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Preparation and Intestinal Immunostimulating Activity of Low Molecular Weight Alginate from Saccharina (Laminaria) Species in Japan

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Abstract: We studied the structure of alginates extracted from five commercial Saccharina (Laminaria) species including three varieties (eight samples in all) harvested in Hokkaido, Japan. The algae used were Saccharina japonica, S. japonica var. diabolica, S. japonica var. ochotensis, S. japonica var. religiosa, S. longissima, S. coriacea, S. angustata, and S. sculpura (Kjellmaniella crassiforia). These alginates have molar fractions of mannuronic acid (FM) ranging from 0.68 to 0.76 and weight average molecular weights (Mw) ranging from 511,000 to 616,000. Alginate samples from both S. angustata (FM = 0.76) and S. longissima (FM = 0.68) showed intestinal immunological activity through Peyer’s patch cells of C3H/HeJ mice. Low molecular weight S. angustata alginate (FM = 0.75, Mw = 70,000) degraded using a wet pulverizing system showed higher activity than the native one.

Key words: alginate, Saccharina (Laminaria) japonica, mannuronic acid content, intestinal immunological activity, wet pulverizing system

INTRODUCTION

Seaweeds have traditionally been used as food in Asian countries, and more recently as health food in the west. Among the edible brown seaweeds, genera Saccharina (Laminaria), Undaria, and Hizikia are most popular. In Japan, twelve species of Saccharina are grown along the northern coast (mainly in Hokkaido island and northern Tohoku) and nine of them are used for food.1) In particular, the following eight seaweeds (five species including three varieties), which are called kombu in Japanese, are the most popular edible Saccharina: S. japonica, S. japonica var. diabolica, S. japonica var. ochotensis, S. japonica var. religiosa, S. longissima, S. coriacea, S. angustata, and S. sculpura (Kjellmaniella crassiforia).

Alginate, a linear polysaccharide consisting of 1,4-linked β-D-mannuronic acid and α-L-guluronic acid residues, is one of the main components of brown algae. The mannuronic acid content of alginate varies with the species, age, and tissue of seaweed2) and season.3) Previously we reported on the effect of both mannuronic acid content and molecular weight on intestinal immunological activity through Peyer’s patch cells of C3H/HeJ mice.4) Over most of the range of weight average molecular weight, Mw (30,000–690,000), alginates with high mannuronic acid content (high-M alginates, Fm 0.69–0.86) showed immunological activity, but alginates with Fm lower than 0.31 did not. For high-M alginates with Fm = 0.78 prepared from S. japonica, those with Mw lower than 200,000 showed the highest activity. Although there have been some reports about the mannuronic acid content of alginate extracted from S. japonica,4) there have been no studies about the structure and effect on intestinal immunological activity of alginates extracted from other commercial edible Saccharina. In this paper we will show that alginates extracted from our commercial Saccharina seaweed samples harvested in Hokkaido are high-M type and the activity of high-M alginate from Saccharina seaweed with Mw > 200,000 can be improved by reducing their molecular weight.

Ultrasonic depolymerization of schizophyllan5) and the other polysaccharides has been known. But this method is not suited for industrial depolymerization of a large bulk of polysaccharide, because of its low efficiency and contamination from horn of sonicator. The wet pulverizing system is used to prepare nanofibers of cellulose6) and chitosan.7) This method overcomes the disadvantages, such as low efficiency and contamination, as seen in the ultrasonic depolymerization method. It is known that polysaccharide is depolymerized by forcing a solution in a solvent through a capillary at a high shear rate.8) But the wet pulverizing system has not applied to depolymerize the polysaccharide.

