Carboxymethylation of Enteromorpha Polysaccharide and Its Antioxidant Activity (Postprint)

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Date: 2018-10-26T00:00:00+00:00

Abstract

Carboxymethylated Enteromorpha prolifera polysaccharide was prepared using a sodium hydroxide-chloroacetic acid chemical reaction system to obtain carboxymethylated Enteromorpha prolifera polysaccharides with different degrees of substitution. The degree of substitution was influenced by sodium hydroxide concentration, reaction temperature, and reaction time. When the sodium hydroxide concentration was 20%, the reaction temperature was 60°C, and the reaction time was 3 h, the maximum degree of substitution obtained for carboxymethylation was 0.781. The antioxidant activity of different carboxymethylated Enteromorpha prolifera polysaccharides was evaluated through in vitro antioxidant assays. At a concentration of 1.6 mg · mL ^{1}, the carboxymethylated Enteromorpha prolifera polysaccharide exhibited hydroxyl radical and superoxide anion radical scavenging capacities of 44.45% and 51.98%, respectively, and its DPPH radical scavenging rate and reducing power were 16.75% and 0.4576, respectively. Compared with the unmodified polysaccharide, the scavenging capacities for hydroxyl radicals and superoxide anion radicals were significantly enhanced, while carboxymethylation modification had a diminishing effect on the DPPH radical scavenging capacity and reducing power of Enteromorpha prolifera polysaccharide. These results indicate that the structural changes in Enteromorpha prolifera polysaccharide induced by carboxymethylation modification can enhance its antioxidant activity.

Full Text

Carboxymethylation Modification and Antioxidant Activity of Enteromorpha intestinalis Polysaccharides

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Abstract

Carboxymethylated Enteromorpha intestinalis polysaccharides (EIPC) were prepared using a sodium hydroxide-chloroacetic acid chemical reaction system to obtain derivatives with varying degrees of substitution. The degree of substitution was influenced by sodium hydroxide concentration, reaction temperature, and reaction time. Under optimal conditions of 20% sodium hydroxide concentration, 60°C reaction temperature, and 3 h reaction time, the maximum degree of substitution reached 0.781. The antioxidant activity of different carboxymethylated polysaccharides was evaluated through in vitro assays. At a concentration of 1.6 mg·mL ^{1}, the hydroxyl radical and superoxide anion radical scavenging capacities were 44.45% and 51.98%, respectively, while DPPH radical scavenging and reducing power were 16.75% and 0.4576, respectively. Compared with the native polysaccharide, the scavenging abilities for hydroxyl radicals and superoxide anions were substantially enhanced, whereas carboxymethylation diminished DPPH radical scavenging and reducing power. These results demonstrate that structural changes induced by carboxymethylation can enhance the antioxidant activity of Enteromorpha intestinalis polysaccharides.

Keywords: Enteromorpha intestinalis, polysaccharide, carboxymethylation modification, degree of substitution, antioxidant activity

CLC number: Q71
DOI: 10.11931/guihaia.gxzw201807003

Enteromorpha is a large green macroalga with significant economic value, commonly known as "taitiao" or "haitai" in folk tradition, belonging to the family Ulvaceae, order Ulvales, phylum Chlorophyta. Approximately 40 species of Enteromorpha have been identified worldwide, with about 11 species found in China, primarily including Enteromorpha tubulosa, Enteromorpha intestinalis, Enteromorpha clathrata, and Enteromorpha compressa, all of which grow in intertidal zones (孙士红, 2007). The dried algal thallus can be used as traditional Chinese medicine, food, or feed additive. Enteromorpha contains abundant nutrients, including essential vitamins, amino acids, minerals, and fatty acids (吴闯等, 2013). Polysaccharides from Enteromorpha exhibit diverse biological activities, with documented effects including antioxidant, immunomodulatory, hypolipidemic, and antitumor properties (Kim et al., 2011; 石学连等, 2009; Jiao et al., 2009; 林文庭和张智芳, 2009; 陈芳容等, 2012; Li et al., 2013; Jiao et al., 2010).

Appropriate chemical modification of polysaccharides can alter their biological

activities (Wang et al., 2013). Current modification methods include chemical, physical, and biological approaches, with carboxymethylation being a widely used chemical method in industrial applications (申林卉, 2013). Studies have shown that carboxymethylation significantly improves polysaccharide water solubility and enhances biological activity or imparts novel bioactive properties. Parvathy et al. (2005) reported substantially improved solubility of carboxymethylated galactomannan. Wang et al. (2000) prepared carboxymethylated Pleurotus tuber-regium polysaccharide, increasing its solubility from virtually insoluble to over 30 $mg \cdot mL^{1}$, thereby enhancing absorption. The modified polysaccharide also significantly inhibited Fe^{2} -VC-induced lipid peroxidation in rat liver mitochondria, prevented membrane fluidity reduction, and suppressed mitochondrial damage. This study investigates carboxymethylation of Enteromorpha intestinalis polysaccharides to obtain derivatives with different degrees of substitution and explores how structural modifications affect in vitro antioxidant activity, providing a theoretical foundation for developing the bioactive potential of Enteromorpha polysaccharides.

