be seen that DSA-1 showed the biggest diameter of inhibition zone (1.1cm) below 50℃. But when the temperature exceeded 55℃, the diameter of inhibition zone decreased obviously. However, it seemed that the diameter of inhibition zone tended to be a stable value and thus DSA-1 showed certain stability to high temperature.

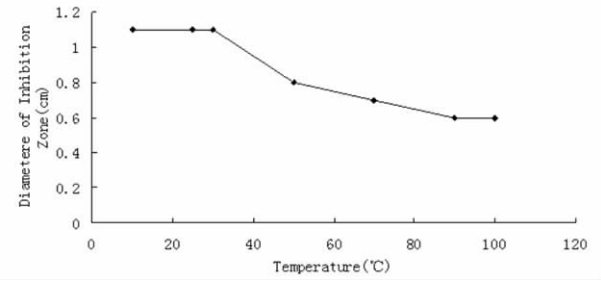

Fig.8 Influence of temperature on DSA-1's antimicrobial activity to Staphyloccocus aureus

Influence of organic acids on antibacterial activity of DSA-1 was shown in Table.1. It can be seen that 120mg/mL DSA-1 and 5% EDTA all presented antimicrobial activity to Staphyloccocus aureus, and 5% citric acid did not indicate any antimicrobial activity to Staphyloccocus aureus. However, when DSA-1 mixed with 5% EDTA or 5% citric acid, the diameter of inhibition zone was much bigger than that of single DSA-1. The results revealed that there was synergistic effect for 5%EDTA or 5% citric acid on antibacterial activity of DAS-1.

Table 1 Influence of organic acids to the DSA-1'S antimicrobial activity to Staphyloccocus aureus

	DSA-1	5%EDTA	DSA-1+5%EDTA	5% Citric Acid	DSA-1+ 5%Citric Acid
inhibition zone diameter (cm)	1.1	0.8	2.4	-	1.9

Influence of vitamins on the antibacterial activity DSA-1 was shown in Table.2. 5%Vc showed antimicrobial activity to Staphyloccocus aureus, but neither 25%DMSO nor 5%VE presented the antimicrobial activity. However, when DSA-1 mixed with 5%Vc or 25%DMSO, the diameter of inhibition zone was bigger than that of single DSA-1. When 5%VE +25% DMSO was added to DSA-1 solution, the diameter of inhibition zone was smaller than that of single DSA-1. Therefore, there was synergistic effects observed in combination of DSA-1 + 5%Vc or DSA-1+ 25%DMSO, but there was antagonistic effects in combination of DSA-1 + 5% VE+ 25%DMSO.

Table 2 Influence of vitamins on DSA-1'S antimicrobial activity to Staphyloccocus aureus

	DSA-1	5%Vc	DSA-1+ 5%Vc	25%DMSO	DSA-1+25% DMSO	5%VE	DSA-1+5 % VE+25%DMSO
inhibition zone diameter (cm)	1.1	1.3	1.6	-	1.3	-	1.0

3 Discussion

With the development of the biology technology, more and more antibacterial polypeptides were discoverded. P. haitanensis , as a important economic corp, has the wide planting area in our country and is loved by people in their daily diet. The technology of organic solvent precipitation is the common technology in extracting protein. In this research, water-soluble protein from P. haitanensis was obtained by procedures of water extraction and organic solvent precipitation,. Different enayme has the different condition, in which it has the best hydrolysis. When the reaction condition is set in 37℃ and PH in 1.8, the pepsin has the best hydrolysis. So in this research, the condition above-mentioned was set as the pepsin reaction condition, and the best pepsin hydrolysis