The aim of this study is to find a way to process large amounts of high-activity (immunostimulating) alginate using a wet pulverizing system. First, we characterized the structure and molecular weight of alginates extracted from eight edible Saccharina seaweeds using NMR and SEC-MALLS measurements. Then, to improve their immunostimulating activity, we
MATERIALS AND METHODS

Materials. Eight dried algae samples, *S. japonica*, *S. japonica* var. *diabolica*, *S. japonica* var. *ochotensis*, *S. japonica* var. *religiosa*, *S. longissima*, *S. coriacea*, *S. angustata*, and *S. sculpera* (*Kjellmaniella crassiforia*), were provided by Fujicco Co., Ltd. (Kobe, Japan). All samples are commercially used as human food and were harvested in the northern island of Japan, Hokkaido in July and August. Alginate extraction was done according to a previously reported method.2,11) The dried seaweed was ground and passed through a 2 mm-mesh. The seaweed material (15 g) was swollen in 75 mL of 5 % formaldehyde and suspended in 750 mL of 0.2 M HCl. The suspension was filtered, and the residue was washed with distilled water and suspended in 1.5 L of 0.02 M NaOH while keeping the pH above 7. After filtering the suspension, NaCl was added to the supernatant to a final concentration of 1 %, and alginate was precipitated by adding an equal volume of ethanol. The precipitant was washed with ethanol and ether, and dried at room temperature.

Nuclear magnetic resonance (NMR) spectroscopy. We measured the proton spectra at 80 °C using a JNM-AL400 spectrometer (JEOL Ltd., Tokyo, Japan). Before NMR measurement, we reduced the viscosity of the high molecular weight alginites by mild acid hydrolysis according to the method of Ertesvåg and Skjåk-Bræk.12) The alginate hydrolyzate was neutralized, freeze-dried and dissolved in D2O. Five microliters of 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt were used as the internal standard for the chemical shift. 20 µL of 0.3 M triethylene tetra-amine hexa-acetate (TTHA) was added to chelate the remaining Ca2+.

Size-exclusion chromatography-multi angle laser light scattering (SEC-MALLS). We made SEC-MALLS measurements on an HPLC system equipped with three straight connected columns: TSKgel G6000PWXL, G5000PWXL, and G4000PWXL (7.5 mm i.d. × 600 mm, Tosoh Corporation, Tokyo, Japan). The column outlet was connected to a Dawn Heleos-II multiangle laser light scattering spectrometer (Wyatt Technology Corp., Santa Barbara, USA) (λ = 658 nm) which was connected to an Optilab rEX differential refractometer (Wyatt Technology Corp.). The mobile phase was 0.05 M Na2SO4/0.01 M EDTA (pH 6). The flow rate was 0.7 mL/min. The injection volume was 100 µL, and the sample concentration was 0.2 or 0.4 % for appropriate light scattering intensity. The sample solution was filtered with a 0.22 µm filter before injection. The value of the refractive index increment (dn/dc) for the alginate was 0.150 mL/g, according to a previous report.13) Data from the light scattering and the differential refractometers were collected and processed using Astra (v. 5.3.4.14) software (Wyatt Technology Corp.).

Preparation of low molecular weight alginate. Alginate from *S. angustata* was degraded using a wet pulverizing system (Star Burst mini HJP-25001H, Sugino Machine Ltd., Japan). The nozzle diameter was 100 µm. Repeatedly forcing the sample solution through a nozzle at a high shear rate depolymerized the polysaccharides. First, the effects of charge pressure and the number of pulverizing cycles on molecular weight were examined. 20 mL of 0.5 % alginate aqueous solution was pulverized 30 times for each chamber pressure of 50, 150, and 245 MPa (Exp. 1). Intermediate aliquots were taken and molecular weights measured by SEC-MALLS.

Based on the results of Exp. 1, we prepared a sample with *Mw < 100,000* by pulverizing 200 mL of 0.5 % alginate aqueous solution for 5 cycles with a chamber pressure of 245 MPa (Exp. 2).

