Macrophage Stimulation Activity of the Polysaccharide Fraction from a Marine Alga (Porphyra yezoensis): Structure–Function Relationships and Improved Solubility

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The polysaccharide fraction from Porphyra yezoensis (PASF) has already been shown to stimulate murine phagocytic functions in vivo and in vitro [Y. Yoshizawa et al., Biosci. Biotechn. Biochem., 57, 1862–1866 (1993)]. In this study, various treatments were applied to PASF to assess its structure–function relationships. Desulfation of PASF decreased in vitro macrophage-stimulation activity, while further sulfation of PASF did not change the activity. Among 7 fractions obtained by anion-exchange chromatography of PASF, stronger activity was found in the fractions having a lower or higher sulfate content than in those having a medium sulfate content. Digests of PASF with β-agarase showed higher activity and solubility, and lower viscosity, than undigested PASF. These results indicate that the sulfate groups in PASF, probably porphyran, contributed to the macrophage stimulating activity, although a larger number of sulfate groups did not always cause stronger activity.

Sulfated polysaccharides have been shown to possess several biological activities, heparin and some others having clearance of lipemia and anticoagulant activity.¹,² It has been reported that semisynthesized sulfated polysaccharides such as dextran sulfate, pentosan polysulfate,³ lentigin sulfate,⁴ and ribofuranran sulfate⁵ inhibited HIV (human immunodeficiency virus)-induced cytopathic effects in vitro. Hatanaka et al. have shown that the anticoagulant and anti-HIV activities of ribofuranran sulfate depended on the degree of sulfation.⁵ Brown and red algae contain a large amount of such sulfated polysaccharides as fucan sulfate, carrageenan, and galactan sulfate, fucan sulfate from brown algae particularly being shown to have anticoagulant activity⁶ and antitumor activity.⁷ These facts suggest that the sulfate groups in polysaccharides may play a role in these biological activities, although structure–function relationships have not been well characterized.

We have previously reported that two discrete immunomodulating fractions that had been extracted from the edible red alga Porphyra yezoensis stimulated murine phagocyte functions in vitro and in vivo.⁸ One was the Porphyra water-soluble fraction (PWSF), which had been extracted with hot water from the whole body of the alga and precipitated with ethanol, and the other was the Porphyra acid-soluble fraction (PASF), which had been extracted with acid from the residue of the hot-water extract and precipitated with ethanol. These components may be available for developing physiologically functional foods endowed with immunopotentiating activity towards humans and domestic animals.

Porphyran is known to be predominantly included in Porphyra and to have a basic disaccharide repeating unit of 3-O-(3,6-anhydro-β-D-galactopyranosyl)-β-D-galactopyranose, which could be substituted with a methyl group (C-6 of the D unit) or with a sulfate group (C-6 of the L unit).⁹ The large contents of 3,6-anhydrogalactose (3,6-AG) and sulfate groups in both PWSF and PASF indicated that their major constituent was porphyran. However, no report has been presented yet about murine macrophage stimulation activity by porphyran. In this study, we examined structure–function relationships by treating PASF with further sulfation, desulfation, fractionation, and digestion, and we discuss characteristics of the immunomodulating components derived from the marine alga. PWSF and PASF have high viscosity predictable from their high molecular weights, the mean molecular masses being 1730 and 400 kDa for PWSF and PASF, respectively. Digestion of PASF with β-agarase reduced the viscosity, while improving the solubility and increasing the relative macrophage-stimulating activity.

Materials and Methods

Preparation of PASF. P. yezoensis was obtained from Shin-Futtsu Fisherman's Union (Chiba, Japan). PASF was prepared from P. yezoensis as previously described.⁹ Briefly, the residue of the hot-water extract from P. yezoensis dry powder was suspended in distilled water, adjusted to pH 2.0 with HCl, and left overnight at room temperature. After filtering and neutralizing, the filtrate was precipitated with ethanol, redissolved in water, and lyophilized.