1. Materials and Methods

1.1 Reagents and Equipment

1.1.1 Materials and Reagents

Enteromorpha intestinalis was collected from Hangzhou Bay, Zhejiang, China, and identified by Professor Zhu Wenrong of Ningbo University. Sodium hydroxide, isopropanol, chloroacetic acid, concentrated hydrochloric acid, concentrated sulfuric acid, pyrogallol, trichloroacetic acid, ferric chloride, hydrogen peroxide, sodium dihydrogen phosphate, disodium hydrogen phosphate, thiobarbituric acid, ascorbic acid, and ethylenediaminetetraacetic acid disodium salt were all analytical grade reagents from domestic sources. DPPH was purchased from Tokyo Chemical Industry Co., Ltd.

1.1.2 Instruments and Equipment

Temperature-controlled magnetic stirrer (Shanghai Zhicheng Electrical Appliance Co., Ltd.), rotary evaporator (Shanghai Yarong Biochemical Instrument Factory), electronic balance (Mettler-Toledo Instruments Co., Ltd.), thermostatic magnetic stirrer (Gongyi Zihua Instrument Co., Ltd.), pH meter (Sartorius Scientific Instruments, Beijing), DL-5-8 centrifuge (Shanghai Anting Scientific Instrument Factory), UV760CRT UV-Vis spectrophotometer (Shanghai Aopu Analytical Instrument Co., Ltd.), Fourier transform infrared spectrometer (Thermo Fisher Scientific, USA), and freeze vacuum dryer (Beijing Boyikang Experimental Instrument Co., Ltd.).

1.2 Experimental Methods

1.2.1 Extraction of Enteromorpha intestinalis Polysaccharides

Fresh Enteromorpha intestinalis was washed, dried at 50°C, pulverized, and passed through a 20-mesh sieve. The powder was extracted three times with

80% ethanol at 85°C under reflux for 2 h each time. The residue was separated and dried. Five grams of this residue were mixed with distilled water at a specific solid-liquid ratio, extracted in a water bath, filtered, and centrifuged to obtain the supernatant. The supernatant was concentrated, precipitated with four volumes of absolute ethanol, stored at 4°C for 48 h, then centrifuged at 4000 r · min ^{1} for 15 min. The precipitate was dried to yield crude Enteromorpha intestinalis polysaccharide (EIP) (刘玉凤等, 2016).

1.2.2 Carboxymethylation of Enteromorpha intestinalis Polysaccharides

Carboxymethylation was performed using the sodium hydroxide-chloroacetic acid method (Silva et al., 2004). Briefly, 120 mg of EIP was mixed with 10 mL of 20% NaOH and 25 mL of isopropanol, then stirred in an ice bath for 3 h to form a homogeneous suspension. A separate mixture (25 mL isopropanol containing 3 g chloroacetic acid and 10 mL of 20% NaOH) was slowly added dropwise to the reaction system, which was gradually heated to 60°C and stirred for 3 h. The reaction was terminated and cooled to room temperature, then adjusted to pH 7 with 1 mol · L ^{1} HCl. The solution was dialyzed against running water and distilled water for 48 h each, concentrated by rotary evaporation, and freeze-dried to obtain carboxymethylated Enteromorpha intestinalis polysaccharide (EIPC).

1.2.3 Infrared Spectroscopy Analysis

Two milligrams of EIP or EIPC were mixed with 100 mg of dry KBr in an agate mortar, ground uniformly, and pressed into pellets. Infrared spectra were recorded in the range of 4000–400 cm ^{1}.

1.2.4 Single-Factor Experiments for Carboxymethylation

To optimize the degree of substitution (DS), single-factor experiments were conducted by varying one parameter while keeping others constant. The parameters examined were: NaOH concentration (10%, 15%, 20%, 25%, 30%) with fixed chloroacetic acid (3 g), temperature (60°C), and time (3 h); reaction temperature (40°C, 50°C, 60°C, 70°C, 80°C) with fixed chloroacetic acid (3 g), 20% NaOH, and time (3 h); and reaction time (1 h, 2 h, 3 h, 4 h, 5 h) with fixed chloroacetic acid (3 g), 60°C, and 20% NaOH.