time of water-soluble protein was set at 3h. In spread plate method, the larger inhibition zone implicates the strong antibacterail activity. The hydrolysates thus obtained showed strong antimicrobial activity to Staphyloccocus aureus by Spread Plate Method in this research. Fraction (molecular weight range: >50, 000) obtained by ulrofiltration from the hydrolysates was further purified by Bio-Gel P-10 and DEAE Sephadex A-50 chromatography.Different polypeptides were separated according to the molecular weight by Bio-Gel P-10 and according to the electric charge by DEAE Sephadex A-50 chromatography. SDS-PAGE results showed that the molecular weight range of the antibacterial peptides from P. haitanensis was between 43.0KD and 66.2KD. Furthermore, the antibacterial peptides' antimicrobial activity to Staphyloccocus aureus was affected by temperature intensively, high temperature commonly can cause degradation of peptides. On the other hand, the synergistic effects were observed in all combination of DSA-1 + 5%EDTA, DSA-1+ 5% citric acid, DSA-1 + 5%Vc or DSA-1+ 25%DMSO, and antagonistic effects were observed in combination of DSA-1 + 5%VE+ 25%DMSO. Therefore, though there are still much work to do about the further purification of antibacterial peptides in the future, research on antibacterial peptides from Porphyra haitanensis as a kind of important economic seaweed, has a bright future in medicine as well as in diet nutrition.

References

[1] Zhang QB, Li N, Zhou GF, et al. In vivo antioxidant activity of polysaccharide fraction from Porphyra haitanesis (Rhodephyta) in aging mice [J].Pharmacological Research, 48 (2003): 151-155

[2] Guo TT, Xu HL, Zhang LX, et al. In vivo protective effect of Porphyra yezoensis polysaccharide against carbon tetrachloride induced hepatotoxicity in mice [J]. Regulatory Toxicology and Pharmacology, 49 (2007): 101-106

[3] Zhao TT, Zhang QB, Qi HM, et al. Extension of life span and improvement of vitality of Drosophila melanogaster by long-term supplementation with different molecular weight polysaccharides from Porphyra haitanensis [J]. Pharmacological Research, 57(2008): 67-72

[4] Qua WJ, Ma HL, Pan ZL, et al. Preparation and antihypertensive activity of peptides from Porphyra yezoensis [J]. Food Chemistry 123 (2010):14-20

[5] Peng CZ, Li L, Liu ZX. Comparison and Analysis Rhizophora 0f Pr0tein Extraction from apiculata BI [J]. Journal of tropical organisms, 2010, 1 (1): 12-15

[6] Li MM, Jiang JZ, Wang HX. Comparison of Protein Extraction M ethods from Potato Tuber [J]. Anhui Agri. Sci. Bull, 2009, 15 (23):37-38

[7] Li L, Zhang YH, Cheng HG, et al. Studying on Extracting Method of Protein Electrophoresis in Gentiana macrophylla Pal1 [J].Gansu Agr, Sci.and Techn, 2008 (7): 23-25

[8] Ebbo S. Determination of the pepsin activity in human gastric juice, using defined oligopeptides as substrates [J]. Clinical Biochemistry, 1989, 22(22):91-98

[9] Wang H, Liu JH, Li KW.The study on I'he extraction of fish scale collagen with pepsin [J]. Food research and development, 2007, 28 (4): 134-136

[10] Xu J, Yuan YJ, Tu CY. Preparation of oligo-peptide by pepsin hydrolyzation of proteins isolated from mung bean [J]. Chinese Journal of Bioprocess Engineering, 2008, 6(4): 31-35

[11] Guo DS, Wei YX, LiL, et al. Purification of antimicrobial protein from plasma of the mussel Scapharca subcrenata and its antimicrobial activity [J].Marine science, 2005, 29 (3): 25-29

[12] Bulet CS, Dimarcq J. Isolation from a coleopteran insect of a novel inducible antibacterial peptide and of new members of the insect defensin family [J]. J Biol Chem, 1991, 266(36): 520- 525

[13] Ni GH, Song HL.Chooseing Absorption Wavelength in Hydrolysates from Chicken [J]. Food research and development, 2002, 23 (5): 73-75

