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Selenylation Modification of Degraded Polysaccharide from *Enteromorpha prolifera* and Its Biological Activities

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Abstract Polysaccharide extracted from *Enteromorpha prolifera* possessed excellent biological activities, but its molecular weight was greatly high which influenced the activity. Organic Se had higher biological activities and was safer than inorganic Se species. In the present study, *Enteromorpha* polysaccharide was degraded to low molecular weight by free-radical degradation method of H_2O_2 and ascorbic acid. By single factor and orthogonal experiments, the optimal degradation conditions were reaction time of 2 h, reaction temperature of 50℃, H_2O_2/ascorbic acid (n/n=1:1) concentration of 15 mmol L^{-1}, and solid-liquid ratio of 1:50 (g mL^{-1}). Then, the degraded polysaccharide was chemically modified to obtain its selenide derivatives by nitric acid-sodium selenite method. The selenium content was 1137.29 μg g^{-1}, while the content of sulfate radical had no change. IR spectra indicated that the selenite ester group was formed. Degraded polysaccharide selenide was characterized and evaluated for antioxidant, antifungal and antibacterial activities. The results showed that degraded polysaccharide selenide had strong capacity of scavenging DPPH and ·OH free radical. It had significant antibacterial properties for *Escherichia coli*, *Bacillus subtilis* and *Salmonella* spp., and it also had significant antifungal properties for *Apple anthrax*. The result ascertained degradation and selenylation modification did not change the main structure of polysaccharides. It was possible that free-radical degradation was an effective way for enhancing antioxidant activity to decrease molecular weight of polysaccharides.

Key words *Enteromorpha prolifera*; polysaccharide; free-radical degradation; selenylation modification; biological activity

1 Introduction

Enteromorpha prolifera is one of the most common fouling green algae, which distributed worldwide from the intertidal to the upper subtidal zones. It possesses high nutrient value and therapeutical properties (Zhao *et al*., 2011). *Enteromorpha* polysaccharide, a group of sulfated heteropolysaccharides, is one of the main biologically active substances in *Enteromorpha prolifera*. It possessed various bioactive functions, such as blood lipid reduction, immunity, antitumor, and anti-inflammatory (Jiao *et al*., 2010; Teng *et al*., 2013; Zhang *et al*., 2013). However, the high molecular weight and high viscosity limited its pharmaceutical application. Therefore, it was necessary to obtain new pharmacological agents by degrading the *Enteromorpha* polysaccharide to improve the activity (Xu *et al*., 2015; Zhang *et al*., 2013). Among the methods of polysaccharides degradation, oxidation method was used more and more recently (Zhang *et al*., 2014). To our knowledge, no paper had reported *Enteromorpha polysaccharide* was degraded by H_2O_2-Vc method.

Selenium (Se) was a very important essential trace element for biological systems and has been used to nourishment and medicine (Ferri *et al*., 2003). Recently, selenium polysaccharides were attracting more and more attention due to their excellent high biological activity and low toxicity. It possessed more or stronger biological activities in comparison with selenium-free polysaccharide (Hu *et al*., 2010). It was also more easily absorbed by organism (Liu *et al*., 2015; Zhao *et al*., 2013). Thus, the selenylation modification became intriguing focus in the polysaccharide research field. However, little information has been obtained regarding selenylation modification of the degraded polysaccharide (Lv *et al*., 2014). In this paper, H_2O_2–Vc method was introduced to degrade polysaccharide from *Enteromorpha prolifera*. The antioxidant, antifungal, and antibacterial activities were investigated. It was expected that this investigation would provide encouragement for further exploration into selenylation modification of degraded polysaccharide.

2 Materials and Methods

2.1 Materials

Enteromorpha prolifera was obtained from Qingdao Huiquan waters. Hydrogen peroxide, ascorbic acid (Vc), salicylic acid, ferrous sulfate, sodium selenite, anhydrous

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ethanol, methanol, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), H_2O_2 and other reagents were of analytical grade. Dialysis membrane was produced by Spectrum Co., and molecular weight was cut off at 500 Da. Diethylaminoethyl-cellulose (DEAE-52) was purchased from Beijing Leybold Cable Technology Co., Ltd.

