Immunomodulation and Antitumor Activities of Degraded Polysaccharide from Marine Microalgae *Sarcinochrysis marina* Geitler

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Abstract: *The polysaccharide (SMP) from Sarcinochrysis marina Geitler were degraded using ascorbate and hydrogen peroxide in combination by ultrasonic assistant, and the degraded fractions were purified by gelchromatography. Then three different uniform fragments (SMP1, SMP2 and SMP3) were obtained with molecular weight of 452.79, 168.66 and 8.69 kDa. Their effects on peritoneal macrophage and spleen lymphocyte activation in vitro and the growth of mouse transplantable tumor S180 were measured. The results showed that the degraded fragments could evidently increased phagocytosis and proliferation of macrophage, stimulate NO emission to different extents, and promote propagate of lymphocytes. The immunocompetence had a direct proportion to molecular weight of the samples. The low molecular weight fraction (SMP3) had the strongest immunity activity. Different doses of samples could significantly inhibit the growth of S180 solid tumors, increase the thymus index and spleen index, and promote splenocyte proliferation of tumor-bearing mice. The effect is dose-dependent in a linear fashion. SMP2 had the strongest antitumor activity, and the tumor inhibition index of 200 mg/kg/day dose was 58.51%. All of above results indicated that the antitumor activity of SMP might be achieved by improving body immune response.*

Keywords: *Microalgae, Polysaccharide, Degradation, Immuloregulation, Antitumor.*

Introduction

Microalgae are important sources of natural bioactive compounds, including polysaccharides, with different physiological efffects on human health. In particular, *Marine microalgae* polysaccharides have received special attention due to their potential biological and pharmacological activities including antioxidant, antiviral, anti-irradiation, and reducing blood cholesterol level [4-6, 16]. Some marine microalga polysacchairde such as from *Spirulina plantent*, *Dunaliella salina* and *Porphyridium* sp have been separated and prepared, and their bioactivities have been clarified [2, 12, 16]. In our previous study, we reported the antitumor effects of marine microalgae *Porphyridiun cruentum*, and verified that the anti-tumor effect of microalgae polysaccharide is achieved by improving immune response [14].

In this study, we probe into the immunomodulation and antitumor activities of polysaccharide from another marine microalgae *Sarcinochrysis marina* Geitler (*S. Marina*), which belong to *Chrysophyta*, *Pelagophyceae*, *Sarcinochrysidales* and *Sarcinochrysidaceae*. Up to present, little research has been conducted on the bioactivity of polysaccharide from *S. Marina* besides the report regarding the fatty acid of *S. Marina* [11]. We report here results of

physicochemical properties analysis of degraded polysaccharides. Then the immunity regulation and anti-tumor activity of different molecular weight polysaccharides of *S. Marina* were studied. The study will lay a good foundation for application of microalga polysaccharides as functional material in food or pharmacy industry.

Materials and methods

Materials

Original microalgae strains *Sarcinochrysis marina* Geitler (*S. Marina*) were provided by Ocean University of China Algae Seed Library, Chinese Academy of Sciences. The experimental strains were cultured by Microalgae Biotechnology lab of Yantai University.

Preparation of low-MW polysaccharide

Crude polysaccharide of *S. Marina* (SMP0) with 3644.66 kDa molecular weight (MW) was provided by the Microalga Biotechnology Lab of Yantai University. The SMP was degraded by free radical induced using H_2O_2-Vc system, and the degradation condition was ascorbic acid 8 mmol/L, hydrogen peroxide 8 mmol/L, Action temperature 50°C, and action time 1.5 h. Liquid of degraded polysaccharides were neutralized with 0.01 mol/L NaOH. A slight precipitate was filtered. Then the filtrate was purified further by dialyzing, condensing and purified with Sepherose-6B. Finally, three relatively homogeneous polysaccharide fragments (SMP1, SMP2 and SMP3) were obtained.

Physicochemical properties analysis of degraded polysaccharide

Sulfate groups content of polysaccharide was analyzed by the barium sulfate turbidity method [20]. Uronic acid content was measured by the carbazole-sulfuric acid method using glucuronic acid as standard [10]. The average MW of polysaccharides was determined by high-performance gel permeation chromatography (HPGPC), and the monosaccharide components were determined by gas chromatography (GC) (Agilent 6820, USA) equipped with an FID detector on an SE-54 capillary column (50 m×0.2 mm×0.25 μm) [15].

