Food Chemistry 111 (2008) 503–507

Contents lists available at ScienceDirect

Food Chemistry

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

Analytical Methods

Determination of physicochemical properties of sulphated fucans from sporophyll of *Undaria pinnatifida* using light scattering technique

Chen Yang, Donghwa Chung, SangGuan You *

Department of Marine Food Science and Technology, Faculty of Marine Bioscience and Technology, Kangnung National University, Gangneung, Gangwon 210-702, Republic of Korea

ARTICLE INFO

Article history:
Received 6 March 2008
Received in revised form 26 March 2008
Accepted 31 March 2008

Keywords:
Fucoidan
Undaria pinnatifida
Weight average molecular weight
HPSEC-MALLS-RI
Microwave heating

ABSTRACT

The weight average molecular weight (M_w) of the intact sulphated fucans extracted with water from the sporophyll of *Undaria pinnatifida* without acid treatment was determined using the high performance size exclusion chromatography coupled to multiangle laser light scattering and refractive index detection (HPSEC-MALLS-RI) system. The effects of different heating conditions on the determination of M_w were investigated. The extracted intact fucoidans mostly consisted of carbohydrates (54.9%) and sulphate (41.5%) with monosaccharide composition of 78.8% fucose and 21.2% galactose. The M_w of the intact fucoidans was reduced from 23,600 to 5200 kDa when heated in boiling water for 1–15 min. Microwave heating for 30 s decreased the M_w of fucoidans to 2400 kDa despite no significant polymer degradation. The results indicate that the 30 s-microwave heating yielded a more accurate M_w value of the intact fucoidans than any other heat treatments used in this study.

1. Introduction

Fucoidan is a soluble and sulphated polysaccharide of complicated chemical structure, commonly found in brown seaweeds. The backbones of fucoidan from brown seaweeds, such as *Chorda filum*, *Fucus evanescens* and *Ecklonia stolonifera*, mainly consist of fucose molecules linearly linked by α-(1 → 3), α-(1 → 3)-(1 → 4) and α-(1 → 3)-(1 → 2), respectively, and also contain minor amounts of other sugars like xylose, galactose, mannose and glucuronic acid (Bilan et al., 2002; Chizhov et al., 1999; Duarte, Cardoso, Noseda, & Cerezo, 2001; Lee, Jin, Kim, & Ryu, 1995; Percival & Ross 1950). The type of glycosidic linkages of fucoidan monosaccharides in the backbones is closely related to the species of brown seaweeds (Bilan et al., 2005; Chizhov et al., 1999; Partankar, Oehninger, Barnett, Williams, & Clark, 1993). The backbones of fucoidan polymers from *C. filum* are substituted by single-fucoside unit mainly at C-2 position (Chizhov et al., 1999), whereas those from *Ascophyllum nodosum* and *Fucus serratus* L. have substitutions at C-2 or C-4 position with multi-fucoside units (Bilan, Grachev, Shashkov, Nifantiev, & Usov, 2006; Marais & Joseleau, 2001). Sulphate groups were also found at O-2 and/or O-4 positions of the fucoidan backbones from *Cladosiphon okamuranus* and *F. serratus* L. (Bilan et al., 2005; Nagaoka et al., 1999).

The molecular weight of fucoidan polymers has also been studied and values ranged from 21 to 1600 kDa, exhibiting significant variations (Lee, Jin, Kim, & Ryu, 1995; Li, Xue, Xue, Li, & Fu, 2006; Nagaoka et al., 1999; Rioux, Turgeon, & Beaulieu, 2007; Rupérez, Ahrazem, & Leal, 2002). This considerable difference in the molecular weight may be due to differences in not only the species of brown seaweeds but also extraction methods, which are often varied and complicated. In addition, acid treatments are generally used to precipitate and remove alginic acid during the extraction. This often causes difficulty in measuring the molecular weight of intact fucoidan due to the acid-induced depolymerization. Fucoidan polymers extracted from *Fucus vesiculosus* with HCl treatment at 35 and 70 °C showed the molecular weights of 1600 and 877 kDa, respectively (Rioux et al., 2007; Rupérez et al., 2002). These suggest that the molecular weight of fucoidan is significantly influenced by the acidic treatment conditions. To the best of our knowledge, the molecular weight of intact fucoidan polymers, which were obtained without acid treatments, has not yet been accurately measured. It is necessary to obtain the molecular weight of intact fucoidan polymers because the slight reductions and/or changes in the molecular weight of the polysaccharides could lead to substantial changes in their biological activities.