Cytospora sp., *Apple anthrax*, *Fusarium oxysporum vasinfectum*, *Colletotrichum capsici*, *Alternaria brassicae*, *Staphlococcus aureau*, *Eschetichia coli*, *Bacillus pumilus*, *Bacillus subtilis*, *Salmonella*, *Micrococcus luteus* were provided by Bionic Test Center in Qingdao Agricultural University.

2.2 Analytical Methods

2.2.1 Chemical analysis

Total sugar content was analyzed with the phenol-sulfuric acid method. Selenium content analysis was performed by graphite furnace atomic absorption spectrometer WFX-210. The content of sulfate radical was analyzed with the reported method (Xiong *et al*., 2013). Molecular weight was determined by Waters 1525 Gel Permeation Chromatography system with refractive index detector.

2.2.2 Structure analysis

UV spectra were measured by *shimadzu* UV 2550 from 200 to 400 nm. Fourier transform infrared (FT-IR) spectra were measured by Thermo Scientific Nicolet iS10 FT-IR spectrometer (Waltham, MA) in KBr disks from 500 to 4000 cm^{-1}.

2.3 Preparation of Degraded Polysaccharide (LEP) and Degraded Polysaccharide Selenide (Se-LEP)

The extraction and purification of polysaccharide (EP) from *Enteromorpha prolifera* was performed according to the method that we previously reported (Lü *et al.* 2013).

2.3.1 Selection of degradation reagent

Three kinds of degradation reagent were investigated to achieve efficient degradation of polysaccharide, (1) 5 mL 10 mmol L^{-1} H_2O_2, (2) 5 mL 10 mmol L^{-1} ascorbic acid , (3) 5 mL 10 mmol L^{-1} H_2O_2 and 5 mL 10 mmol L^{-1} ascorbic acid. The antioxidant activity was used to evaluate the effect of degradation by DPPH free radical scavenging assay (Lv *et al*., 2014). Since the antioxidant activity of the purified polysaccharide was often evaluated in vitro by hydroxyl free radical (·OH) scavenging assay, ·OH was chosen as evaluating the effects of degradation.

2.3.2 Optimizing preparation of low-molecular-weight polysaccharide (LEP)

Polysaccharides have shown many biological activities, such as antitumor, antioxidation, immune-stimulating, and neuroprotecting effects. Degradation of polysaccharide into LEP was expected to improve its biological activity. In this paper, the antioxidant activity of LEP was used to evaluate the effect of degradation by DPPH free radical scavenging assay. LEP was obtained by varying reaction time, reaction temperature, H_2O_2/Vc (n/n=1:1) concentration, and solid-liquid ratio. An orthogonal design L_9 (3^4) was adopted to select the optimum experimental conditions (shown in Table 1). 0.1 g polysaccharide was used to prepare LEP in each experiment. The values for different variables were chosen by the single factor experiments.

Table 1 Orthogonal experimental results for the preparation of LEP

Test No.	Reaction temperature (℃)	Reaction time (h)	H_2O_2/Vc (n/n=1:1) concentration (mmol L^{-1})	Solid-liquid ratio (g mL^{-1})	Scavenging rate (%)
1	70	1	20	1:50	41.58
2	70	2	15	1:100	43.08
3	70	3	25	1:150	29.58
4	50	1	15	1:150	54.17
5	50	2	25	1:50	53.42
6	50	3	20	1:100	46.58
7	60	1	25	1:100	45.08
8	60	2	20	1:150	47.17
9	60	3	15	1:50	61.58
K_1	114.24	140.83	135.33	156.58	
K_2	154.17	143.67	158.83	134.74	
K_3	153.83	137.74	128.08	130.92	
R	39.93	5.93	30.75	25.66	

2.3.3 Preparation of degraded polysaccharide selenide (Se-LEP)

The preparation of Se-LEP was performed according to the method that we previously reported (Lü *et al*., 2014). Briefly, 0.1 g LEP was put into a 50 mL round-bottomed flask, to which 10 mL 1.0% nitric acid solution and 0.1 g sodium selenite were added. The mixture was reacted at 60℃ for 5 h. After centrifuged, the residual sodium selenite in the supernatant was dialyzed with distilled water. And then vacuum freeze-dried, Se-LEP was obtained.