1.2.5 Determination of Degree of Substitution

The degree of substitution was determined by the method of Eyler et al. (1947). In a test tube, 0.25 mL of 0.5 mg · mL ^{1} EIPC solution was mixed with 0.25 mL of concentrated sulfuric acid, heated at 125°C for 3 h, then cooled. Two milliliters of 2,7-dihydroxynaphthalene solution were added, mixed, heated in a boiling water bath for 20 min, cooled to room temperature, and diluted with 2 mL distilled water. The absorbance at 520 nm was measured against a blank. Glycolic acid was used as a standard to calculate the grams of glycolic acid per gram of polysaccharide (A). The degree of substitution was calculated using the formula: DS = 162A/(76 - 80A), where DS is the degree of substitution and A is the grams of glycolic acid per gram of sample.

1.2.6 Antioxidant Activity Assays

The antioxidant activity of EIPC was evaluated by measuring its scavenging capacity against DPPH radicals, superoxide anion radicals, and hydroxyl radicals, as well as its reducing power (刘玉凤等, 2016). To compare the antioxidant activities of EIPC with different degrees of substitution, samples were prepared at concentrations of 0, 0.1, 0.2, 0.4, 0.8, and 1.6 mg · mL ^{1}.

(1) DPPH Radical Scavenging Assay: Two milliliters of sample solution were mixed with 2.0 mL of 0.04 mg · mL ^{1} DPPH solution (in absolute ethanol), reacted in the dark at room temperature for 30 min, and the absorbance (Ai) was measured at 517 nm. The absorbance of ethanol mixed with DPPH (Ac) and ethanol mixed with sample (Aj) were also measured. The scavenging rate was calculated as: K (%) = [1 - (Ai - Aj)/Ac] × 100.

(2) Superoxide Anion Scavenging Assay: Four point five milliliters of Tris-HCl buffer (50 mmol · L ^{1}, pH 8.2) were preheated at 25°C for 20 min, then 0.3 mL of pyrogallol solution (25 mmol · L ^{1}) and 0.2 mL of sample were added, mixed quickly, and transferred to a cuvette. Absorbance (At) was measured at 319 nm every 30 s for 8 measurements. Deionized water served as a blank control to determine the autoxidation rate of pyrogallol (V0). Plotting At versus time yielded the slope Vt. The scavenging rate was calculated as: K (%) = (1 - Vt/V0) × 100.

(3) Hydroxyl Radical Scavenging Assay: In a stoppered test tube, 0.2 mL of FeSO4-EDTA mixture (10 mmol · L ^{1}), 0.2 mL of -deoxyribose solution (20 mmol · L ^{1}), and 0.2 mL of sample were combined, diluted to 1.8 mL with phosphate buffer (0.2 mol · L ^{1}, pH 7.4), then 0.2 mL of H2O2 (10 mmol · L ^{1}) was added. After incubation at 40°C for 1 h, the reaction was terminated with 1 mL of 2.8% trichloroacetic acid, followed by addition of 1 mL of 1% thiobarbituric acid. The mixture was heated in a boiling water bath for 10 min, cooled, and the absorbance (AS) was measured at 532 nm. Deionized water (negative control) and ascorbic acid (positive control) were used to obtain absorbance values A0 and AC, respectively. The scavenging capacity was calculated as: K (%) = [1 - (AS - A0)/(AC - A0)] × 100%.

(4) Reducing Power Assay: Five hundred microliters of carboxymethylated polysaccharide solution were mixed with 0.5 mL of 0.2 mol · L ^{1} PBS buffer (pH 6.7) and 0.5 mL of 1% potassium ferricyanide solution, incubated at 50°C for 20 min, then cooled. After adding 0.5 mL of 10% trichloroacetic acid, the mixture was diluted with 2 mL distilled water and 0.5 mL of 0.1% FeCl3 solution, mixed thoroughly, and allowed to stand for 10 min before measuring absorbance at 700 nm.

2. Results and Discussion

2.1 Infrared Spectroscopy Analysis

EIP and EIPC samples were characterized using a Nicolet iS 10 Fourier transform infrared spectrometer in the wavelength range of 4000–400 cm ^{1} [Figure 1: see original paper]. The carboxymethylated product exhibited characteristic polysaccharide absorption peaks at 3441 cm ^{1} (O-H), 1260 cm ^{1}, 1204 cm ^{1}, and 1077 cm ^{1} (C-O-H). Notably, enhanced absorption was observed at 1611 cm ^{1} (asymmetric C=O stretching of carboxyl groups, -COOH), 1422 cm ^{1} (C-H bending of methyl groups attached to carboxyl groups, -CH3), and 1328 cm ^{1} (symmetric C=O stretching), confirming the presence of carboxymethyl groups (-CH2-COOH) in EIPC.