Animals and cell lines

Female Kunming mice aged 7~8 weeks old were obtained from LvYE Pharm. Co. Ltd (Permit No. SYXK (Lu) 20030020) and housed under standard conditions at 24 ± 1°C and humidity of 50 ± 10% with an alternating 12 h light and dark cycle. Adequate sterile water and rodent chow were allowed. All the mice were acclimatized for 1 week before the start of each experiment. The sarcoma 180 (S180) cells were provided by Pharmacy School of Yantai University. Mouse macrophage cell line Raw264.7 (ATCC No. TIB-71) was provided by Shanghai institute of cell biology (Shanghai, China). All experimental procedures performed were approved by the Institutional Animal Care and Use Committee of National Institute Pharmaceutical Education and Research.

In vivo antitumor activity of polysaccharides

Under sterile condition, 0.2 ml of S180 cell suspension was inoculated subcutaneously to mouse front leg armpit. A normal control group was used in this experiment. Other one hundred and forty mice inoculated were divided into four groups: S180-bearing model group, 20 mg/kg/day cyclophosphamide (CTX) and 50, 100, 200 mg/kg/day polysaccharides treatment groups. Normal control and S180-bearing model groups received the same volume of normal saline. CTX was intraperitoneal injection and polysaccharides were administrated p.o. once daily. Did the administration at a fixed time every day and recorded the activities of mice, a continuous 10 d. After the last administration, all mice were weighed and killed by

cervical dislocation. The antitumor inhibitory rate was calculated based on Eq. (1), and spleen index and thymus index were expressed as the organ weight relative to body weight [14]:

$$[(A - B)/A] \times 100\% \quad (1)$$

where A and B were the average tumor weight of the model groups and treatment groups, respectively.

Effect of lymphocyte proliferation from tumor-bearing mice

Spleen were taken under aseptic conditions from tumor-bearing mice at 24 h after last drug administration, prepared single cell suspension and adjusted the cell concentration of 8-10×10^6 cells/mL with complete RPMI-1640 medium (FBS 10%). Splenocytes were seeded in 96-well plate with 100 μL/well. Then 100 μL different polysaccharides (100 μg/mL) and ConA (10 μg/mL) were added, and the plates were incubated at 37°C in a humid atmosphere with 5% CO_2. The absorbance was determined by MTT described at 490 nm by ELISA reader [3]. Each test was repeated four wells. The proliferation index (PI) was calculated based on the following Eq. (2):

$$PI = A_{sample} / A_{blank} \quad (2)$$

In vitro immune activity of polysaccharides

Proliferation detection of Raw264.7 cells

The mouse monocyte-macrophage Raw264.7 cells were trained to the logarithmic phase and cells were collected after digestion with trypsin. Adjust the cells concentration to 1×10^6 cells/mL with RPMI-1640 medium containing 10% fetal bovine serum (FBS). Then cell suspension were seeded in 96-well plate with 100 μL/well and allowed to attach 2 h. Different dosages of four kinds of SMP on the selected cell lines were 12.5, 25, 50, 100, 200 μg/mL while the positive control was treated with the LPS (4 μg/mL) and the complete RPMI 1640 medium was incubated as normal control. After 44 h incubation at 37°C in a humid atmosphere with 5% CO_2, the proliferation was determined using the colorimetric MTT method. All tested samples were carried out in four times.

Neutral red uptake by Raw264.7 cell line

Adjust the concentration of Raw264.7 cells at 1×10^6 cells/mL, seed them in each well of a 96-well plate. 100 μL of samples (12.5~200 μg/mL) were added to each well, and 100 μL of LPS (2 μg/mL) and RPMI-1640 medium (10% FBS) were set as a positive and a negative control, respectively. The cultivation of Raw264.7 cells as above, disposed the supernatant after 22 h culture. Replacing per well with 100 μL of 0.08% neutral red solution, and cultured for 20 min. Then wells were washed 3 times with PBS and added 200 μL cell lysis solution each hole at room temperature overnight. After the cells being dissolved, the absorbance was measured at 540 nm.