2.4 Antioxidant Activity Assay

2.4.1 Scavenging activity of DPPH radicals

2 mL 0.1 mmol L^{-1} DPPH was added to 1 mL various doses of polysaccharide (0.2, 0.4, 0.6, 0.8 and 1 mg mL^{-1}) in 10 mL test tubes, respectively. The mixture was shaken

vigorously, and kept at room temperature for 30 min. Then, the absorbance was obtained at 517 nm by UV-visible spectrophotometer. The DPPH radical scavenging activity was calculated as:

$$SE_{DPPH}=[1-(A_1-A_2)/A_0]\times 100\%,$$

where SE_{DPPH} was DPPH scavenging effect, A_0 was the absorbance of the blank group (distilled water + DPPH), A_1 was the absorbance of the sample reaction (sample + DPPH), and A_2 was the background absorbance of the sample (distilled water replaced DPPH).

2.4.2 Scavenging activity of hydroxyl radical

2 mL various doses of polysaccharide (0.25, 0.5, 1.0, 2.0, 4.0 mg mL^{-1}) in 10 mL test tube were mixed with 1 mL $FeSO_4$ (9 mmol L^{-1}) and 1 mL salicylicacid-ethanol solution (9 mmol L^{-1}), respectively. Then, 1 mL H_2O_2 (8.8 mmol L^{-1}) was added before incubation at 37℃ for 60 min. The hydroxyl radical scavenging activity was calculated as:

$$SE_{hydroxyl}=[1-(A_1-A_2)/A_3]\times 100\%,$$

where $SE_{hydroxyl}$ was hydroxyl scavenging effect, A_1 was the absorbance of the sample, A_2 was the background absorbance of the sample, A_3 was the absorbance of blank.

The antioxidant activity assays were performed in triplicate and results are expressed as mean values ± standard deviations (SD).

2.5 Antifungal and Antibacterial Activities

Staphlococcus aureau, *Eschetichia coli*, *Bacillus pumilus*, *Bacillus subtilis*, *Salmonella*, and *Micrococcus luteus* were cultured in LB/MH broth at 37℃, which were used to test the antifungal activities. *Cytospora* sp., *Apple anthrax*, *Fusarium oxysporum vasinfectum*, *Colletotrichum capsici*, and *Alternaria brassicae* were cultured in PDA broth at 28℃, which were used to test the antibacterial activities.

The minimal inhibitory concentration (MIC) values were determined based on a micro-well dilution method as previously described with modifications (Sarker *et al*., 2007). The 96-well plates were prepared by dispensing, into each well, 5 μL of sample, and 95 μL of the inoculants in nutrient broth. After incubation under aerobic conditions for 24 h at 37℃ for bacteria, and 48 h at 28℃ for fungus, MIC was defined as the lowest concentration of sample at which no visible growth could be detected. Sample free solutions were used as blank controls. The antifungal and antibacterial activity assays were performed in triplicate.

3 Results and Discussion

3.1 Selection of Degradation Reagent

When H_2O_2, Vc, and H_2O_2/Vc were tested as the degradation reagent, their extraction rates of degraded polysaccharide were 76.30%, 77.10%, and 73.10%, and their scavenging rates of hydroxyl free radical were 41.16%, 40.22% and 56.35%, respectively. H_2O_2/Vc was the best degradation reagent.