Desulfation of PASF. Desulfated PASF was prepared from PASF by the procedure of Gretz et al.¹⁰ with a modification. Briefly, PASF (100 mg) was dissolved in distilled water (50 ml) and to this solution, NaBH₄ (150 mg) was added. After the solution was left at room temperature for 3 h, an equal weight of NaBH₄ and 2 g solid NaOH were added. The resulting solution was heated at 80°C, and 10-m1 portions of the reaction mixture were collected at 0, 15, and 45 min, before being neutralized with 6.0 M HCl in an ice bath. Desalting of the product was done in a PD-10 column (Pharmacia LKB Biotechnology, Uppsala, Sweden), and the high-molecular-weight fractions were lyophilized.

Sulfation of PASF. Further sulfation of PASF was done with piperidine-N-sulfonic acid.¹¹ Briefly, PASF (20 mg) was dissolved in 4 ml of dimethyl sulfoxide. Piperidine-N-sulfonic acid (0.14 g) was added to the solution, which was heated for 1 h at an appropriate temperature while being stirred. After being neutralized, the polysaccharide was precipitated with ethanol, redissolved in water, desalted in the PD-10 column, and lyophilized.

Anion-exchange chromatography of PASF. PASF (500 mg) was put on a DEAE-Toyopearl 650M (Tosoh, Tokyo, Japan) column (2.6 × 70 cm) that
had been equilibrated with 0.1 N HCl. After the column was washed with distilled water, the absorbed materials were eluted with a linear gradient of NaCl (0-0.5 M) at a flow rate of 2 ml/min, the absorbance being monitored at 254 nm. The fractions containing sugar detectable by the phenol-sulfuric acid method were collected into 7 fractions (F1 to VII) and lyophilized, after being desalted in the PD-10 column.

**Enzymatic degradation of PASF.** The low-molecular-weight substances in PASF were eliminated by Sephadex G-25M (Pharmacia LKB Biotechnology) column chromatography before degradation with β-agarase (Sigma, St. Louis, MO, U.S.A.). β-Agarase (2 unit/mg of PASF) was added to a 0.1 % PASF solution in water, and the mixture incubated at 30°C for an appropriate time. After this incubation, the reaction was stopped by heating, and the mixture was lyophilized.

**Chemical analyses.** The content of 3,6-AG was measured with the resorcinol reagent, using neogarobiose (Sigma) as a standard. The total sugar content was measured by the phenol-sulfuric acid method and is expressed as the polysaccharide content. Galactose (Wako Pure Chemicals, Osaka, Japan) and neogarobiose (Sigma) were used as standards because the molar extinction of 3,6-AG is different from that of galactose.

The protein content was measured by the procedure of Lowry et al., using bovine serum albumin (Sigma) as a standard. Sulfate was measured with an ion chromatograph system (IC-500, Yokogawa Hokushin Electric, Tokyo, Japan) after hydrolyzing the polysaccharides with 1 N HCl at 110°C for 8 h.

The reducing power of enzyme-degraded PASF was measured by the Somogyi-Nelson method, using neogarobiose as a standard. The viscosity of the enzyme-degraded PASF solution (1.0 %) was measured by a rotational viscometer (type BL, Tokimec, Tokyo, Japan) at 25°C.

**Macrophase-stimulating assay.** The in vitro macrophase stimulating activity was evaluated by measuring the glucose consumption and nitrite production of proteose peptone-induced peritoneal macrophages from female C57BL/6 mice. The cells suspended in an RPMI1640 medium containing 10% fetal calf serum were incubated with PASF, PASF-derived materials or lipopolysaccharide (LPS, Sigma) for 72 h. Glucose and nitrite in the macrophase culture supernatants were measured by the Glucose B-test Wako assay kit (Wako Pure Chemicals) and by the reaction of nitrite with the Griess reagent to form a chromophore absorbing at 540 nm, respectively.

