Carbohydrate Polymers 92 (2013) 2084–2087

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Degradation and the antioxidant activity of polysaccharide from *Enteromorpha linza*

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ARTICLE INFO

Article history:
Received 14 October 2012
Received in revised form
16 November 2012
Accepted 30 November 2012
Available online 10 December 2012

Keywords:
Enteromorpha linza
Polysaccharide
Molecular weight
Antioxidant activity

ABSTRACT

Polysaccharide extracted from *Enteromorpha linza* possesses excellent antioxidant activities, but its molecular weight was greatly high which influences the activity. In this study, the combination of ascorbic acid and H_2O_2 was used as degradation reagents in order to obtain the lower molecular weight product. The results suggested that the most effective molar ratio of the two reagents was 1. Three degraded polysaccharides were selected to evaluate their antioxidant activities *in vitro* and characterized the relationship between antioxidant activity and chemical characteristics. It was found that the degraded sample with lower molecular weight possessed the higher antioxidant activities.

1. Introduction

Nature polysaccharides are widely distributed in animals, plants, and microorganisms, and possess marked immunological properties ranging from nonspecific stimulation of host immune system, resulting in anti-tumor, anti-viral, and anti-infective effects, to antioxidant, anti-mutagenic or hematopoietic activity (Bohn & BeMiller, 1995; Guo et al., 2006; Kennedy, 1989; Kennedy & White, 1983). However, the high molecular weight and high viscosity of many polysaccharides limited their pharmaceutical application. And therefore, chemical modification of polysaccharides provided an opportunity to obtain new pharmacological agents with possible therapeutic uses (Franz & Alban, 1995; Zhang et al., 2012). Generally speaking, the activity of polysaccharide depends on several structural parameters such as degree of substitution of function groups, the molecular weight, type of sugar and functional groups (Melo, Feitosa, Freitas, & de Paula, 2002). And furthermore, many studies have demonstrated that molecular weight distributions of polysaccharides have great influence on their biological activities (Chen, Lu, Cheng, & Wang, 2005; Im et al., 2005; Schepetkin, Faulkner, Nelson-Overton, Wiley, & Quinn, 2005; Zhao et al., 2006). Therefore, it is necessary to prepare different molecular weight polysaccharides to reduce the viscosity and improve the activity.

Enteromorpha linza is common green seaweed residing in the upper region of the intertidal zone or in eutrophicated coastal waters. In our previous study, polysaccharide from *E. linza* was isolated, purified and evaluated as a novel antioxidant and humectant (Shi et al., 2010). In addition, two derivatives by means of oversulfation and acetylation coupling were synthesized and investigated their antioxidant activities (Zhang et al., 2011). But the molecular weight of this polysaccharide and derivates is also greatly high. In order to enquiry study the structure–function relationship of polysaccharide from *E. linza*, in this study, the combination of ascorbic acid and H_2O_2 was used as degradation reagent to obtain product with different molecular weights. Three degraded polysaccharides were selected to evaluate their antioxidant activities *in vitro* and characterized the relationship between antioxidant activity and chemical characteristics.

2. Materials and methods

2.1. Materials

E. linza was collected on the coast of Ningbo in October 2011. The nature polysaccharide was extracted from *E. linza* in hot water and precipitated by alcohol to give a main fraction, which was named after EP.

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http://dx.doi.org/10.1016/j.carbpol.2012.11.096

Ethylene diamine tetraacetic acid (EDTA), ascorbic acid, H_2O_2, sodium citrate and other reagents were of analytical grade. Dialysis membranes were produced by Spectrum Co., and molecular weight was cut off at 3600 Da.

2.2. Analytical methods

Total sugar content was determined by phenol–sulfuric acid method using rhamnose as standard (Dubois, Gillis, Hamilton, Rebers, & Smith, 1956). Sulfate content was determined by barium chloride method (Kawai, Seno, & Anno, 1969).

Infrared spectra were measured by a Nicolet Magna-Avatar 360 with KBr disks.

The intrinsic viscosity (η_r) of reaction mixtures was determined in an Ubbelohde viscosimeter at 25 °C. The result was measured as the following equation [$\eta_r = (\ln t/t_0)/c$], where t is the solution flow time (s), t_0 is the solvent flow time (s) and c is the concentration of solution in distilled water (g/mL).

Molecular weight of all samples was determined by HP-GPC on a Waters 515 GPC system at 35 °C, where 0.7% Na_2SO_4 solution was used as mobile phase with a flow rate of 0.5 mL/min. TSK G3000 column (300 mm × 7.8 mm) and 2140 refractive index detector were used. A series of different molecular weight dextrans purchased from the National Institute for the Control of Pharmaceutical and Biological Products (China) were used as standard.