Traditionally, size exclusion chromatography (SEC), for which the calibration of SEC column is required, has been used in measuring the relative molecular weight values of polysaccharides. Recently, high performance SEC coupled to multiangle laser light scattering and refractive index detection (HPSEC-MALLS-RI), for which the column calibration is not necessary, has become popular for measuring the absolute values of weight average molecular weight (M_w) and the radius of gyration (R_g) of polysaccharides. Rioux et al. (2007) used the HPSEC-MALLS-RI system to determine the M_w of fucoidan polymers extracted with acid treatments from

* Corresponding author. Tel.: +82 33 640 2853; fax: +82 33 640 2340.
E-mail address: umyousg@kangnung.ac.kr (S. You).

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doi:10.1016/j.foodchem.2008.03.085

various species of brown seaweeds. However, the authors simply dissolved the extracted fucoidan polymers in NaCl solution without further heating or mechanical treatments. A simple dissolution is often not adequate for determining the accurate M_w of polysaccharides because the polymer chains can be aggregated by secondary interactions through hydrogen bondings (Fishman & Hoagland, 1994; Hanselmann, Burchard, Ehrat, & Widmer, 1996). Therefore, additional heating and/or mechanical treatments, such as autoclaving, microwaving, homogenization and sonication, have been applied to break the secondary interactions and thus to obtain a better dissolution of polysaccharides (Bello-Perez, Roger, Baud, & Colonna, 1998; You & Izydorczyk, 2002; You & Lim, 2000).

The objective of this study was to determine the M_w of fucoidan polymers extracted from the sporophyll of *Undaria pinnatifida* using the HPSEC-MALLS-RI system. The extraction was performed without acid treatment to obtain intact fucoidan polymers. Heating with boiling water or microwave was applied for different times to break up the secondary interactions between fucoidan chains in distilled water. The effect of different heating conditions on the determination of M_w was investigated in order to obtain the accurate M_w of fucoidan polymers.

2. Materials and methods

2.1. Materials

The dried sporophyll of brown seaweed (*U. pinnatifida*), originated from the coast of Youngdukgun, Kyoungbuk province, Korea, was purchased, milled using a blender, sieved (<0.5 mm), and then stored at −20 °C before analyses. All chemicals and reagents used were of analytical grade.

2.2. Isolation of fucoidan

The milled sample (20 g) was treated with 85% ethanol (EtOH, 1 L) with constant mechanical stirring (12 h) at room temperature to remove pigments and proteins, washed with acetone, centrifuged at 1800*g* for 10 min, and then dried at room temperature. The dried biomass (5 g) was extracted with distilled water (100 mL) at 65 °C with stirring for 1 h. The extraction was conducted twice, and the extracts were combined. The extracts were centrifuged at 18,500*g* for 10 min, and the supernatant was collected. The supernatant was mixed with 1% $CaCl_2$ and the solution was kept at 4 °C overnight to precipitate alginic acid. The solution was centrifuged at 18,500*g* for 10 min, and the supernatant was collected. EtOH (99%) was added into the supernatant to obtain the final EtOH concentration of 30%, and then the solution was placed at 4 °C for 4 h. After centrifugation at 18,500*g* for 10 min, more EtOH (150 mL) was added to the collected supernatant to obtain the final EtOH concentration of 70%, and the solution was placed at 4 °C overnight. The intact fucoidan was obtained by the filtration of the solution with a nylon membrane (0.45 μm pore size, Whatman International Ltd., Maidstone, UK), followed by washing with EtOH (99%) and acetone, and then dried at room temperature overnight. The yield of fucoidan was calculated based on the dried biomass obtained after the treatment of the milled sample with 85% EtOH.