Effect of NO release in macrophage Raw264.7

Adjust the concentration of Raw264.7 cells at 2.5×10^5 cells/mL, seed them in each well of a 96-well plate. Samples (12.5~200 μg/mL) and LPS (2 μg/mL) were added as above. After 48 h incubation, 50 μL of supernatant from each well was transferred into a new 96-well plate, and then 50 μL N-(1-naphthyl) ethylenediamine dihydrochloride solution and 50 μL sulfonic acid solution were added to each well at room temperature in the dark for 10 min. The absorbance was detected at 540 nm within 30 min [13].

Statistical analysis

The data were expressed as means ± standard deviation (SD) and examined for their statistic significance of difference with ANOVA and *t*-test using SPSS16.5 software. $p < 0.05$ indicated significant difference; $p < 0.01$ indicated the very significant difference.

Results and discussion

Physicochemical properties of polysaccharide

The polysaccharide and the degraded fragments from *S. Marina* all consisted of three monosaccharides: arabinose, D-fructose and glucose. The molar ratio of the three degraded polysaccharides was 2.90:1:4.83, 1:2.51:4.11 and 1:1.61:2.07, respectively.

The sulfate content of SMP1, SMP2 and SMP3 were 13.99%, 17.28% and 25.35%, respectively. The uronic acid content of them was 19.21%, 9.99% and 0.03%. The average molecular weight was determined as 452.79, 168.66 and 8.69 kDa, respectively.

Anti-tumor effects of polysaccharides in vivo

The Mouse activity record showed that the hair growth and activity of tumor-bearing mice in the model group were normal before tumor generation, but the hair became disordered, the body weight sharply declined and the activity became slower after tumorigenesis. Mouse hair in the positive control group was disorganized, activities were sluggish, and the physique was emaciated. The food intake of mice was significantly decreased compared with that in the model group, although the hair, activities and eating conditions of mice in the polysaccharide treatment group did not differ from those in normal mice.

As shown in Table 1, compared with the model group, all degraded polysaccharides (SMP0, SMP1, SMP2 and SMP3) caused a significant inhibit the growth of S180 solid tumor ($p < 0.01$ or $p < 0.05$) at given drug concentration. The inhibition rates of them were related to the dosage of them. Compared with normal group, the spleen index and thymus index of model group had no evident change ($p > 0.05$), while the spleen index and thymus index of polysaccharides group (200 and 100 mg/kg/day) were increased significantly ($p < 0.01$ or $p < 0.05$). CTX suppressed tumor cell growth obviously, meanwhile, body weight, spleen index and thymus index of CTX-treated group were marked reduction ($p < 0.05$). SMP-2 had the strongest antitumor effect, inhibition rates of 200 mg/kg/day dose was 58.51% and lower 16.39% than that of CTX-treatment group.

Lymphocyte proliferation of tumor-bearing mice

This assay compared the effect of different MW polysaccharides on immune function of mouse by determining the proliferation of tumor-bearing mice spleen lymphocytes (SP) (Fig. 1). The results showed that the absorbance of spleen lymphocytes was markedly decreased ($p < 0.05$) in S180-bearing mice with cyclophosphamide treatment. However, the SP proliferation index obviously increased in SMP-0, SMP-1, SMP-2 and SMP-3 treatment groups under the concentration of 100 and 200 mg/kg/day ($p < 0.05$ or $p < 0.01$). Compared with CTX-treated group, SP proliferation of each dosage polysaccharides group were effectively increased ($p < 0.01$).

Immunomodulation effects of polysaccharides in vitro

Effect of SMP on mice macrophage Raw264.7 cells proliferation

During the concentration range 12.5-200 μg/mL, SMP0 and its degraded fragments SMP1, SMP2 and SMP3 all could objective boost proliferation of peritoneal macrophages Raw264.7,

and degraded fragments had better effect than SMP0 (Fig. 2). Compared with the LPS group, when the concentration of SMP1 was 100 μg/mL, the promotion ability on proliferation had significant differences ($p < 0.01$), and when the concentration of SMP2 and SMP3 was 50 μg/mL, the promotion ability were much higher ($p < 0.01$).