2.3. Selection of degradation reagent

Three experiments were conducted in order to select appropriate degradation reagent. The reaction took place in a glass beaker that immersed in a thermostat bath at 25 °C. A solution of raw polysaccharide EP (2.0 g) in distilled water (200 mL) was added in 10 mM ascorbic acid, 10 mM H_2O_2 and a mixed solution of 10 mM ascorbic acid and 10 mM H_2O_2, respectively. Then the solution was stirred and the viscosity was timing measured per 10 min after reaction starting. The time of reaction was 2 h.

2.4. Determination of the concentration of degradation reagent

After the degradation reagent was definitive, two experiments were carried out: (1) H_2O_2 with different concentrations (1, 5, 10, 15 and 20 mM) was introduced into polysaccharide solution containing 10 mM ascorbic acid and (2) ascorbic acid with different concentrations (1, 5, 10, 15 and 20 mM) was introduced into polysaccharide solution containing 10 mM H_2O_2. Then, the optimal concentration of degradation reagent was determined through the change of viscosity with the above method.

2.5. Sampling

After reaction, the solution was precipitated by 75% alcohol and centrifuged to obtain the degraded product. Three samples were selectively prepared and studied the antioxidant activities in two systems.

2.6. Antioxidant activities

2.6.1. Hydroxyl radical assay

The reaction mixture, containing all different samples (0.6–7.0 mg/mL), was incubated with EDTA–Fe^{2+} (2 mM), saffron (360 μg/mL), and H_2O_2 (3%) in potassium phosphate buffer (150 mM, pH 7.4), was incubated for 30 min at 37 °C (Wang et al., 1994). The absorbance was read at 520 nm against a blank. Hydroxyl radical bleached the saffron, so decreased absorbance of the reaction mixture indicated a decrease in hydroxyl radical scavenging ability. The capability of scavenging hydroxyl radical was calculated using the following equation:

$$\text{Scavenging effect}\,(\%) = \left[\frac{A_0 - A_1}{A_0}\right] \times 100$$

where A_0 is the absorbance of the control (without samples) and A_1 is the absorbance of the mixture containing sample.

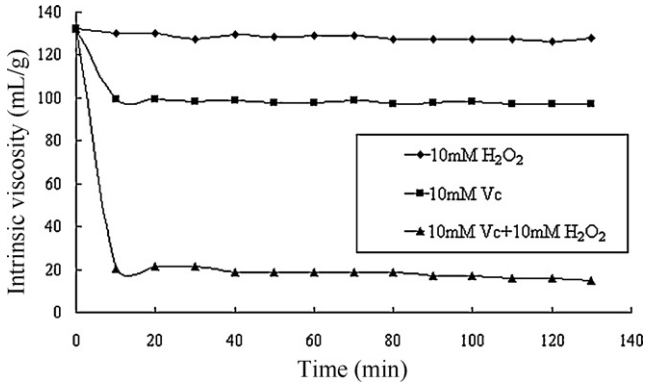

Fig. 1. The change of viscosity of polysaccharide solution with different degradation reagents (ascorbic acid, Vc).

2.6.2. Reducing power assay

The reducing power was determined as described previously by Yen and Chen (1995). Briefly, 1.0 mL different concentrations of samples (0.47–6.0 mg/mL) in phosphate buffer (0.2 M, pH 6.6) were mixed with 1.0 mL potassium ferricyanide (1%, w/v), and were incubated at 50 °C for 20 min. Afterwards, 2.0 mL trichloroacetic acid (10%, w/v) was added to the mixture to terminate the reaction. Then the solution was mixed with 1.2 mL ferric chloride (0.1%, w/v) and the absorbance was measured at 700 nm. Increased absorbance of reaction mixture indicated increased reducing power.

3. Results and discussion

3.1. Determination of the degradation reagent

In recent years, there are many researches on polysaccharides degradation, such as physical method (Stahmann et al., 1995), chemical method (Meng et al., 2012) and enzyme method (Yoshizawa et al., 1995). But there were many limitations in these methods. As a matter of fact, oxidation method was used more and more recently (Hjerde, Stokke, Smidsrød, & Christensen, 1998; Wang, Hollingsworth, & Kasper, 1999). In this method, there was only one degradation reagent, H_2O_2 or O_3, which was unstable and unrepeatable. And therefore, in this study, H_2O_2 and ascorbic acid were tried to be degradation reagent, which was based on the mechanism of action of ascorbic acid and H_2O_2 appears to be *via* •OH since they fulfilled the requirements for a Fenton reaction in plants (Fry, 1998).

In this study, the concentration of polysaccharide solution was selected to be 1% (m/v). Fig. 1 shows the change of viscosity of polysaccharide solution under the effect of different degradation reagents. From the figure, there was no significant decrease in intrinsic viscosity when 10 mM ascorbic acid (Vc) or 10 mM H_2O_2 was used alone. It means that the single reagent has no effect to the degradation. However, when 10 mM ascorbic acid was associated with 10 mM H_2O_2 in degradation reaction, the viscosity decreased quickly in 10 min after the reaction starting. It suggested that these two reagents should be used simultaneously as degradation reagent.