**Results**

**Chemical composition of PASF**

The chemical analysis results for PASF are shown in Table I. Although the preparation protocol was the same, PASF prepared in this study had lower 3,6-AG and higher sulfate contents than the previously reported preparation.8)

**Desulfation and sulfation of PASF**

It is known that the sulfate ester in galactose-6-sulfate can be eliminated with concomitant formation of 3,6-AG by an alkali treatment. The degree of desulfation was monitored by a chemical analysis, the number of sulfate groups per sugar unit being calculated as the molar ratio of sulfate groups per sum of the galactose and 3,6-AG units. By treating for 15 and 45 min, we obtained 2 fractions of desulfated PASF which had 65% and 49% degrees of native sulfate, respectively. The increase in 3,6-AG content in desulfated PASF was also confirmed (data not shown). Further sulfation of PASF was done with piperidine-N-sulfonic acid, and sulfated PASF having 1.5- and 2.1-fold the number of sulfate groups compared with native PASF were obtained by treating at 60°C and 70°C, respectively.

The macrophase-stimulating activity of these desulfated and sulfated PASF samples was determined by measuring the glucose consumption and nitrite production in the culture supernatants of intraperitoneal cells with these samples. Desulfation of PASF decreased both the glucose consumption and nitrite production by macrophages (Fig. 1), while sulfated PASF had unchanged activities when compared with native PASF (data not shown).

**Table I. Chemical Composition of Native PASF**

<table>
<thead>
<tr>
<th>Component</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total sugar</td>
<td>74.2%</td>
</tr>
<tr>
<td>Protein</td>
<td>1.7</td>
</tr>
<tr>
<td>3,6-AG</td>
<td>14.9</td>
</tr>
<tr>
<td>Sulfate</td>
<td>6.4</td>
</tr>
</tbody>
</table>

Data are presented as the percentage of dry material.

**Fig. 1.** Macrophase-stimulating Activity of Desulfated PASF. Peritoneal exudate cells, which were obtained from mice 3 days after an intraperitoneal injection of proteose peptone, were cultured at 2 x 10⁶/well in a microplate to obtain macrophase monolayers. The cells were incubated with alkaline-eliminated PASF for 72 h. The glucose and nitrite concentrations in triplicate culture supernatants were measured, and the mean±SD is shown.

**Fig. 2.** Chromatography of Native PASF by DEAE-Toyopearl 650M (700 x 26 mm). PASF (500 mg) dissolved in water was put on and eluted with a linear gradient of a 0-0.5 M NaCl solution.
Macrophone-stimulating Polysaccharide from Porphyra

Fig. 3. Macrophone-stimulating Activity of the Fractions from Ion-exchange Chromatography.

Macrophone monolayers (4 x 10^6/well) obtained as indicated in the legend to Fig. 1 were incubated without (indicated as "Control") or with stimulus for 72 h, and the glucose and nitrite concentrations in triplicate culture supernatants were measured. The results are expressed as the mean ± SD.

Table II. Yield and Chemical Composition of the Fractions from Ion-exchange Chromatography.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Yield (mg)</th>
<th>Total sugar (%)</th>
<th>Protein (%)</th>
<th>3,6-AG (%)</th>
<th>Sulfate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FI</td>
<td>34.2</td>
<td>12.1</td>
<td>5.0</td>
<td>1.6</td>
<td>ND</td>
</tr>
<tr>
<td>FII</td>
<td>12.1</td>
<td>87.7</td>
<td>4.3</td>
<td>23.4</td>
<td>2.8</td>
</tr>
<tr>
<td>FIII</td>
<td>16.3</td>
<td>103.1</td>
<td>1.2</td>
<td>31.2</td>
<td>5.2</td>
</tr>
<tr>
<td>FIV</td>
<td>52.2</td>
<td>87.8</td>
<td>0.7</td>
<td>24.7</td>
<td>6.5</td>
</tr>
<tr>
<td>FV</td>
<td>120.4</td>
<td>77.7</td>
<td>0.4</td>
<td>17.6</td>
<td>8.7</td>
</tr>
<tr>
<td>FVI</td>
<td>49.2</td>
<td>78.8</td>
<td>0.7</td>
<td>12.5</td>
<td>13.5</td>
</tr>
<tr>
<td>FVII</td>
<td>17.5</td>
<td>64.8</td>
<td>1.9</td>
<td>9.0</td>
<td>11.0</td>
</tr>
</tbody>
</table>