3.2 Preparation of LEP and Se-LEP

LEP was prepared by varying reaction time, reaction temperature, H_2O_2/Vc (n/n=1:1) concentration, and solid-liquid ratio. The scavenging rate varied from 29.58% to 61.58%. According to the statistical analysis, the optimum conditions of LEP were reaction time of 2 h, reaction temperature of 50℃, H_2O_2/Vc (n/n=1:1) concentration of 15 mmol L^{-1}, and solid-liquid ratio of 1:50 (g mL^{-1}). Under these conditions, the scavenging rate was 64.71%, which was higher than those in Table 1. The yield of LEP was 58.33%. The selenium content of Se-LEP was 1137.29 μg g^{-1}, while the selenium content in Se-EP was only 493.5 μg g^{-1}. The content of sulfate radical was 22.44%, 22.82%, 22.84% and 22.96% for EP, Se-EP, LEP, and Se-LEP, respectively. After degradation or selenylation, the content of sulfate radical has no change. The bioactivities of sulfate radical in different polysaccharide did not change.

3.3 Structure Spectroscopic Analysis

From the UV spectra, there was no absorption at 280 and 260 nm, which indicated protein and nucleic acid were not observed and their contents were very low. There was no absorption appeared at 210 nm, indicating the absence of SeO_3^{2-}. However, there was a strong absorption at 196.50 nm for Se-LEP, and no absorption for LEP. Thus, selenite ester groups might be formed in Se-LEP, which demonstrated further by IR.

3.3.1 IR spectroscopic analysis

The wide and strong absorption peak at 3430.62 cm^{-1} corresponded to the stretching vibrations of hydrogen bonded OH groups. The band at around 2946.57 cm^{-1} was C-H stretching vibration. The bands attributed to C=O stretching vibrations appeared at 1635.1 cm^{-1}, which indicated the existence of uronic acids. The bands attributed to C-O stretching vibrations appeared at 1417.41 cm^{-1}. The bands attributed to S=O stretching vibrations at 1226.58 cm^{-1}, and the bands attributed to C-O-S stretch

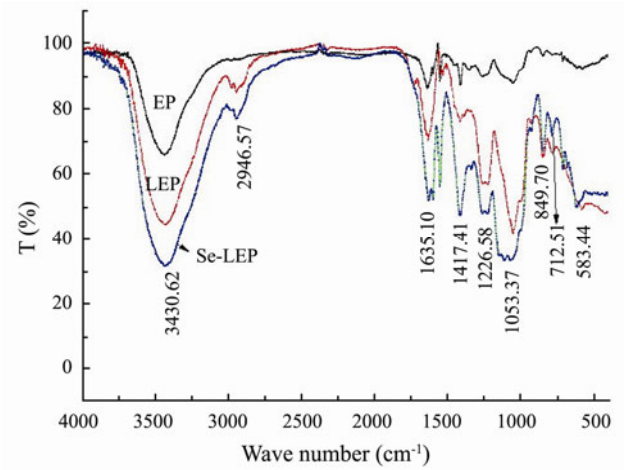

Fig.1 IR spectra of EP, LEP and Se-LEP.

ing vibrations at 849.70 cm^{-1}, which indicated the existence of sulfate radical. The bands attributed to C-O-C stretching vibrations of at 1070.60 cm^{-1}, which indicated the existence of pyranose ring. The FT-IR spectroscopy of Se-LEP presented one characteristic absorption band at 712.51 cm^{-1} describing stretching vibration of Se=O. As compared with the spectrogram of EP and LEP, Se-LEP was succeeded in selenylation modification. The conjugation of selenium did not affect the main structure of the polysaccharide.

