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Carboxymethylated degraded polysaccharides from *Enteromorpha prolifera*: Preparation and *in vitro* antioxidant activity

Mei-Jia Shi [a], Xiaoyi Wei [b], Jie Xu [a], Bing-Jie Chen [a], De-Yin Zhao [a], Shuai Cui [a], Tao Zhou [a,*]

[a] School of Food Science and Biotechnology, Zhejiang Gongshang University, 18 Xuezheng Street, Xiasha, Hangzhou, Zhejiang 310018, PR China
[b] College of Tourism & Food, Shanghai Business School, Shanghai 200235, PR China

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ABSTRACT

In order to improve the bioactivities of the polysaccharide from *Enteromorpha prolifera* (PE), crude PE (Mw 1400 kDa) was degraded to low molecular weight polysaccharide (44 kDa) in the presence of hydrogen peroxide/ascorbic acid, followed by carboxymethylation. The reaction conditions for carboxymethylation of degraded polysaccharide (DPE) were optimized by Response Surface Methodology. The carboxymethyled degraded polysaccharide (CDPE) obtained under optimized conditions, with a degree of carboxymethylation of 0.849, was characterized by FT-IR and ^{13}C NMR. The molecular weight of CDPE was measured to be 53.7 kDa. CDPE was evaluated for its antioxidant activity by determining the ability to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl and superoxide anion radicals, and by determining the ferric reducing power. The antioxidant activity of CDPE was found to be greatly improved in comparison with degraded polysaccharide (DPE) and crude polysaccharide from *Enteromorpha prolifera* (PE).

1. Introduction

Enteromorpha prolifera, one of the most common fouling green algae, is distributed worldwide from the intertidal to the upper subtidal zones. It possesses high nutrient value and therapeutical properties (Lin, Shen, Wang, & Yan, 2008; Zhao et al., 2011). Several polysaccharides from this species have been extracted and their bioactivities, including blood lipid reduction (Teng, Qian, & Zhou, 2013), immunity (Zhang, Wang, Zhao, Yu, & Qi, 2013), and anti-inflammatory (Jiao et al., 2009) have been reported. However, the biological activities of polysaccharide isolated from *E. prolifera* (PE) are of insufficient potential for a range of purposes in the food and pharmaceutical industries. Attempts have been made to enhance the activities of PE, and these are mainly focused on degradation and chemical modification of polysaccharide. Degradation of polysaccharide in principle can lead to the exposure of more active moieties. Indeed, it has been reported that the antioxidant activity of polysaccharides from *Enteromorpha* species can be enhanced by hydrolysis (Li et al., 2013; Zhang, Wang, Mo, & Qi, 2013). Introduction of functional groups on to polysaccharides by chemical modification has also been demonstrated to be an efficient protocol for the improvement of some bioactivities (Du et al., 2014). For example, it was reported that sulfation, acetylation and phosphorylation of polysaccharide from *Enteromorpha* species markedly increased the antioxidant activity (Wang, Zhang, Yao, Zhao, & Qi, 2013a, 2013b; Zhang et al., 2011). Selenylation of *Enteromorpha prolifera* also improves antibacterial activity (Lv, Gao, Shan, & Lin, 2014). In continuation of interests in enhancing the biological activities of polysaccharides (Xu et al., 2015, 2016), the effects of the carboxymethylation of degraded polysaccharide isolated from *Enteromorpha prolifera* on its antioxidant activity are herein reported.

2. Materials and methods

2.1. Materials and reagents

E. prolifera was harvested in February 2013 in Zhoushan, Zhejiang, China. The raw sample was rinsed carefully with fresh water and air-dried. The dried seaweed was milled with a blender, sieved (0.125 mm) and stored at 20 °C before use.

1,1-Diphenyl-2-picrylhydrazyl (DPPH), and ascorbic acid were purchased from Aladdin Chemical Reagents Co. (Shanghai, China). Riboflavin, DL-methionine and nitrotetrazolium blue chloride were purchased from Sinopharm Chemical Reagents Co. (Hongkong,

* Corresponding author.
E-mail address: taozhou@zjgsu.edu.cn (T. Zhou).

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China). Dextran standards were purchased from Sigma Co. (USA). The solvents and other chemicals used in this work were of an analytically pure reagent grade.

2.2. Preparation of the degraded polysaccharides

The extraction of crude polysaccharide from *E. prolifera* was carried out according to a previous report (Xu et al., 2015). Degradation of crude PE was achieved in the presence of hydrogen peroxide and ascorbic acid according to Zhang's method, with slight modification (Zhang et al., 2013). Briefly, a solution of crude PE (1 g) in distilled water (100 ml) was heated to 30 °C, hydrogen peroxide and ascorbic acid (molar ratio 1:1) were then added (final concentration of the both: 0, 3, 6, 9, 12, 15 mmol/l). After the resulting mixture was stirred for 2 h, the reactant was concentrated under reduced pressure, followed by the precipitation with 4 folds volume of 95% ethanol, and then allowed to stand at 4 °C overnight. After centrifugation, the obtained precipitate was redissolved in distilled water. The resulting solution was dialyzed (MW cut off 3500) against distilled water for 48 h, then lyophilized, yielding the degraded polysaccharide from *E. prolifera* (DPE). The total antioxidant activity of DPE was assayed by FRAP method using a test kit (Luo, Li, & Kong, 2012). DPE possessing the best total antioxidant activity was used for carboxymethylation.