[14] Politi L, Moriggi M, Nicoletti R, et al. Absorption chromatography on Bio-Gel P-2 [J]. Journal of Chromatography A, 1983, 267: 403-408

[15] Vagenina IV, Kozlovsky E., Mustaeva LG, et al. Size exclusion chromatography of polypeptides on Bio-Gel P2 in the dynamic axial compression mode [J]. Journal of Chromatography, 1999, 840 (2): 281-288

[16] Yi G, Wang KY, Chen DF, et al. Isolation, purification and characteristics of the hemolysin produced by Stenotrophomonas maltophilia from Channel catfish[J]. Freshwater Fisheries, 2010, 40 (1): 61-65

[17] Yasha K, Qayyum H Direct immobilization of peroxidase on DEAE cellulose from ammonium sulphate fractionated proteins of bitter gourd (Momordica charantia) [J]. Enzyme and Microbial Technology, 2006, 38: 470-477

[18] Xu MQ, Gu YX, SunY, et al. Isolate hypoderm otin a by negative ion exchane cn rom atography [J]. Chinese Journal of Veterinary Parasitology, 2003, 11 (1): 1-3

[19] Yu WH, Jiang XW. Isolation of Skin Anti-bacteria Peptide of Chinese Forest Frog and Research of Its Partial Character [J]. Heilongjiang Journal of Animal Science and Veterinary Medicine, 2002(5): 8-9

[20] Xu NJ, Fan X, Han LJ, et al. Screening marine algae from ShanDong coast for anittumor acttvtty [J]. Oceanologla ET Limnologla Sinica, 2001, 32 (4): 408-413

[21] Guan SY, Lin JQ, Yu Z, et al. Study of the anti-bacteria activity of the extracts from the leaves of five herbs [J]. Jouranl of GuangDing College of Pharmacy, 2008, 24(3): 269-271

坛紫菜(Porphyra Haitanensis)中抑菌活性肽的制备与初步纯化 *

刘 蕾[1,2] 魏玉西[1△] 刘 淇[2△] 赵 玲[1] 王凌燕[1]

(1 青岛大学生物系 山东 青岛 266071;2 中国水产科学研究院黄海水产研究所 山东 青岛 266071)

摘要 **目的:** 提取坛紫菜中水溶性蛋白,并对其进行初步纯化和抑菌活性影响因素研究。**方法:** 坛紫菜水溶性蛋白胃蛋白酶在37℃、pH1.8 条件下酶解 3h,再经超滤、Bio-Gel P-10 和 DEAE Sephadex A-50 层析纯化步骤得到一定分子量范围的多肽混合物。采用平板打孔法和对金黄色葡萄球菌的抑制作用,跟踪测定活性多肽纯化过程及其活性影响因素。**结果:**SDS-PAGE 测定结果表明该抗菌多肽分子量介于 43.0KD~66.2KD 之间。它对金黄色葡萄球菌生长的抑制作用随着温度的升高逐渐减弱,5%EDTA、5%柠檬酸、5%维生素 C 及 25%二甲基亚砜对它的抑菌活性有协同作用,而 5%维生素 E 则对它的抑菌活性有拮抗作用。**结论:**从坛子菜水溶性蛋白中初步纯化得到的分子量介于 43.0KD~66.2KD 的多肽,对金黄色葡萄球菌的生长有明显地抑制作用,并且它的抑菌活性受到温度,部分有机酸和维生素的影响。

关键词:坛紫菜;水溶性蛋白;抗菌肽;纯化;影响因素

中图分类号:282.71 **文献标识码:**A **文章编号:**1673-6273(2011)03-476-06

* 基金项目:农业部公益性行业专项(200903030)

作者:刘蕾 (1985-), 女, 硕士, 主要从事海洋微生物检测与控制方面的研究,E-mail: lioulei19852004@163.com

△通讯作者:魏玉西, E-mail:yuxiw729@163.com; 刘淇, E-mail: liuqi@ysfri.ac.cn

(收稿日期:2010-10-07 接受日期:2010-10-31)