3.3.2 Molecular weight analysis

The molecular weight determination was measured by high-performance gel permeation chromatography (HPGPC). Samples (10.0 mg) were dissolved in distilled water (10.0 mL), passed through a 0.45 μm filter and applied to a gel-filtration chromatographic column of Ultrahydrogel TM Linear (300 mm×7.8 mm, Waters, USA). 3 mmol L^{-1} sodium acetate was used as the flow phase at a flow rate of 0.8 mL min^{-1}. The temperature of the column was maintained at 25℃ and the injection volume was 20 μL. Preliminary calibration of the column was carried out using glucan standards with different molecular weights (2500, 4600, 7100, 21400, 41100, 133800 Da). According to the standard curve

$$\lg M_W = -0.3456Rt + 9.2227 \ (R^2 = 0.9999),$$

where *Mw* represented relative molecular weight, Rt represented retention time), the average molecular weights of EP and Se-LEP were calculated to be about 8.4×10^4 Da, and 5.4×10^4 Da, respectively.

3.3.3 Separation and purification by DEAE-52 column

Se-LEP was sequentially purified by DEAE-52 column according to the reported methods with minor modifycation (Dubois *et al*., 1956). The Se-LEP solution (2 mL, 20 mg mL^{-1}) was applied to a column (1.6 cm× 50 cm) of DEAE-52 cellulose. Then, the column was stepwise eluted with 0, 0.1, 0.3 and 0.5 mol L^{-1} NaCl solutions at a flow rate of 0.5 mL min^{-1}. The resulting elute (10 mL/tube) was collected and the polysaccharides were detected at 490 nm by the phenol-sulfuric acid method. Five fractions of Se-LEP were obtained. Each fraction was collected, concentrated, and further purified through a column (1.5 cm×100 cm) of Sephadex G-100, resulting in six purified fractions. Finally, six fractions were lyophilized for further investigation.

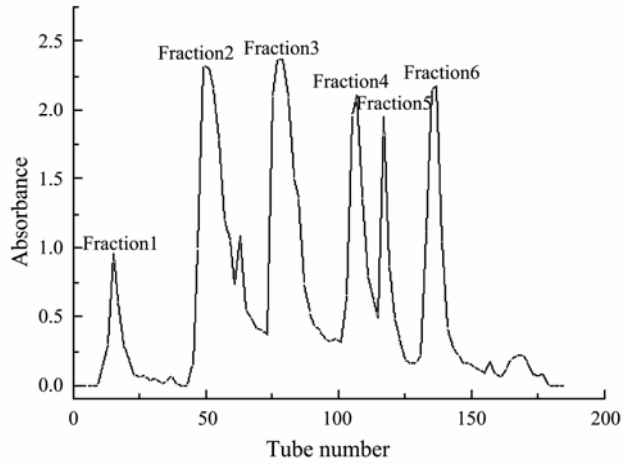

Fig.2 Elution curve of Se-LEP on DEAE-52 column.

3.4 Antioxidant Activity *in vitro*

3.4.1 Scavenging activity of DPPH radical

DPPH was a free radical compound and had been used widely to test the free radical scavenging ability of various samples. With the color of solution changing from purple to yellow, the radical would be scavenged and the absorbance reduced. The DPPH free radical scavenging effects of EP, LEP, Se-EP and Se-LEP were measured and the results were given in Fig.3. It was due to the hydrogen-donating ability that the higher the scavenging rate was for DPPH free radical, the higher antioxidant activity. Scavenging effect of polysaccharide on DPPH radical increased with the increasing of concentration. Among EP, Se-EP, LEP, and Se-LEP, Se-LEP has the strongest scavenging capacity.

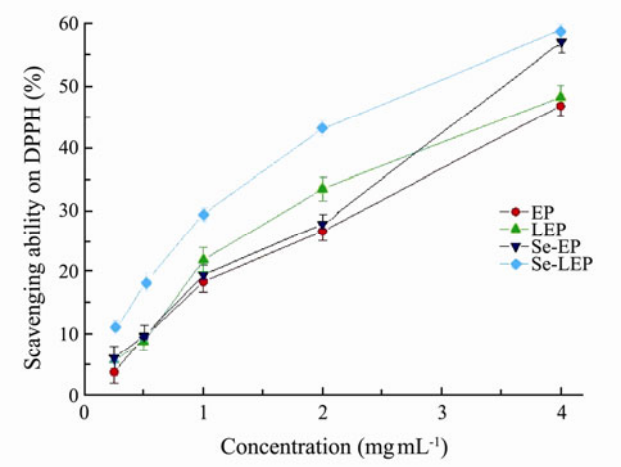

Fig.3 Scavenging effects on DPPH radical. Results are expressed as means values ± standard deviation (SD). All assays were performed in triplicate.