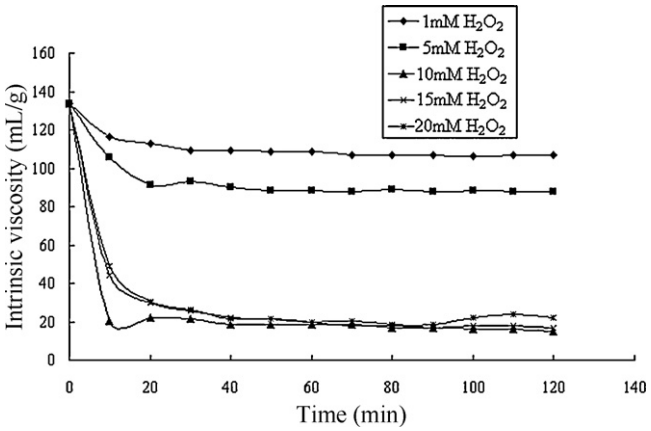

Fig. 2. The change of viscosity of polysaccharide solution with the different concentrations of H_2O_2 and 10 mM ascorbic acid (Vc).

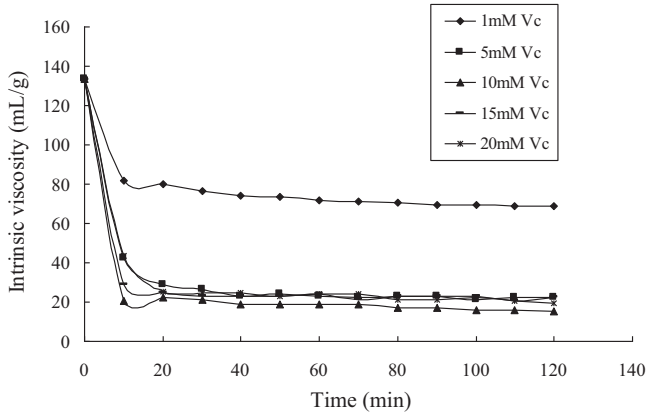

Fig. 3. The change of viscosity of polysaccharide solution with the different concentrations of ascorbic acid (Vc) and 10 mM H_2O_2.

3.2. Influence of concentration of the degradation reagent

The dependence of viscosity on the concentration of H_2O_2 for 2 h is shown in Fig. 2. From the figure, the intrinsic viscosity of solution decreased markedly in first 20 min and slowly after that. And in addition, when the concentration of H_2O_2 was 1 and 5 mM, the viscosity changed a little. But, when the concentration of H_2O_2 was close to or higher than that of ascorbic acid (10 mM), no significant decrease in intrinsic viscosity was observed. It showed that when $c(H_2O_2)/c$ (ascorbic acid) > 1, the decrease of viscosity was strongly dependent on the concentration of H_2O_2.

Fig. 3 shows the effect of concentration of ascorbic acid on degradation in 10 mM H_2O_2 solution. As shown in figure, when the concentration of ascorbic acid was higher than 5 mM, the intrinsic viscosity of solution decreased markedly. From Figs. 2 and 3, when $c(H_2O_2)/c$ (ascorbic acid) = 1, the viscosity of solution was the lowest. Based on these results, it was concluded that it would be more efficiency to obtain lower molecular weight polysaccharide by keeping the molar ratio of the two reagents at 1.

The test was conducted that the change of intrinsic viscosity of solution when the molar ratio of ascorbic acid and H_2O_2 was at 1. Obviously, from Table 1, the higher the absolute concentrations of two reagents were, the lower the viscosity of solution was. The intrinsic viscosity of solution reduced 79.7% at the higher concentration 20 mM. It suggested that the degradation was successful.

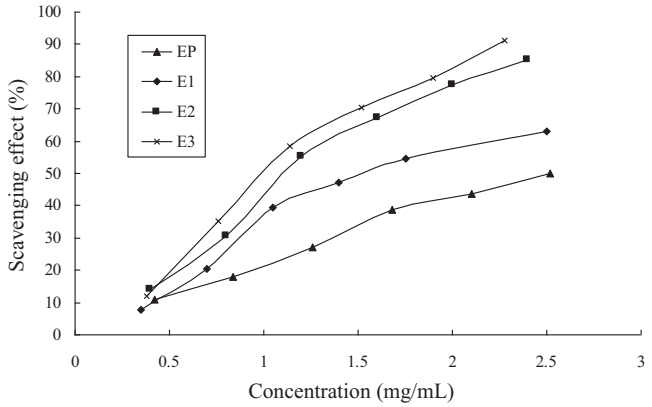

Fig. 4. Scavenging effects of the sample on hydroxyl radical. Values are means ± S.D. (n = 3).