Table 1. Effects of SMP on gain of body weight, spleen and thymus index and tumor weight in S180 bearing mice ($n = 10$, ± s)

Groups	Dose, (mg/kg/day)	Body weight, (g)	Spleen index, (mg/g)	Thymus index, (mg/g)	Tumor weight, (g)	Inhibition rate, %
Normal	-	7.77±1.10	7.28±2.48	2.89±0.550	-	-
Model	-	7.15±1.18	6.86±1.16	2.82±0.631	1.46±0.290	-
CTX	20	6.54±1.79	$4.49±0.84^{**\Delta}$	$2.22±1.16^{*\Delta}$	$0.366±0.201^{\Delta\Delta}$	74.90
SMP-0	200	7.53±2.16	$8.04±1.33^{*\Delta}$	$3.17±0.723^{\Delta}$	$0.676±0.155^{\Delta\Delta}$	53.67
	100	7.68±1.49	7.49±1.28	2.90±0.564	$0.883±0.135^{\Delta}$	39.54
	50	8.03±1.74	$8.00±1.45^{*\Delta}$	2.85±0.672	$1.04±0.168^{\Delta}$	28.52
SMP-1	200	7.94±1.55	$7.94±1.97^{\Delta}$	$3.22±0.717^{*\Delta}$	$0.651±0.336^{\Delta\Delta}$	55.39
	100	7.66±0.94	7.50±1.90	$3.31±0.855^{*\Delta}$	$0.799±0.090^{\Delta}$	45.09
	50	7.83±2.58	7.63±2.78	2.88±0.594	$0.956±0.035^{\Delta}$	34.42
SMP-2	200	7.66±2.02	$8.11±2.12^{*\Delta}$	$3.51±0.726^{*\Delta}$	$0.605±0.157^{\Delta\Delta}$	58.51
	100	7.77±0.983	$8.42±1.79^{**\Delta\Delta}$	2.96±0.644	$0.800±0.133^{\Delta\Delta}$	45.14
	50	7.36±1.21	$8.06±2.50^{*\Delta}$	2.65±0.291	$0.887±0.092^{\Delta}$	39.18
SMP-3	200	8.06±0.868	$8.15±1.56^{*\Delta}$	$3.30±0.707^{*\Delta}$	$0.708±0.187^{\Delta\Delta}$	51.48
	100	7.58±1.40	$8.51±2.57^{**\Delta\Delta}$	2.86±0.989	$0.847±0.320^{\Delta\Delta}$	40.09
	50	7.37±1.72	7.74±2.54	2.80±0.862	$1.03±0.175^{\Delta}$	29.04

Note: * $p < 0.05$, ** $p < 0.01$ compared with normal control; $^{\Delta}$ $p < 0.05$, $^{\Delta\Delta}$ $p < 0.01$, compared with model.

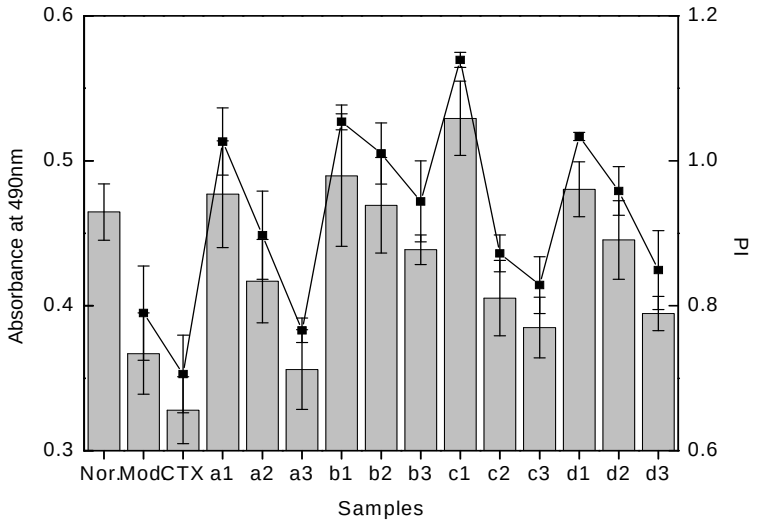

Fig. 1 Effects of SMP on splenocyte proliferation in S180-bearing mice (a, b, c, d represent SMP0, SMP1, SMP2, SMP3; 1, 2, 3 identify the concentration of SMP – 50, 100 and 200 mg/kg/day, respectively)