2.3. Preparation of carboxymethylated derivative of DPE (CDPE)

Carboxymethylation of DPE was performed based on literature methods with modifications (Wang, Zhang, & Zhao, 2015; Yang et al., 2011). A solution of DPE (0.3 g) in DMSO (12.5 ml) and NaOH (20%, 5 ml) was stirred at 40 °C for 3 h, then chloroacetic acid solution in DMSO (12.5 ml) and NaOH (20%, 5 ml) was added (the final concentration of chloroacetic acid was 1.0, 1.5, 2.0, 2.5, 3.0 mol/l). The resulting solution was heated to a definite temperature (45, 50, 55, 60, 65 °C) for a definite time (2, 3, 4, 5, 6 h). The reaction mixture was cooled to room temperature and adjusted to pH 7 with 0.5 M HCl, diluted with water, and dialyzed (MW cut off 3500) in distilled water for 48 h, then lyophilised, yielding carboxymethylated extract (CDPE).

The effect of chloroacetic acid concentration, reaction time and temperature on the degree of carboxymethylation (DS) was investigated. Monofactor tests indicated that the optimal chloroacetic acid concentration, reaction time and temperature were 2 mol/l, 4 h and 55 °C, respectively (detailed data shown in Supplementary materials). On the basis of the results of monofactor tests, the reaction conditions for carboxymethylation of DEP were further optimized by employing response surface methodology (RSM). A Box-Behnken design (BBD) was used to survey effects of independent variables (reaction temperature (A), reaction time (B) and chloroacetic acid concentration (C)) at three levels on the dependent variable (DS). Based on the results of preliminary experiments, a total of 17 randomised experiments, including 12 factorial and 5 zero point tests, were designed (Table 1).

Table 1
Central-composite experimental design of the independent variables along with the observed values for the degree of carboxymethylation.

Experimental code	Temperature (°C)	Time (h)	Concentration (mol/l)	Degree of carboxymethylation (DS)
1	50	4	2.5	0.744
2	60	5	2	0.802
3	55	5	2.5	0.785
4	55	4	2	0.851
5	60	3	2	0.691
6	55	3	2.5	0.689
7	55	5	1.5	0.618
8	55	4	2	0.870
9	60	4	2.5	0.737
10	55	3	1.5	0.629
11	50	4	1.5	0.625
12	55	4	2	0.855
13	55	4	2	0.866
14	50	5	2	0.736
15	55	4	2	0.827
16	50	3	2	0.773
17	60	4	1.5	0.657

2.4. Characterization of polysaccharide

2.4.1. Analysis of chemical compositions

The total sugar contents of PE and DPE were analyzed with the phenol-sulfuric acid method, using glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Uronic acid was estimated using a modified sulfuric acid-carbazole method with D-glucuronic acid as the standard (Bitter & Muir, 1962). Coomassie brilliant blue reaction was used to determine protein concentration (Bradford, 1976). Sulfate content was determined according to Kawai's method (Kawai, Seno, & Anno, 1969).

2.4.2. Determination of the monosaccharide composition

The monosaccharide composition was determined using Gas Chromatography–Mass Spectrometry (GC–MS). The samples were hydrolyzed by trifluoroacetic acid to monosaccharides. Then, the monosaccharides were derivatised to acetylated aldononitriles. Xylose, arabinose, glucose, galactose, mannose and rhamnose standards were also derivatised. Acetyl inositol was used as the internal standard. 10 mg samples were hydrolyzed with 2 M trifluoroacetic acid (4 ml) at 110 °C for 6 h in a sealed tube. The solution was mixed with 3 ml of methanol, and transferred to a glass tube. The solution was evaporated with nitrogen at 40 °C five times. Hydroxylamine hydrochloride (10 mg) and anhydrous pyridine (0.5 ml) were added to the tube. The sealed tube was immersed in a water bath at 90 °C for 30 min. Then, 0.5 ml of acetic anhydride was added. The mixture was kept at 90 °C for 30 min. The residue was dissolved in 1.0 ml chloroform, then inositol hexacetate was added. The resulting solution was analyzed using GC–MS.

GC–MS was performed on a gas chromatography/mass spectrometer (Trace GC Ultra DSQ II, Thermo Fisher Scientific, Waltham, MA, USA) equipped with a TR-5MS capillary column (30 m × 0.25 mm × 0.25 μm). The carrier gas was high purity nitrogen. The temperature program was set as follows: the initial temperature of the column was 80 °C and held for 3 min, then increased to 200 °C at 15 °C/min, and held for 1 min at 200 °C, and then increased to 250 °C at 10 °C/min, and held for 5 min at 250 °C. The flow rate was 1.0 ml/min. The injection temperature was 250 °C. The EI ion source of the mass spectrometer was set at 250 °C. Operational conditions for the mass spectrometer were 70 eV. EI source with a mass range between 33 and 500 Da. The split ratio was 10:1.

2.4.3. Molecular weight analysis

Molecular weight determination of polysaccharides was measured using high-performance gel permeation chromatography (HP-GPC) which was undertaken on a Waters 1000 HPLC system, with a Grace 3300 Evaporative light Scattering Detector, ELSD. 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™ Linear (300 mm × 7.8 mm. Waters., USA). Deionized water was used as the flow phase at a flow rate of 0.5 ml/min. The temperature of the column was maintained at 30 °C and the injection volume was 10 μl. Preliminary calibration of the column was carried out using Dextran standards with different molecular weights.