2.3. Determination of sulphate, total carbohydrate, and protein contents

The sulphate content of fucoidan polymers was determined by the $BaCl_2$ gelatin method using K_2SO_4 as a standard after hydrolyzing the polysaccharide (2.5 mg) in 6 mL of 0.5 M HCl (Dodgson & Price 1962). The contents of total carbohydrate and proteins were determined by the phenol–sulfuric acid method using fucose as a standard (Dubois, Gilles, Hamilton, Rebers, & Smith 1956), and by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) using DC Protein assay kit (Bio-Rad, Hercules, CA, USA), respectively.

2.4. Determination of monosaccharide composition

Quantitative determination of the monosaccharide composition of fucoidan polymers was performed using a HPLC system, consisting of a pump (Waters 510, Waters, Milford, MA, USA), an injection valve (Model 7010, Rheodyne, Rohnert Park, CA, USA) with a 20 L sample loop, a column (Carbohydrate analysis column, 4.6 × 250 mm, Waters, Milford, MA, USA) and a RI detector (Waters 410), after 90 min-hydrolysis of the polysaccharide (6.3 mg) in 300 μL of 2 M trifluoroacetic acid (TFA) at 120 °C. After removing TFA in the sample solution with a dried stream of nitrogen, the hydrolyzed polysaccharide sample was injected into the HPLC system. The mixture of acetonitrile and water (90:10) was used as a mobile phase at a flow rate of 2 mL/min.

2.5. Determination of weight average molecular weight

To measure the weight average molecular weight (M_w) as well as radius of gyration (R_g) of fucoidan polymers, 20 mg of fucoidan was dissolved in 2 mL of distilled water. The fucoidan solution was heated in boiling water for 1, 5, 10 or 15 min, or in a microwave oven (RE-552W, SamSung, Seoul, Korea) using a microwave bomb (#4872, Parr Instrument Co., Moline, IL, USA) for 30, 60, 90 or 120 s. The heated fucoidan solution was filtered through a cellulose acetate membrane (3.0 μm pore size, Whatman International Ltd.) before injection to a high performance size exclusion chromatography coupled to multiangle laser light scattering and refractive index detection (HPSEC-MALLS-RI) system.

The HPSEC-MALLS-RI system consisted of a pump (model 321, Gilson, Middleton, WI, USA), an injector valve with a 100 μL sample loop (model 7072, Rheodyne), a guard column (TSK PWxl, TosoBiosep, Mongomeryville, PA, USA), three SEC columns (TSK G5000 PW, 7.5 × 600 mm; TSK G3000 PWxl, 7.8 × 300 mm; TSK G2500 PWxl, 7.8 × 300 mm; TosoBiosep, Mongomeryville, PA, USA), a multiangle laser light scattering detector (HELEOS, Wyatt Technology Corp, Santa Barbara, CA, USA) and a refractive index detector (RI-150, Thermo Electron Corp., Yokohama, Japan). The SEC columns used in this study are capable of separating the polymers having the M_w below 1000 kDa. In order to reduce the ionic interactions between fucoidan polymers, the aqueous solution of 0.15 M $NaNO_3$ and 0.02% NaN_3 was used as a mobile phase at a flow rate of 0.4 mL/min. The normalization of MALLS detector and the determination of volume delay between MALLS and RI detectors were carried out with bovine serum albumin (BSA). The d*n*/d*c* value was set to 0.129 for fucoidan polymers (Rioux et al., 2007). The calculations of M_w and R_g were carried out using ASTRA 5.3 software (Wyatt Technology Corp.). Molecular conformation of fucoidan polymers was examined using the following relationship: $R_g = KM_w^{\alpha}$, where K is an optical constant and α is the slope of the plot between $\log R_g$ versus $\log M_w$ (sphere if $\alpha < 0.3$; random coil if $0.3 \leqslant \alpha < 0.5$; rod if $\alpha \geqslant 0.5$) according to Roger, Bello-Perez, and Colonna (1999).