3.4.2 Scavenging activity of hydroxyl radical

The hydroxyl radical possessed extremely high reactivity and could induce severe damage to functioning biomolecules in living cells. In principle, this could be prevented and/or inhibited by antioxidants. ·OH was generated by mixing H_2O_2 and Fe^{2+}, and it had a high reactive behavior but short survival time. If salicylic acid was added, ·OH could be effectively captured, and colored substances were produced. The substances had a strong absorption at 510 nm wavelength. However, when adding an analyte with scavenging function of ·OH in the reaction system, it would compete with salicylic acid for ·OH, which would reduce the generation of the amount of colored substances. Using the method of fixing reaction time, different amounts of analyte in the same volume of reaction system were added to determine the absorbance at 510 nm. In this way, the scavenging capacity of the ana-

lyte on ·OH could be determined. Fig.4 showed EP, Se-EP, LEP and Se-LEP had strong scavenging effect on ·OH, and the scavenging capacity was proportional to the quantity of materials, and at the same conditions, Se-LEP had the strongest scavenging capacity.

These results indicated that degradation polysaccharides should be explored as potential antioxidants.

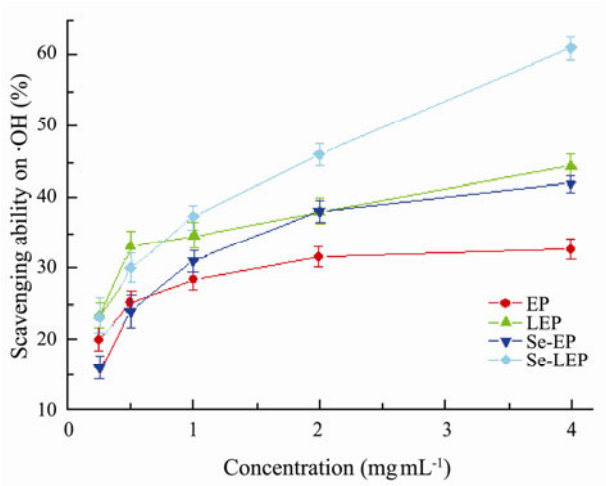

Fig.4 Scavenging effects on hydroxyl radical. Results are expressed as means values ± standard deviation (SD). All assays were performed in triplicate.

3.5 Antifungal and Antibacterial Activities

Under the experimental concentrations of 50 mg mL^{-1}, the antibacterial and antifungal effects of EP, Se-EP and LEP were not obvious. However, Se-LEP showed significant antibacterial properties for *Escherichia coli*, *Bacillus subtilis* and *Salmonella* spp. with the MIC of 50, 25, 25 mg mL^{-1}, respectively. Se-LEP showed significant antifungal properties for *Apple anthrax* with the MIC of 25 mg mL^{-1}.

The antifungal and antibacterial activities results established the antifungal and antibacterial potency of Se-LEP. However, the structure of Se-LEP was very complex, and its biological activity was usually not a function of one single factor but rather a combination of factors. The relationship between the structure of Se-LEP and antifungal, antibacterial mechanism required further studies.

4 Conclusions

In the present study, an efficient degradation method of *Enteromorpha* polysaccharide by free radicals method with H_2O_2 and Vc was developed. Se-LEP was synthesized with high Se content, while the content of sulfate radical did not change. Se-LEP had significant antioxidant, antifungal, and antibacterial activities. However, the mechanism of antioxidant activities *in vivo* and the safety of sulfated polysaccharides from *Enteromorpha prolifera* for human metabolism should be further researched.

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