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Study of antioxidant activities of sulfated polysaccharides from *Laminaria japonica*

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Abstract The composition, molecular weight and in vitro antioxidant activity of various sulfated polysaccharides obtained by anion exchange chromatography, acid hydrolysis and radical process degradation of the crude sulfated polysaccharide extracted from *Laminaria japonica* were compared. The low sulfated F-A2, with a peak-molecular weight (Mp) of 5–15 kDa, 14.5% sulfated ester and 21.8% glucuronic acid, exhibited a very strong antioxidant activity on superoxide and hydroxyl radicals, with activity even higher than that of large molecular weight fractions F-A and F-B. However, highly sulfated fractions with a peak-molecular weight below 15 kDa had much lower antioxidant activities than other fractions. These results indicated that the sulfate group of the low molecular weight fractions represents a physical block for the reaction with oxygen radicals. The chemical properties and antioxidant activities of sulfated polysaccharide fractions obtained by radical process degradation of crude sulfated polysaccharide were quite different from those obtained by acid hydrolysates. By radical process degradation, the high molecular weight was decreased to give LM2 (Mp 8 kDa) and LM1 (Mp 1.5 kDa), with a yield of 40% and 15%, respectively. LM2 was enriched with fucose and sulfated ester, while containing low amounts of glucuronic acid. The antioxidant activity showed that LM2 was unable to scavenge either superoxide or hydroxyl radical, which suggested that radical process degradation targeted mainly ascopyllan-like species rich in glucuronic acid, while the fraction rich in sulfated L-fucose remained unchanged. However, LM1 with Mp 1.5 kDa still retained apparent scavenging ability for superoxide radical, although it contained no glucuronic acid and certain amounts of galactose and mannose as main neutral sugars. These result suggest that the antioxidant activity of sulfated polysaccharides is apparently related not only to molecular weight and sulfated ester content, as previously determined, but also to glucuronic acid and fucose content.

Keywords *Laminaria japonica* · Sulfated polysaccharide · Free radical · Antioxidant · Radical process degradation

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Introduction

The sulfated polysaccharides of brown algae are known to contain L-fucose as the main sugar constituent, and sulfate ester. Their structures vary according to the season, age of population, species and geographic location. Over the past decade many studies have demonstrated that the sulfated polysaccharides from brown algae possess excellent biological properties, including anticoagulant, anti-inflammation and anti-tumor activities (Pereira 1994; Nishino et al. 1989; Omata et al. 1997; Zhang et al. 1995). Studies on the relationship between biological activity and structure have revealed that several structural parameters, such as molecular weight, degree of sulfation (DS), sulfation position, type of sugar, and glycosidic branching, are required for anticoagulant and antitumor activities (Manish et al. 1993; Pereira et al. 2002). Moreover, fucoidan has the additional advantage of the absence of potential risk of contamination by animal viruses. As a by-product of idione and alginate production, sulfated polysaccharides extracted from *Laminaria japonica* represent a source of marine compounds with potential applications in medicine.

In recent years, sulfated polysaccharides from the marine algae *Prophyra haitanesis* (Zhang et al. 2003), *Ulva pertusa* (Qi et al. 2005a, b), *Fucus vesiculosus* (Ruperez et al. 2002), *Laminaria japonica* (Xue et al. 2000; Zhang et al. 2004) and *Ecklonia kurome* (Hu et al. 2001) have been demonstrated to have antioxidant activities. However, there are few reports on the relationship between structure and antioxidant activity of sulfated polysaccharides from marine alga. Tsiapali et al. (2001) investigated the free radical scavenging activities of glucan and nonglucan polymers and found that polyelectrolytes such as glucan sulfate or phosphate might increase scavenging activity. Qi (2005a, b) found that a polysaccharide from *U. pertusa* with low sulfate content and low molecular weight had a stronger reducing power and free radical scavenging effect than other sulfated polysaccharides. Liu et al. (1997) found that the presence of proteinaceous substances in polysaccharide molecules potentiated their free radical scavenging activity. However, other structural parameters, such as the the degree of sulfation, sulfation position, type of sugar, and glycosidic branching, are required for antioxidant activity and the mechanism of action of the antioxidant activities of sulfated polysaccharides remains unclear.

In the present study, we attempted to determine the relationship between the chemical properties of different sulfated polysaccharide fractions and their antioxidant activities on oxygen free radicals, and to study the mechanism of action of sulfated polysacchrides' antioxidant activity. For this purpose, we prepared various sulfated polysaccharide fractions by anion exchange chromatography, acid hydrolysis and radical process degradation of a crude sulfated polysaccharide from *L. japonica*. The in vitro antioxidant activity of the sulfated polysaccharide fractions was examined using the chemiluminescence of superoxide and hydroxyl radicals.