3. Results and discussion

3.1. Compositional analysis

The yield (8.8%) of the fucoidan polymers extracted in this study was close to the value (9.4%) reported by Nishide, Anzai, and Uchida (1987) for the sporophyll of the same species, which was quite

larger than the values (1.1–4.8%) reported for other brown seaweeds (Lee et al., 1995; Rioux et al., 2007; Rupérez et al., 2002; Yamamoto, Takahashi, Tamura, Maruyama, & Mori, 1984). The extracted fucoidan polymers consisted of mostly carbohydrates (54.9%) and sulphates (41.5%) with a small amount of proteins (2.8%), in a good agreement with the constituents of fucoidan polymers from *F. evanescens* (Bilan et al., 2002). It was reported that the fucoidan polymers from other sources had comparable amounts (42–66%) of carbohydrates with smaller amounts (11.5–34.2%) of sulphates, and their protein contents varied from 0% to 12.4% (Bilan et al., 2006; Lee et al., 1995; Marais & Joseleau, 2001; Ponce, Pujol, Damonte, Flores, Stortz, 2003; Rioux et al., 2007; Rupérez et al., 2002). Monosaccharide composition analysis showed that fucose (78.8%) was the major sugar in the extracted fucoidans and considerable amount of galactose (21.2%) was also contained. Other monosaccharides such as glucose, xylose and mannose were not detected in the current study. Fucoidan polymers in the literatures showed significant differences in their monosaccharide compositions. In general, the chemical composition of fucoidan polymers is significantly different depending on species, anatomical regions, growing conditions, extraction procedures and analytical methods (Bilan et al., 2002, 2004, 2006; Chizhov et al., 1999; Duarte et al., 2001; Lee et al., 1995; Marais & Joseleau, 2001; Ponce et al., 2003).

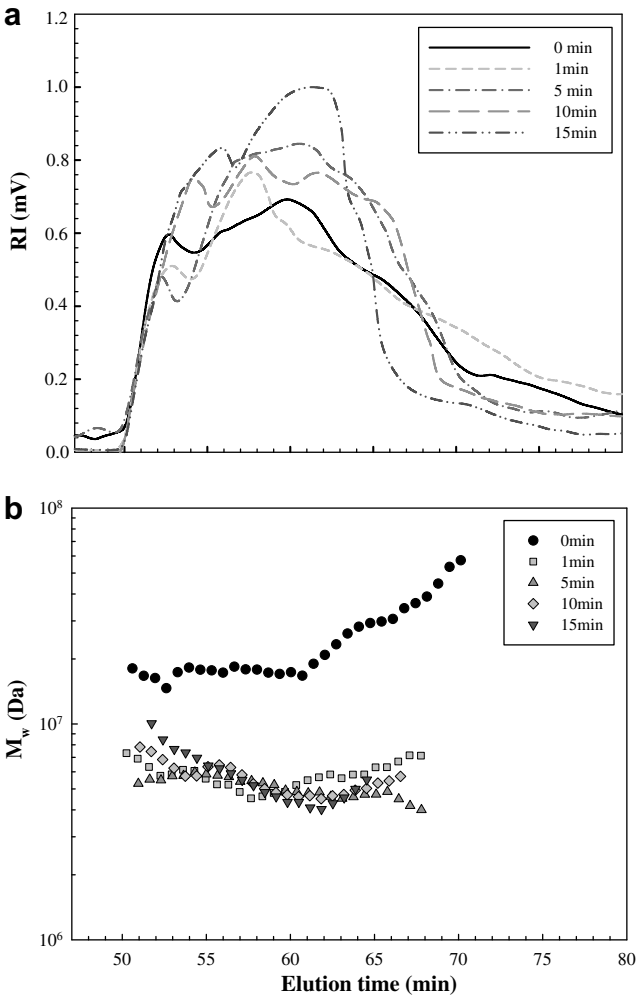

Fig. 1. The HPSEC chromatograms and the weight average molecular weight (M_w) of fucoidans dissolved in distilled water heated by boiling water at different times (0, 1, 5, 10 or 15 min).