Intestinal immunological activity. The intestinal immunological activity of three alginate samples (two native alginites from *S. longissimi* and *S. angustata* and degraded *S. angustata* alginate (Sa-d6)) was measured by measuring the enhanced proliferation activity of bone marrow cells according to a method described previously.1,3,4) In brief, suspensions (2.0 × 10⁵ cells/mL in RPMI 1640 medium, 2.7 mL) of Peyer’s patch cells from the small intestine of C3H/HeJ mice (female, 8 weeks old) were cultured with sample solutions (0.3 % alginate solution, 200 µL) and sterilized water (100 µL), placed in a Petri dish and cultured for five days at 37 °C in a humidified atmosphere of 5 % CO2:95 % air. Sterilized water was used as a control instead of adding 0.3 % alginate solution. The resulting culture supernatants (50 µL) were further cultured with 100 µL of bone marrow cell suspension (5.0 × 10⁵ and 3.0 × 10⁵ cells/mL) and 50 µL of normal horse serum in a 96-well plate for six days at 37 °C in a humidified atmosphere of 5 % CO2:95 % air. Proliferation of bone marrow cells was measured by Alamar Blue reduction assay as described previously.4,5) The statistical significance of differences between samples and control were tested by the Tukey-Kramer test.

RESULTS AND DISCUSSION

Alginates preparation. The yield, molecular weight and mannuronic acid content of alginites extracted from the eight seaweed samples are listed in Table 1. Yields ranged from 18.5 % (*S. japonica* var. *diabolica*) to 32.7 % (*S. sculpera*). All alginate samples showed high mannuronic acid content ranging from 0.68 (S. longissima) to 0.76 (*S. angustata*). All samples showed molecular weights ranging from 511,000 to 616,000.

The mannuronic acid content of alginate varies with the species of seaweed.2,1) *S. japonica* has been used as a source for production of low G type alginate in industrial markets.6) Honya et al.3) also report that alginate from *S. japonica* contains more mannuronic acid than guluronic acid. Our alginate samples extracted from the eight *Saccharina* seaweeds were low G type as in the case in *S. japonica*. There was little variation in the Fm of these alginates.

Alginate from *S. angustata* had the highest mannuronic acid content among the samples examined. According to our previous study, both Fm and molecular weight affect immunological activity. For high-M alginates, those with *Mw* lower than 200,000 show the highest activity. Thus the immunological activity of *S. angustata* alginate should be enhanced by lowering its molecular weight. We prepared low-molecular weight samples of *S. angustata* alginate using a wet pulverizing system.
Preparation of low molecular weight alginate.

Figure 1 shows a SEC-MALLS chromatogram of _S. angustata_ alginates which were degraded using a wet pulverizing system with a chamber pressure of 245 MPa in Exp. 1. Table 2 lists the _M_w_ and the ratio of _M_w_ and _M_n_ (_M_w/_M_n_) (where _M_n_ is the number average molecular weight) which is the index for molecular weight distribution of the degraded samples determined by SEC-MALLS. The samples pulverized for 1, 5, 10, 20, and 30 cycles (denoted as ’number of pulverizing cycles’) are designated Sa-d1, Sa-d2, Sa-d3, Sa-d4, and Sa-d5, respectively. _M_w_ decreased with increasing number of cycles. The molecular weight distributions of degraded samples were narrower than that of the native sample. Figure 2 shows the effect of pressure and number of pulverizing cycles on the molecular weight of the alginate. Higher pressure made lower molecular weight alginate. The lowering rate of molecular weight gradually decreased, and after 20 cycles, it was almost flat. Among the pressures examined, 245 MPa yielded samples with _M_w_ under 100,000 with the fewest pulverizing cycles. Based on this result, 200 mL of alginate aqueous solution was pulverized for 5 cycles at a chamber pressure of 245 MPa to prepare a large amount of low molecular weight sample (Exp. 2). The _M_w_ of the resulting sample, designated as Sa-d6, was 70,000 (Table 2). Figure 3 shows the 1H NMR spectrum of Sa-d6. Peaks were assigned according to a previous report. The spectrum was similar to that of the native one, and the _F_M_ was almost unchanged (_F_M_ = 0.75) from that of native one (_F_M_ = 0.76). This shows that the wet pulverizing treatment can depolymerize alginate without changing _F_M_.

This study shows that a wet pulverizing system is also useful to reduce the molecular weight of alginates.