Materials and methods

The brown alga *Laminaria japonica* Areschoug was collected in Rongcheng, Shandong, China, in May 2005 and stored at −4°C. Luminol (5-amino-1, 2, 3, 4-tetrahydrophthalazin-1, 4-dion) was purchased from Merck (Darmstadt, Germany). Carnosine was from Wako Pure Chemistries (Tokyo, Japan).

The crude sulfated polysaccharide of japonica was prepared according to Nishino et al. (1989) and further fractioned by anion exchange chromatography on Q-Sepharose FF column (Amersham Pharmacia Biotech, Piscataway, NJ). The low sulfated fraction F-A was prepared by elution with 1.0 mol L^{-1} NaCl and the high sulfated fraction F-B was obtained by elution with 2.0 mol L^{-1} NaCl.

F-A and F-B (3 g) were each dissolved in 100 mL 80 mmol L^{-1} H_2SO_4 and hydrolyzed at 80°C for 9 h. Low molecular weight fractions F-A1 (<5 kDa), F-A2 (5–15 kDa), F-B1 (<5 kDa) and F-B2 (5–15 kDa) were then obtained by ultrafiltration with 15 kDa and 5 kDa cut-off membranes (Shanghai Institute of Applied Physics, Chinese Academy of Sciences). The molecular weights and chemical composition of F-A, F-B as well as their acid hydrolysates are shown in Table 1.

The radical process degradation of fucoidan proceeded through the formation of hydroxyl radicals from the hydrogen peroxide-cupric redox system. The crude fucoidan (1.0 g) was mixed with 0.4 mmol copper acetate monohydrate at pH 7.5 and degraded at 60°C for 5 h in a temperature control reactor with addition of hydrogen peroxide solution (9% v/v) at a flow rate of 12 mL h^{-1}. Low molecular weight fractions LM1 (<5 kDa) and LM2 (5–15 kDa) were prepared by ultrafiltration with 15 kDa and 5 kDa cut-off membranes in turn. The molecular weights and chemical compositions of LM1 and LM2 are shown in Table 2.

The molecular weights of fucoidan fractions (F-A and F-B) were determined by high-performance steric exclusion chromatography (HPSEC) in 0.2 mol L^{-1} NaCl using a TSK-gel G4000Pwxl column (TOSHO, Japan) with G1362A RID as a detector. The molecular weights of F-A1, F-A2, F-B1, F-B2, LM1 and LM2 were determined by HPSEC using a TSK-gel G3000Pwxl column (TOSHO, Japan). The columns were calibrated with blue dextran, dextran sulfates with molecular weight of 5 kDa, 8 kDa, 10 kDa and fucose (164.16 Da) (Sigma, St. Louis, MO). Peak-molecular weight (Mp), weight-average, number-average weight and polydispersity (I= weight-average / number-average weight) were determined using the Agilent ChemStation.

Uronic acid content was determined by the modified carbozole method of Bitter and Muir (1962), using D-glucuronic acid as standard (Sigma). Neutral sugar compo-

Table 1 Molecular weight and contents of sulfate ester and glucuronic acid of F-A, F-B and their acid hydrolysates from *Laminaria japonica*

Fraction	F-A	F-A1	F-A2	F-B	F-B1	F-B2
Molecular weight (kDa)	742	<5	5–15	175.9	<5	5–15
Polydispersity	2.2	1.8	1.7	2.0	1.8	1.5
Sulfated ester (%)[a]	16.5	12.8	14.5	33.5	25.7	30.5
Glucuronic acid (%)[a]	21.4	20.8	21.8	8.9	7.8	8.9

[a] Of polysaccharide dry weight

Table 2 Molecular weights and chemical composition of LM1 and LM2 by radical process degradation of crude fucoidan extracted from *Laminaria japonica*. *I* = Dispersity

Fraction	Molecular weight	I	Sulfated ester (%)[a]	Glucuronic acid (%)[a]	Neutral sugars (%)		
					Fucose[b]	Galactose[b]	Mannose[b]
LM1	1.5 kDa	1.2	23.4	2.7	20.6	42.8	25.6
LM2	8.0 kDa	1.8	36.5	5.3	57.8	10.6	11.5

[a] Calculated on polysaccharide dry weight
[b] Calculated from gas-liquid chromatograms, taking total area of the peaks as 100%

nent was determined by gas-liquid chromatography after acid hydrolysis of the sample (20 mg) was carried out with 2 mol/L trifluoroacetic acid at 100°C for 6 h. Sulfate content was deduced from the method of Dodgson and Price (1962).