3.2. Effect of boiling water heating on molecular weight

The HPSEC chromatograms of fucoidan polymers, which were dissolved in distilled water and heated by boiling water for various times, are shown in Fig. 1a. Unheated fucoidan polymers (0 min) were eluted from the SEC columns between elution times of 50 and 76 min and showed only one broad peak with some shoulders, indicating that they had a continuous molecular weight distribution. The fucoidans heated by boiling water showed not significantly different HPSEC chromatograms. This suggests that the molecular weight of fucoidan polymers was not significantly affected by the heat treatments and considerable polymer degradation did not occur. However, the weight average molecular weight (M_w) of each HPSEC fraction, obtained by multiangle laser light scattering (MALLS) technique that can provide the absolute molecular weight of polymers, showed that the values of M_w drastically decreased after 1 min of heat treatment (Fig. 1b). This may be because of the disruption of the secondary interactions between fucoidan chains by the heat treatment. With further increase of heating time up to 15 min, only slight reductions in the M_w were observed for all HPSEC fractions. The M_w calculated from entire HPSEC fractions also markedly decreased from 23,600 to 5900 kDa when the heating was applied for 1 min and remained almost the same with further increase of heating time up to 15 min (Table 1). In general, SEC separates polymers based on their size, therefore, larger molecules, commonly having higher M_w values, are eluted first from the column, followed by smaller molecules. It was shown from Fig. 1b that such trend was weakly observed only for fucoidan polymers heated for 10 and 15 min. For the fucoidan polymers heated for 1 and 5 min, the values of M_w were similar regardless of elution time and those of unheated fucoidans even increased with elution time after 62 min. This is probably because the size of fucoidan polymers, primarily affecting the elution time, was not directly related to their molecular weight due to the compact polymer aggregation through the secondary interactions. In the study of hyaluronic acids using the HPSEC-MALLS-RI system, it was also shown that the molecules of different M_w were eluted at the same elution time, indicating no direct relationship between the size and the molecular weight of the polymers (Soltes, Mendichi, Lath, Mach, & Bakos, 2002). Radius of gyration (R_g) was also calculated from entire HPSEC fractions in order to estimate the approximate size of fucoidan polymers (Table 1). The result showed that the value of R_g drastically decreased from 163.9 to

Table 1
The weight average molecular weight (M_w) and radius of gyration (R_g) of fucoidans dissolved in distilled water by heating in boiling water and a microwave at different times

Heating in boiling water			Heating in a microwave		
Heating time (min)	M_w (kDa)	R_g (nm)	Heating time (s)	M_w (kDa)	R_g (nm)
0	23,600 ± 1.2	163.9 ± 8.2	0	23,600 ± 1.2	163.9 ± 8.2
1	5900 ± 0.3	103.5 ± 5.2	30	2400 ± 0.1	74.8 ± 3.7
5	5200 ± 0.3	101.2 ± 5.1	60	2100 ± 0.1	65.2 ± 3.3
10	5500 ± 0.3	94.0 ± 4.7	90	1900 ± 0.1	64.5 ± 3.2
15	5500 ± 0.3	100.0 ± 5.1	120	500 ± 0.03	47.3 ± 2.4

103.5 nm after 1 min-heating, which might be also due to the disruption of the secondary polymer interactions, and did not significantly change with further increase of heating time. The above results suggest that the boiling water-heating applied in the current study significantly improved the dissolution of fucoidan polymers in distilled water without considerable polymer degradation and enabled to obtain more accurate M_w of the intact fucoidan polymers extracted without acid treatment.