2.2 Single-Factor Optimization of Carboxymethylation

The effects of NaOH concentration (10–30%), reaction temperature (40–80°C), and reaction time (1–5 h) on the degree of substitution were investigated [Figure 2: see original paper]. The degree of substitution initially increased then decreased with rising NaOH concentration, reaching a maximum of 0.701 at 20% NaOH. Higher NaOH concentrations promote polysaccharide swelling and increase active sites for etherification, but excessive NaOH causes side reactions with chloroacetic acid, reducing substitution efficiency. Therefore, 20% NaOH was selected as optimal.

Similarly, the degree of substitution increased with temperature up to 60°C (maximum DS = 0.701), then declined at higher temperatures due to increased chloroacetic acid hydrolysis and polysaccharide degradation in alkaline conditions. Thus, 60°C was chosen as the optimal reaction temperature.

The degree of substitution increased with reaction time from 1 to 3 h as more active centers became available and etherification accelerated. Beyond 3 h, side product formation and decomposition of unstable intermediates prevented further increase in substitution, while prolonged heating was time-consuming and energy-intensive. Consequently, 3 h was selected as the optimal reaction time, yielding a degree of substitution of 0.702.

2.3 Antioxidant Activity

2.3.1 DPPH Radical Scavenging Capacity DPPH radical scavenging by EIPC decreased with increasing degree of substitution, showing no clear linear relationship but indicating that higher substitution diminished this activity [Figure 3: see original paper]. Both EIP and EIPC showed concentration-dependent scavenging up to 0.8 mg $\cdot$ mL ^{1}, with maximum scavenging rates of 20.14% and 39.24%, respectively. At higher concentrations, scavenging capacity declined. At any given concentration, EIP exhibited approximately double the scavenging efficiency of EIPC [Figure 4: see original paper].

2.3.2 Reducing Power Reducing power, which correlates positively with absorbance values, decreased with increasing degree of substitution, indicating that carboxymethylation impaired the reducing capacity of Enteromorpha polysaccharides [Figure 3: see original paper]. Both EIP and EIPC showed concentration-dependent reducing power up to 0.8 mg · mL ^{1}, with absorbance values of 1.26 and 0.59, respectively. Beyond 0.8 mg · mL ^{1}, reducing power slightly decreased. Compared with vitamin C, both EIP and EIPC exhibited lower reducing power, with EIPC showing the weakest activity [Figure 4: see original paper].

2.3.3 Hydroxyl Radical Scavenging Capacity Hydroxyl radical scavenging by EIPC increased with degree of substitution, demonstrating that carboxymethylation enhanced this activity [Figure 3: see original paper]. Both EIP and EIPC showed gradual concentration-dependent increases in hydroxyl radical scavenging, plateauing at 1.6 mg · mL ^{1} with values of 19.84% and 44.45%, respectively. At equivalent concentrations, EIPC exhibited nearly double the scavenging capacity of unmodified EIP. Compared with vitamin C, carboxymethylation significantly improved hydroxyl radical scavenging [Figure 4: see original paper].

2.3.4 Superoxide Anion Scavenging Capacity Superoxide anion scavenging by EIPC increased with degree of substitution, similar to the trend observed for hydroxyl radicals [Figure 3: see original paper]. Both EIP and EIPC showed concentration-dependent scavenging, reaching 28.94% and 51.98%, respectively, at 1.6 mg · mL ^{1}. Carboxymethylation substantially enhanced superoxide anion scavenging compared with the native polysaccharide, demonstrating a significant beneficial effect of modification on this activity [Figure 4: see original paper].

Carboxymethylation of Enteromorpha intestinalis polysaccharides via the sodium hydroxide-chloroacetic acid method was influenced by NaOH concentration, reaction temperature, and time. Under optimal conditions (120 mg polysaccharide, 20% NaOH in isopropanol, 3.0 g chloroacetic acid, 60°C, 3 h), the maximum degree of substitution was 0.781. Carboxymethylation substantially enhanced hydroxyl radical and superoxide anion scavenging capacities, increasing from 22.45% to 44.46% and from 28.94% to 51.98%, respectively, at 1.6 mg · mL ^{1}. However, DPPH radical scavenging and reducing power decreased after modification, possibly due to blocking of active sites. While higher substitution degrees correlated with enhanced hydroxyl radical and superoxide anion scavenging, they inversely affected DPPH scavenging and reducing power. These results demonstrate that carboxymethylation can modulate the antioxidant activity of Enteromorpha polysaccharides, though the degree of substitution is not the sole determinant of activity. Chemical modification represents an important strategy for altering polysaccharide bioactivity with significant implications for both applications and mechanistic studies.

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Received date: 2018-07-03
Funding: National Natural Science Foundation of China (3120026); Guangxi Science Foundation (2014GXNSFBA118134) [Supported by the National Natural Science Foundation (3120026); Guangxi Science Foundation (2014GXNSFBA118134)]

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Note: Figure translations are in progress. See original paper for figures.