The scavenging effects of fucoidan fractions on superoxide and hydroxyl radical were assessed by chemiluminescence analysis of a pyrogallol–luminol system and an ascorbic acid–Cu^{2+}–hydrogen superoxide–yeast suspension system, respectively (Zhang et al. 1995). A chemiluminescence detector (WDD-2, Rayleigh, China) was used for the determination of superoxide and hydroxyl chemiluminescence. The original chemiluminescence of the free radicals (C_0) and the final chemiluminescence of free radicals after addition of sulfated polysaccharides (C) were recorded. Scavenging rate on free radicals=$(C_0-C) / C_0 \times 100\%$. IC_{50} is the content of sulfated polysaccharides with 50% scavenging rate on radicals.

Results

The results of HPSEC demonstrated that F-A had a peak-molecular weight of 742 kDa, which is higher than F-B (175.9 kDa) (data not shown). The chemical properties of F-B showed high contents of fucose (49.0%) and sulfated ester (33.5%)—both higher than those of F-A, while the content of glucuronic acid in F-B was only half that of F-A. The acid hydrolysates of F-A and F-B were fractioned using ultrafiltration on the basis of molecular weight (15 kDa and 5 kDa). The chemical properties of each fraction are shown in Table 1. Low molecular weight fractions with molecular weights of 5–15 kDa and below 5 kDa, contained similar proportions of glucuronic acid to those of the parent fractions, while their sulfated ester contents were a little lower, due to desulfation during acid hydrolysis.

The scavenging effects of F-A, F-B and their acid hydrolysates on the superoxide radical and hydroxyl radical

The inhibition of superoxide chemiluminescence due to F-A, F-B and their acid hydrolysates is shown in Figs. 1 and 2, using carnosine as a positive control. As native polysaccharides with high molecular weight, F-A and F-B possessed similar scavenging effects on superoxide radical in a concentration-dependent manner, with IC_{50} = 0.43 and 0.53 mg mL^{-1}, although the sulfated ester content of F-B was much higher than that of F-A. After hydrolysis of F-A, the low molecular weight fraction F-A2 (Mp 5–15 kDa) exhibited a stronger scavenging ability on superoxide radical than F-A or carnosine, with IC_{50} = 0.26 mg mL^{-1}, while F-A1 (Mp<5 kDa) had lower effect on superoxide, with an inhibitory rate of 40.6% at 0.75 mg mL^{-1}. In contrast, the highly sulfated fractions F-B1 and F-B2 exhibited much lower scavenging effects on superoxide radical than native fuoicdan F-B. The inhibitory rates of F-B1 and F-B2 at 0.75 mg mL^{-1} were only 28.8% and 40.6%, respectively. On the basis of molecular weight, F-A1 and F-A2 exhibited higher scavenging abilities on superoxide radical than the highly sulfated fractions F-B1 and F-B2.

Figures 3 and 4 illustrate the scavenging effects on hydroxyl radical of F-A, F-B and their acid hydrolysates. F-A exhibited higher scavenging ability on hydroxyl radical compared to F-B, with IC_{50} = 0.60 and 0.85 mg mL^{-1}, respectively, while both F-A and F-B inhibited the hydroxyl radical chemiluminescence to a much lower extent than

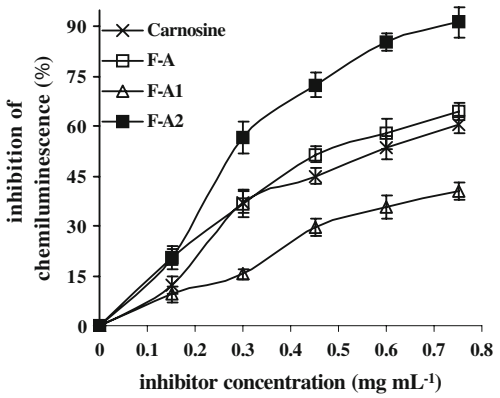

Fig. 1 Inhibition of the chemiluminescence of superoxide radical by carnosine, F-A and its acid hydrolysates F-A1 and F-A2. Values are the mean±SD of at least three experiments