3.3. Effect of microwave heating on molecular weight

The HPSEC chromatograms of fucoidan polymers treated by microwave heating at different times from 0 to 120 s are shown in Fig. 2a. Microwave heating for 30 s did not cause a substantial change in the elution time of major fucoidan fractions. However, this elution time slightly increased from about 57 to 62 min with the increase of heating time from 30 to 90 s, suggesting some degradation of fucoidan polymers by microwave heating. When microwave was applied for 120 s, two distinct peaks were observed at about 61 and 68 min in the HPSEC chromatogram, indicating considerable degradation of fucoidan polymers. It was shown from Fig. 2b that the M_w of each HPSEC fraction significantly decreased after 30 s-microwave heating despite no substantial change in the elution time of major fractions. This is probably because the disruption of the secondary interactions between fucoidan polymers, rather than polymeric degradation, mainly occurred during the heat treatment. Navarro, Flores, and Stortz (2007) also showed that microwave heating for 60 s did not cause considerable degradation of fucoidan polymers. A slight decrease of M_w was observed for all HPSEC fractions with the increase of heating time from 30 to 90 s. A more significant M_w decrease was observed for the HPSEC fractions obtained at the elution time larger than about 65 min when heated for 120 s. This could be due to the occurrence of polymer degradation as suggested from the HPSEC chromatogram (Fig. 2a). Table 1 shows that the M_w calculated from entire HPSEC fractions also markedly decreased from 23,600 to 2400 kDa after 30 s-microwave heating. A slight decrease of the M_w to 1900 kDa was observed with further increase of heating time up to 90 s and the 120 s-microwave heating drastically decreased the M_w to 500 kDa. It was shown from Fig. 2b that for the microwave-heated polymers, higher M_w fractions eluted earlier by following the general separating trend of SEC described above, indicating that polymer molecules of higher M_w were larger than lower M_w polymers. This may be because the secondary polymer interactions were mostly disrupted by any of the microwave heat treatments used in this study. The value of R_g calculated from entire HPSEC fractions drastically decreased from 163.9 to 74.8 nm after 30 s-microwave heating. This might also be due to the disruption of the secondary polymer interactions. A continuous decrease of the R_g up to 47.3 nm was observed with the increase of heating time due to the degradation of fucoidans (Table 1). The above results indicate that only the 30 s-microwave heating was able to significantly enhance the dissolution of fucoidan polymers in distilled water without notable polymer degradation and provide more accurate M_w of the intact fucoidan polymers obtained in this study. However, the M_w (2400 kDa) obtained after 30 s-microwave heating (Table 1) was still higher than the highest M_w (1600 kDa) reported in the literature (Rioux et al., 2007). This may be because the intact fucoidan polymers in the current study were extracted from different seaweed species without an acid treatment that can cause polymeric degradation.

When comparing the M_w values obtained from boiling water-heating and 30 s-microwave heating (Table 1), the value (2400 kDa) from 30 s-microwave heating was much lower than the values (5200–5900 kDa) from boiling water-heating. This may be because of a better dissolution, not the degradation, of fucoidan polymers in distilled water by 30 s-microwave heating. It was also shown in the plot of M_w versus R_g from Fig. 3 that the