3.3. Characteristics of the sample

Three samples were selectively prepared and their chemical analysis results are shown in Table 2. From the table, three degraded products contained similar total sugars and sulfate content to that of natural polysaccharide. It indicated that the degradation could not result in destruction of main chain. For the FT-IR spectra, typical signals of polysaccharide from *E. linza* at 3436, 1623, 1435, 1264, 1054 and 846 cm^{-1} were clear for all the samples (Zhang et al., 2011). These results suggested that no major functional group transformations happened during the degradation. And in addition, the molecular weight of polysaccharide decreased with the more and more concentration of degraded agents. These data demonstrated that high concentrations of H_2O_2 and ascorbic acid could result in intensively degraded polysaccharide from *E. linza*.

3.4. Antioxidant activities

The inhibitory effect on the hydroxyl radical of all the samples is depicted in Fig. 4. It indicated that all the samples exhibited varying degrees of antioxidant activity. Especially, E2 and E3 showed significant scavenging effects. At the concentration of 2.28 mg/mL, the inhibitory effect was 85.1% and 92.2%, respectively. At the concentration over 0.70 mg/mL, the scavenging activities of all the degraded samples were stronger than that of raw polysaccharide EP.

Fig. 5 shows the reducing power of all samples. As shown in the figure, the reducing power of the degraded samples

Table 1
Effect of the concentrations of ascorbic acid and H_2O_2 on polysaccharide degradation.

Samples	$c(H_2O_2)$ (mM)	c (ascorbic acid) (mM)	Intrinsic viscosity (mL/g)	Decrease in viscosity (%)
EP	–	–	137.40	–
1	1	1	89.49	34.9
2	5	5	60.69	55.8
3	10	10	32.88	76.1
4	15	15	30.46	77.8
5	20	20	27.96	79.7

Table 2
The chemical characteristics of all the samples.

Sample	c (ascorbic acid) (mM)	c (H_2O_2) (mM)	Total sugar (%)	Sulfate (%)	M_w (Da)	M_n (Da)
EP	–	–	57.6	20.2	100,519	31,528
E1	1	1	56.2	22.4	46,540	10,198
E2	5	5	54.0	19.5	24,968	6221
E3	10	10	59.8	18.2	18,716	5665

M_w, weight average molecular weight; M_n, number average molecular weight.

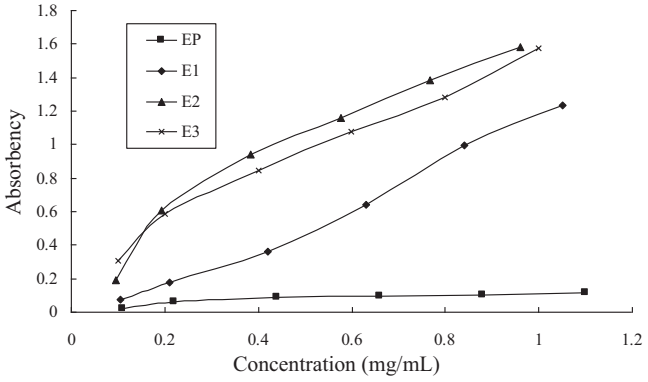

Fig. 5. Reducing power assay of the sample. Values are means ± S.D. (n = 3).

correlated well with increasing concentration, which showed stronger reducing powers. In contrast, the absorbance of EP was only 0.115 at 1.10 mg/mL because of the slow rate of increasing power with increasing concentration.

From the antioxidant activity test, the polysaccharide with different molecular weights showed different antioxidant activities. Because all the samples contain similar chemical compound, the molecular weight plays an important role in antioxidant activity. In our tests the low molecular weight products contain many free hydroxyl groups, which may have a very important effect on the antioxidant activities. It was supposed that the chemical groups in degraded sample have more chance to contact with radical because of the better water-soluble and more surface area. The further studies are needed to improve our understanding of antioxidant activities mechanism.

4. Conclusion

In the present study, the polysaccharide from *E. linza* could be degraded by free radical generated by ascorbic acid and H_2O_2. And then we evaluated their antioxidant activities *in vitro*. The results showed that the molar ratio of the two reagents at 1 is proved to be the optimal manipulation of degradation. And in addition, the antioxidant activities were strongly dependent on the molecular weight. The present findings may be useful in leading to further experiments on bioactivity *in vivo*.

Acknowledgments

This work was supported by the Project of Zhejiang Key Laboratory of Exploitation and Preservation of Coastal Bio-resource (J2012008) and National Natural Science Foundation of China (41206129). We are grateful to Prof. Wu Chen and Technician Mingxing Zhao for their help in this experiment.

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