Fractionation of PASF

To obtain differently sulfated fractions, PASF was put on DEAE-Toyopearl 650M anion-exchange chromatography, and seven fractions were collected (Fig. 2). The chemical composition of each fraction is shown in Table II, their major component being polysaccharide besides FI. The sulfate content gradually increased from FII to FVI, while the 3,6-AG content decreased. FVII contained lower 3,6-AG and sulfate contents than FVI, while both the 3,6-AG and sulfate contents in FII were less than those in FIII. FI, FII, and FVII had higher protein content than that of native PASF.

The macrophone-stimulating activity of each of these fractions was examined (Fig. 3) similarly to the experiments described in Fig. 1. LPS from the microbial cell wall, which is known to incorporate macrophone-activating molecules, was used as a positive control in these experiments. The glucose consumption of macrophages in the presence of FI was similar to that of the control medium. The glucose consumption stimulation by FII to FV was similar to that of native PASF, while FVI and FVII induced slightly higher glucose consumption than did native PASF. The nitrite production pattern by these samples was similar to the glucose consumption pattern, although the difference in nitrite production of PASF and FI from that of the other fractions was more pronounced.

Enzyme degradation of PASF

PASF was degraded with β-agarase from Pseudomonas atlantica to decrease the viscosity of the PASF solutions. Digests were collected at varying times during incubation to determine the reducing sugar content and viscosity (Fig. 4). During the first 4 h of digestion, the reducing power increased rapidly, after which the rate of increase slowed. The decrease in viscosity accompanied the increase in reducing power. Figure 5 indicates the in vitro stimulating activities of these digests obtained by measuring the glucose consumption and nitrite production by macrophages. The macrophone-stimulating activity of the 8-h digested sample was slightly lower than that of untreated PASF, while that of the samples digested by the 24, 48, and 72-h treatments was higher. The highest nitrite production activity was observed for the 48-h treated sample, this being stronger than that of laminarin, which is a macrophone-activating polysaccharide from brown algae.

The solubility of degraded PASF was compared with that of untreated PASF. The 48-h treated PASF sample dissolved rapidly at a concentration of 1% (w/v) within 10 min, while non-treated PASF only swelled during this period (Fig. 6). In these experiments, the macrophone-stimulating activity and solubility of PASF were both improved by enzyme degradation.

Discussion

PASF is the water-soluble polysaccharide fraction from Porphyra yezoensis, a popular dietary seaweed in Japan, and its major constituent can be considered as porphyran
because of its chemical composition, having a basic disaccharide-repeating unit of 3-O-(3,6-anhydro-1-galactopyranosyl)-β-D-galactopyranose, which can be substituted with a methyl group (C-6 of the D unit) or with a sulfate group (C-6 of the L unit). PASF has been shown to increase glucose consumption and nitrite production of macrophages in vitro, and to have macrophage-stimulating activity in mice. Since PASF has high viscosity and is only slightly soluble in cold water, it would be difficult to produce and process PASF for use in food. In this study, we succeeded in increasing the in vitro macrophage-stimulating activity of PASF by ion-exchange chromatography and enzyme degradation, and in improving its viscosity and solubility by enzyme degradation.