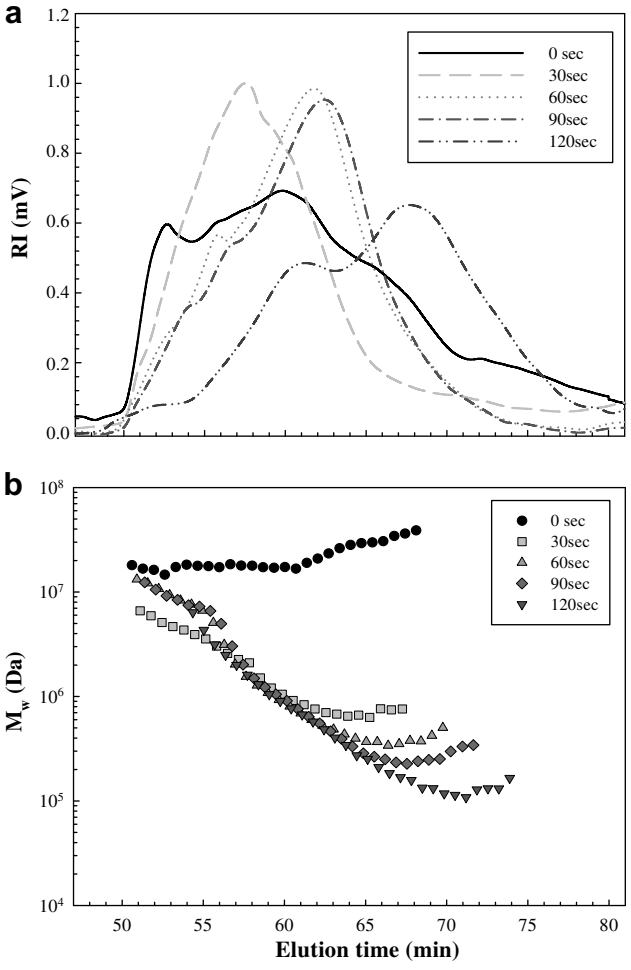

Fig. 2. The HPSEC chromatograms and the weight average molecular weight (M_w) of fucoidans dissolved in distilled water heated by a microwave at different times (0, 30, 60, 90 or 120 s).

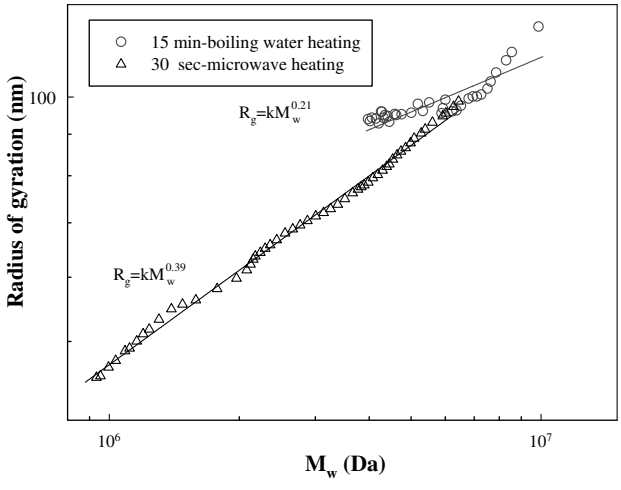

Fig. 3. The plot of weight average molecular weight (M_w) vs radius of gyration (R_g) of fucoidan polymers treated by boiling water (15 min) and microwave (30 s) heatings.

fucoidan polymers heated by boiling water for 15 min had the conformation of compact sphere ($\alpha = 0.21$). It appears that the secondary interactions were not sufficiently disrupted by the boiling water heating for 15 min, resulting in a compact sphere conformation of fucoidan polymers. On the other hand, the polymers heated by microwave for 30 s were random coil ($\alpha = 0.39$), suggesting that the secondary interactions between polymer chains had been mostly destroyed by the 30 s-microwave heating. The above results, therefore, suggest that boiling water heating up to 15 min may not be a suitable method of fucoidan dissolution and microwave heating for 30 s could be employed to obtain accurate molecular weight of intact fucoidan polymers. This microwave heating condition is close to the optimum condition (35 s microwave heating) reported by Bello-Perez et al. (1998) for the dissolution of α-glucans without chain degradation.

4. Conclusions

The HPSEC-MALLS-RI analysis was used to investigate the effects of different heating conditions on the determination of M_w of the intact fucoidans extracted with water from the sporophyll of *U. pinnatifida* without acid treatment. Without any heat treatment, the M_w of the intact fucoidans was measured to be 23,600 kDa. The polymer aggregation caused by the secondary interactions between polymer chains might be responsible for this unreasonably high M_w value. The boiling water-heating from 1 to 15 min could improve the dissolution of fucoidan polymers in distilled water by disrupting some of the secondary interactions without considerable polymer degradation, and thus yielded more accurate M_w values (5200–5900 kDa). The microwave heating was found to be more effective in improving polymer dissolution. The microwave heating for only 30 s could significantly enhance the polymer dissolution without notable polymer degradation, and yielded the most accurate M_w value (2400 kDa) among the eight heat treatments used in this study.

Acknowledgements

This work was supported by Grant No. RTI05-01-02 from the Provincial Technology Innovation Program of the Ministry of knowledge economy. Yang was a recipient of a graduate fellowship provided by the Brain Korea (BK21) program sponsored by the Ministry of Education and Human Resources Development, Republic of Korea.

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