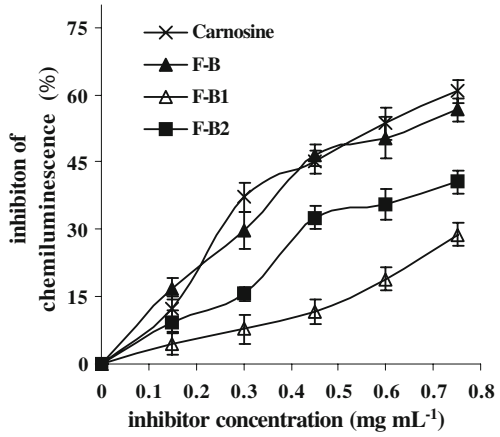

Fig. 2 Inhibition of the chemiluminescence of superoxide radical by carnosine, F-B and its acid hydrolysates F-B1 and F-B2. Values are the mean±SD of at least three experiments

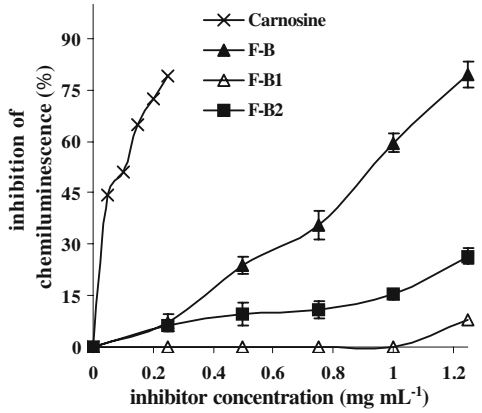

Fig. 4 Inhibition of the chemiluminescence of superoxide radical by carnosine, F-B and its acid hydrolysates F-B1 and F-B2. Values are the mean±SD of at least three experiments

carnosine. After acid hydrolysisof F-A, F-A2 (with a peak-molecular weight of 5–15 kDa) had stronger antioxidant activity as compared to the parent fraction F-A, with IC_{50} =0.3 mg mL^{-1}. In contrast, hydrolysis of F-B reduced its antioxidant capacity. F-B1 and F-B2 had little scavenging effect on hydroxyl radical.

The radical process degradation of sulfated polysaccharides

The radical process degradation proceeded through the formation of hydroxyl radical from the hydrogen peroxide-cupric redox system. At neutral pH, these species were very reactive and degraded the polysaccharide backbone. After 5 h, the high molecular weight decreased to give LM2 (Mp 8 kDa) and LM1 (Mp 1.5 kDa) with a yield of 40 and 15%, respectively. The results of HPSEC demonstrated that LM1 and LM2 had a narrower molecular weight distribution, with dispersity of 1.1 and 1.2 compared to the parent fractions (data not shown). However, the charge group composition of LM1 and LM2 were quite different from that of the starting compounds.

The chemical properties of LM1 and LM2 are shown in Table 2. It is noteworthy that fraction LM2, with a Mp of 8 kDa, was enriched with sulfated ester (36.5%) and fucose (57.8%), to an even higher extent than F-B. In contrast, LM1, with an Mp of 1.5 kDa, had a low amount of sulfate ester (23.4%) and glucuronic acid (2.0%). The neutral sugar composition showed that LM1 contained small amounts of galactose, mannose and fucose.

The scavenging effects of LM1 and LM2 on superoxide radical are shown in Fig. 5. As evidenced by the chemiluminescence of superoxide radical, the highly sulfated LM2 with a fucose content of 57.8% was ineffective as a scavenger of superoxide radical. On the contrary, fraction LM1 with an Mp of 1.5 kDa retained a similar scavenging capacity as crude fucoidan. It is noteworthy that hydroxyl radical degradation of fucoidan abolished the antioxidant activity against hydroxyl radical, as no inhibition was observed for LM1 and LM2 at any concentration.

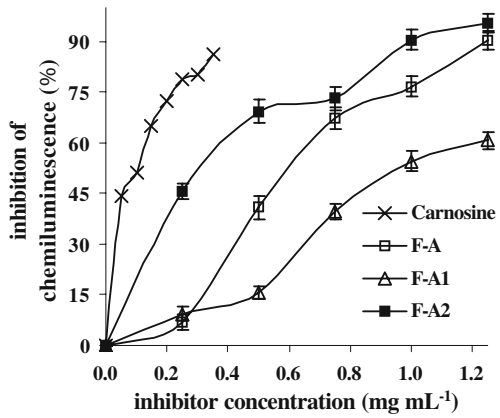

Fig. 3 Inhibition of the chemiluminescence of superoxide radical by carnosine, F-A and its acid hydrolysates F-A1 and F-A2. Values are the mean±SD of at least three experiments