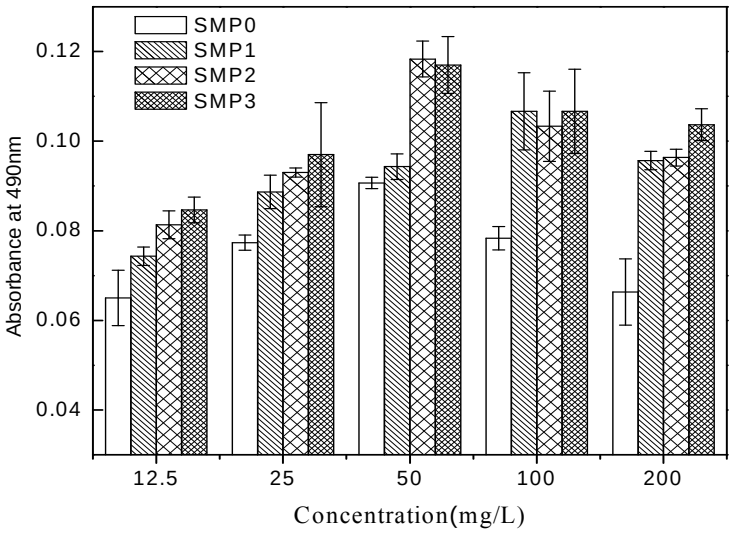

Fig. 2 Effect of the polysaccharide SMP on the proliferation ability of Raw264.7

Effect of mice macrophage Raw264.7 to release NO

As shown in Fig. 3, in the concentration range of 12.5-200 μg/mL, macrophages Raw264.7 could be stimulated by SMP and released NO. At the concentration of 50 μg/mL, the NO burst size of SMP1, SMP2 and SMP3 were significantly higher 1.49, 1.59 and 1.91 times than that of the control group ($p < 0.01$). However, when the concentration was 25 μg/mL, SMP0 had the bigger burst size (81.67 μmol/L), compared with LPS group (37.22 μmol/L).

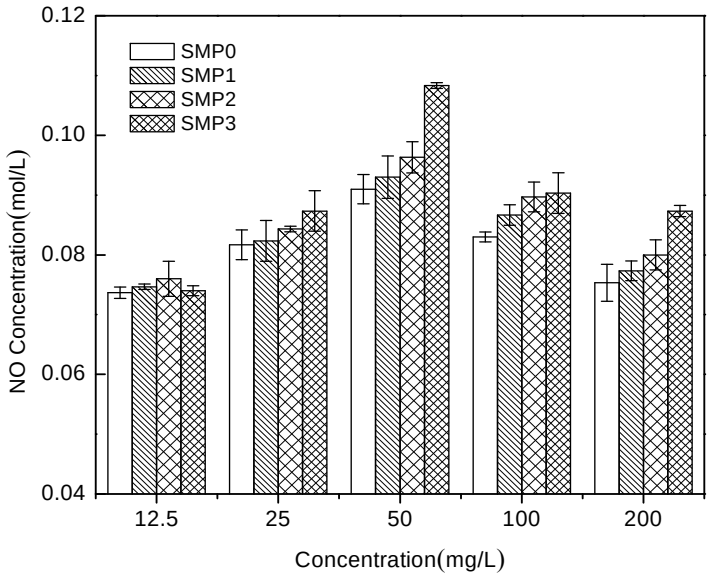

Fig. 3 Effect of the polysaccharide SMP on the release of NO in Raw264.7

Effects of mice macrophage Raw264.7 phagocytosing neutral red

Each polysaccharide had an optimal concentration on effect of phagocytosis, as shown in Fig. 4. The absorbance of SMP1, SMP2 and SMP3 (50 μg/mL) was higher, and there was distinct difference ($p < 0.01$ and $p < 0.05$). However, the absorbance of SMP0 (100 μg/mL) was higher. Compared with positive group, the promotion effect of SMP0-treated group (50-200 μg/mL) had significant difference ($p < 0.01$). The effect of SMP1-treated group (12.5-50 μg/mL) had markedly difference. Besides, low-MW SMP3 had a greater positive effect on proliferation than that of SMP0, SMP1 and SMP2.