We first tried to modify the chemical structure of PASF by desulfation and further sulfation, as is applied to agarose (data not shown). Although neither desulfation nor sulfation improved the activity, the results enable us to discuss the structure-function relationships of PASF. We confirmed that sulfation and desulfation did not cleave the 3,6-AG units, suggesting no side reactions. The C-6 position of an L-galactose unit is known to be fully sulfated, and other L-units formed 3,6-AG in PASF and agarose. It has been reported that the first sulfation to the glucose unit of curdlan occurred at the C-6 position. These facts indicate that any newly introduced sulfate groups in sulfated PASF and agarose were not at C-6 of an L-unit, but were distinct from the native sulfates in PASF, suggesting that C-6 of a D-galactose unit was sulfated. On the other hand, desulfation of PASF is known to have removed sulfates at the C-6 position of an L-unit. The results of the assays for macrophage-stimulating activity show that desulfated PASF had lower activity and that sulfated PASF had the same activity as untreated PASF. Sulfated agarose, having a higher sulfate content compared with that of native PASF, also showed no increase in this activity (data not shown). These results enable us to demonstrate that the sulfated group at C-6 of an L-unit displays the macrophage-stimulating activity.

Ion-exchange chromatography of PASF provided immunopotentiator fractions of much higher relative activity by removing from native PASF the large amount of substances having low or no activity. PASF was divided into 7 fractions according to sulfate content, the activity of each fraction being stronger than that of native PASF, except for FI. In this experiment, the elution pattern of ultraviolet absorption at 254 nm was not consistent with that of sugar detected by the phenol-sulfuric acid method, and the fractions treated with the PD-10 column did not show an absorption at 254 nm (data not shown). In another experiment, we confirmed that the undefined substances absorbing light at 254 nm were eliminated from PASF by gel filtration chromatography with a Sephadex G-25 column. These results indicate that the lower activity of native PASF might be due to those undefined lowmolecular-weight substances that absorbed in the ultraviolet.
spectrum. Therefore, a high-molecular-weight fraction from PASF desalted by Sephadex G-25M column chromatography was used for the enzyme degradation studies.

The strongest activity was shown by FVII, which had the lowest 3,6-AG content and a high sulfate content among the fractions. This strong activity of FVII can be attributed to the large amount of sulfate groups, especially the sulfate at C-6 of the L-unit. On the other hand, FII also showed strong activity, although this had low 3,6-AG and sulfate contents. The activity of FII should be considered to be derived not only from porphyrin but also from other polysaccharides, since glucose, in addition to galactose and methyl galactose, was detected by analyzing the sugar composition after acid hydrolysis of FII (data not shown).

The macrophage-stimulating activity of PASF was improved by ion-exchange chromatography, but the solubility was still low. We finally tried enzyme hydrolysis to simultaneously improve the activity and solubility. Enzyme hydrolysis was performed with β-agarase against desalted PASF. β-Agarase I and II from *Pseudomonas atlantica* have been shown by Morrice *et al.*\(^8\) to degrade porphyrin, and they isolated low-molecular-weight neoagarooligosaccharides, some of their 6-O-methylated derivatives and higher oligomers with some substitution of the anhydro moieties by 6-O-sulfate from the β-agarase I-degradation product.\(^9\) In this study, PASF was revealed to be degraded by β-agarase containing β-agarase I and II.

The digestion proceeded in two stages, the initial rapid stage being attributable to degradation at the unsubstituted agarose sequence, and the latter slow stage to that at the partially substituted sequence, as Morrice *et al.*\(^8\) described. PASF digested with β-agarase for 48h had the strongest macrophage-stimulating activity among several digested and undigested PASF samples. This result is presumable due to a structural change leading to increased accessibility of the active moieties to macrophages, without any destruction of these active moieties by enzyme degradation. The viscosity and the solubility of this 48-h treated sample were both greatly improved in comparison with untreated PASF. The physicochemical characteristics of this PASF digest are regarded as being adaptable for processing as a foodstuff.

In conclusion, the macrophage-stimulating activities of desulfated, sulfated, fractionated, and enzyme-degraded PASF suggest that the sulfate groups in native PASF play a role in macrophage stimulation through direct interaction between the sulfate groups and macrophages, or indirectly by maintaining the conformation of the active moieties. This investigation has also illustrated that improved immunopotentiating activity and solubility in water of the material from the marine alga could be achieved by degrading with a conventional enzyme.

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**References**