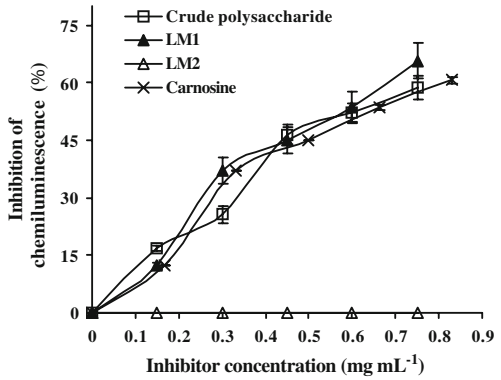

Fig. 5 Inhibition of the chemiluminescence of superoxide radical by carnosine, crude polysaccharide, LM1 and LM2 obtained by radical process degradation. Values are the mean±SD of at least three experiments

Discussion

Two high molecular weight fucoidan fractions were obtained by anion exchange chromatography, with sulfated ester contents of 16.5% and 33.5%, respectively. The two fractions were complex sulfated polysaccharides containing different amounts of glucuronic acid, fucose, mannose, galactose and glucose. However, there was not much difference in their scavenging activities on superoxide and hydroxyl radical. The heterogeneity and the high molecular weight of these compounds made their mechanism of action difficult to study. For this reason, we prepared well-characterized low molecular weight fractions, which may yield some important information necessary to appreciate their antioxidant activities.

The difference in the antioxidant ability of fucoidan fractions on the two oxygen free radicals may be due to their different molecular weights and chemical composition. As high molecular weight fucoidan, F-B and F-A behaved quite differently as sulfated esters, while they exhibited similar scavenging abilities on superoxide and hydroxyl radical. The acid hydrolysates F-A1, F-A2, F-B1 and F-B2 had chemical compositions similar to those of the parent fucoidan fractions F-A and F-B. In our study, the low molecular weight fraction F-A2, containing 14.5% sulfated ester and 21.8% glucuronic acid, exhibited a very strong antioxidant activity on superoxide and hydroxyl radicals—higher than that of F-A and F-B. However, with similar low molecular weights, the highly sulfated fractions F-B1 and F-B2 had lower scavenging abilities on superoxide and hydroxyl radicals than low sulfated fractions. These results indicate that the content of sulfated ester plays a more important role in determining the scavenging ability on oxygen free radical for low molecular weight fucoidan fractions than for high molecular weight fractions. It was deduced that the sulfate group of F-B1 and F-B2 represents a physical block to the reaction with oxygen radicals. It seems likely that the antioxidant activity of fucoidan depends on the chemical composition, notably on the molecular weight, and the contents of sulfated ester and glucuronic acid. However, the mechanism of action of these compounds regarding their antioxidant activity remains unclear. Further studies to study the chemical properties and antioxidant activities of products derived from radical process degradation were then carried out.

Two low molecular weight fucoidan fractions were prepared by radical process degradation from a crude polysaccharide. This method gave high yields and good reproducibility. Chemical analysis showed that the low molecular weight fractions produced by radical process degradation were quite different from those produced by acid hydrolysis. LM2 (Mp 8.0 kDa) was enriched with fucose and sulfated ester, while containing a small amount of glucuronic acid. This result was consistent with the studies of Nardella et al. (1996) and Chevolot et al. (1999). As reported by Nardella, electrophoresis of fucoidan on cellulose acetate membrane showed that radical process degraded mainly the ascopyllan-like species rich in glucuronic acid, while the fraction rich in sulfated L-fucose remained unchanged.

In our experiment, highly sulfated fractions with low molecular weight exhibited little scavenging effect on superoxide and hydroxyl radicals. In particular, the LM2 fraction prepared by radical process degradation had no scavenging effect at all. This result further confirmed the finding that the fraction rich in sulfated L-fucose was hard to degrade by oxygen radical and then had no capacity to scavenge oxygen radical. Nevertheless, fraction LM1 with little glucuronic acid still retained an apparent scavenging effect on superoxide radical. The neutral sugar composition showed that it was a sulfated oligosaccharide, containing mainly galactose and mannose. In light of the results presented here, the ascophyllan-like fraction rich in glucuronic acid and the fraction rich in galactose and mannose are responsible for the scavenging activity on oxygen free radical. Oxygen free radicals reacted most easily with these fractions, cleaving the glycosidic linkages and oxidizing glucuronic acid. As a result, the oxygen radicals were then scavenged by reduction.

As the polysacchride molecule is too large to use as a drug, low molecular weight fucoidan fractions were prepared by acid hydrolysis and radical cleavage. Such low molecular weight polysaccharides, some of which retained antioxidant properties, may be useful as drugs.

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