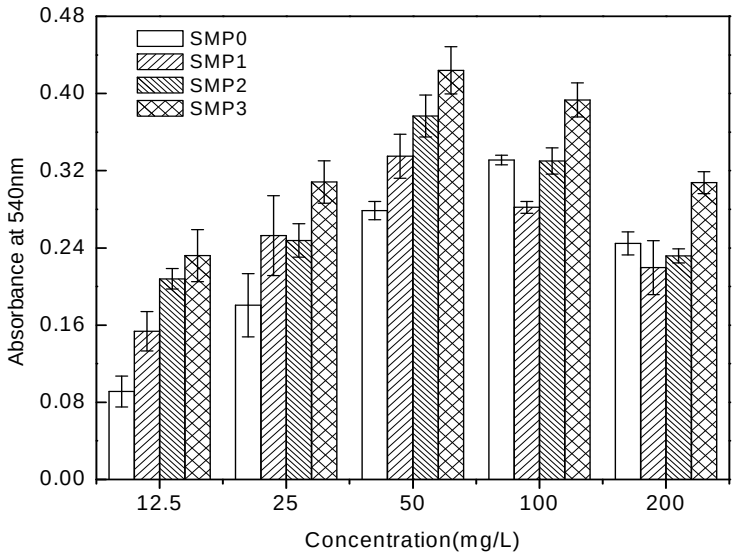

Fig. 4 Effect of the polysaccharide SMP on the Raw264.7 phagocytosis of neutral red

Discussion

Immunoregulatory is an essential activity of polysaccharide compounds. Splenic cell mainly comprises by T and B lymphocytes. Activate T and B lymphocytes can produce various cytokines to enhance immunity, such as TNF2α, IFN2γ and IL22 [17]. Recent years, the molecular research shows that polysaccharides influence the information transmission of lymphocyte process and lymphocytic immune function by combining with polysaccharide receptor on the surface of lymphocytes. Polysaccharide is an important kind of biological response modifier which can not only effectively activate the immune cells and improving immunity, but also have no side effect to host cell. Medium molecule NO participating in the physiological and pathological process has potential immune regulatory effects and might play widely and complicated role in immune modulation [9]. Many studied shows that polysaccharides can recruit macrophages and stimulate NO production [7, 8].

Cell culture *in vitro* empirical study indicated that *S. Marina* polysaccharides could evidently increase phagocytosis and proliferation of macrophage, stimulate NO emission to different extents，and promote the propagation of lymphocytes. SMP1 with minimum molecular weight and SMP3 with the highest sulfuric acid content and have the remarkable immunocompetence. The results proved that the activities of *S. Marina* polysaccharides are dose-dependently acid content and affected with molecular weight and sulfuric.

The results of antitumor effect *in vivo* showed that crude and degraded polysaccharides of *S. Marina* have favorable inhibition effect on implanted S180 solid tumors in mice, and the inhibitory rate of SMP0, SMP1, SMP2 and SMP3 respectively were 53.67%, 55.39%, 58.51% and 51.48% at a concentration of 200 mg/kg/day (Table 1). The tumor inhibition was higher than the efficacy evaluation provisions of Chinese herbal medicine (the tumor inhibitory rate > 30%), which illuminated the polysaccharide from *S. Marina* had distinct antitumor function. The weight of thymus and spleen reflects the immune functional strength to some extent, and thus can be used as drugs on immune function effect index. Spleen index reflects humoral immune status, and thymus index reflects the cellular immune status [1]. Different dose of all samples could increase spleen and thymus index and promoted lymphocyte proliferation, which indicating that their antitumor activity might be achieved by improving immune response. Cyclophosphamide (CTX) can significantly inhibit the growth of transplantable

tumor (inhibitory rate of tumor was 74.9% in 20 mg/kg/day), but it causes the body weight decreased, spleen and thymus function apparently weakened.

All the results suggest low molecular weight and high sulfuric acid content polysaccharides have high activity, but uronic acid content and monosaccharide composition have no effect. They demonstrate that molecular weight and the content of sulfated group in polysaccharides functions importantly on their biological activities. The experimental results basically coincided with the results of studies on polysaccharide from the roots of *Actinidia eriantha* [18], *Spirulina* polysaccharide [19] and *P. cruentum* polysaccharide [14].

Conclusion

Three polysaccharides with different molecular weight from *S. marina* significantly increased lymphocyte proliferation, which indicated the unique mechanism of the antitumor effect of SMP. The conclusion of this experiment offers scientific support for microalga polysaccharides as drugs or health care products that could prevent and treats immune-related disorders. Therefore, further investigation of *microalgae* polysaccharides will have scientific importance and economic value.

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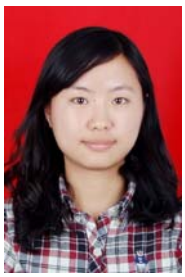

Honghui Liu, undergraduate, engaged in separation and bioactivity research of polysaccharide from marine microalgae.