### Table 1. Mannuronic acid content (_F_M_), weight average molecular weight (_M_w_), polydispersity index for molecular weight distribution (_M_w/_M_n_), and yield (dry basis) of extracted from eight _Saccharina_ seaweed samples.

| Samples           | _F_M_ | _M_w_  | _M_w/_M_n_ | Yield (%) per dried algal sample |
|-------------------|-------|--------|-------------|---------------------------------|
| _S. japonica_     | 0.73  | 599,000| 1.19        | 21.3                             |
| _S. japonica_ var. diabolica_ | 0.72  | 511,000| 1.19        | 18.5                             |
| _S. japonica_ var. religiosa_ | 0.72  | 614,000| 1.32        | 26.9                             |
| _S. japonica_ var. ochotensis_ | 0.70  | 575,000| 1.30        | 29.2                             |
| _S. angustata_    | 0.76  | 584,000| 1.22        | 26.5                             |
| _S. coriacea_     | 0.73  | 575,000| 1.22        | 22.2                             |
| _S. longissima_   | 0.68  | 568,000| 1.26        | 24.7                             |
| _S. sculpera_ (K. crassiforia) | 0.72  | 616,000| 1.23        | 32.7                             |

Fig. 1. SEC-MALLS chromatogram of native and degraded _S. angustata_ alginate.

Samples Sa-d1, Sa-d2, Sa-d3, Sa-d4, and Sa-d5 were degraded by a wet pulverizing system at a chamber pressure of 245 MPa (Exp. 1). Numbers in the figure denote the number of pulverizing cycles.

Fig. 2. Decrease in molecular weight of _S. angustata_ alginate degraded by a wet pulverizing system at chamber pressures of 50, 150, and 245 MPa (Exp. 1).
Table 2. Change in weight average molecular weight ($M_w$), polydispersity index for molecular weight distribution ($M_w/M_n$) of $S. angustata$ alginate degraded by a wet pulverizing system at a chamber pressure of 245 MPa.

| Exp. | Preparation volume (mL) | Degraded alginate | Number of pulverizing cycles | $M_w$  | $M_w/M_n$ |
|------|-------------------------|-------------------|-----------------------------|--------|-----------|
| 1    | 20                      | Sa-d1             | 1                           | 174,000| 1.24      |
|      |                         | Sa-d2             | 5                           | 87,800 | 1.11      |
|      |                         | Sa-d3             | 10                          | 71,500 | 1.09      |
|      |                         | Sa-d4             | 20                          | 57,000 | 1.09      |
|      |                         | Sa-d5             | 30                          | 52,900 | 1.11      |
| 2    | 200                     | Sa-d6             | 5                           | 70,000 | 1.11      |

Fig. 3. $^1$H NMR spectra of native $S. angustata$ alginate and its degraded sample, Sa-d6.

Fig. 4. Bone marrow cell proliferating activity stimulated by the culture medium supernatant of Peyer’s patch cells cultured with 200 µg/mL alginate.

The initial numbers of bone marrow cells were $7.5 \times 10^6$ (■) and $4.1 \times 10^5$ (□) cells/mL. a and b indicate the statistical significance of differences vs. control ($^a p < 0.01$, $^b p < 0.05$) and vs. $S. angustata$ ($^a p < 0.01$) respectively by the Tukey-Kramer test for each group (■ and □).
**Intestinal immunological activity.**

The intestinal immunological activities of native alginates from *S. longissima*, *S. angustata*, and degraded *S. angustata* (Sa-d6) were compared by measuring the bone marrow cell proliferating activity stimulated by Peyer’s patch cell medium cultured with the alginates (Fig. 4). Alginates from *S. longissima* and *S. angustata* increased bone marrow cell proliferation activity, but the increase for the Sa-d6 alginate was significantly greater still. Thus the low molecular weight sample had higher activity than the native one. The active alginate is thought to enhance the proliferation of bone marrow cells by stimulating the production of cytokines such as IL-6 and GM-CSF from immune cells in Peyer’s patches. It doesn’t stimulate proliferation of bone marrow cells directly (data not shown).14,15)

We showed that the alginates extracted from commercial *Saccharina* seaweed are high-M type, and have intestinal immunological activity. Furthermore, *S. angustata* alginate was successfully processed into low molecular weight samples using a wet pulverizing system, enhancing its activity. So if high molecular weight alginates from kombu are to be used for their intestinal immunological activity, they should probably be processed to reduce their molecular weight.

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