

Hyaluronidase-inhibiting Acidic Polysaccharide Isolated from *Porphyridium purpureum*

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Abstract

A polysaccharide derived from the unicellular marine alga, *Porphyridium purpureum*, was purified and investigated for its hyaluronidase-inhibitory effect. Inhibition of hyaluronidase is known to mediate inflammation and tumor metastasis. Here, a polysaccharide prepared from a hot-water extract of *P. purpureum* was purified by cetylpyridinium chloride precipitation and by chromatography. Analysis revealed an acidic polysaccharide with sulfo groups. The molar ratio of the polysaccharide sugars glucuronic acid, galactose, glucose, ribose, and xylose was estimated to be 0.7 : 2.3 : 1.0 : 0.2 : 3.2. The molecular mass of the polysaccharide was estimated to be about 500 kDa by Toyopearl-HW 65 gel chromatography. The purified polysaccharide inhibited hyaluronidase activity ($IC_{50} = 0.210 \text{ mg mL}^{-1}$).

Introduction

Hyaluronidase [EC 3.2.1.35] (hyaluronoglucosaminidase) hydrolyzes hyaluronic acid and chondroitin sulfate, which are constituents of the amorphous substances of connective tissue. Hyaluronic acid is a natural and sugar-like biopolymer consisting of D-glucuronic acid and N-acetyl-D-glucosamine units. These days, hyaluronidase has received much attention due to its ability to abruptly alter hyaluronic acid homeostasis. The interaction of hyaluronidase with hyaluronic acid disrupts basement membrane integrity and produces an angiogenic response. For example, the enzyme mediates inflammation via histamine release from mast cells. Therefore, hyaluronidase inhibitors seem to be effective for suppressing allergies and inflammation (Furusawa *et al.*, 2011). Hyaluronidase has also been reported to be implicated in tumor invasiveness and metastasis (Madan *et al.*, 1999). Hyaluronidase inhibitors are potent regulators that maintain hyaluronidase homeostasis and might serve as anti-inflammatory agents (Girish *et al.*, 2009).

Many studies have reported that polysaccharides have hyaluronidase-inhibitory activity. Furusawa *et al.* (2011) reported that coffee silverskin, an acidic polysaccharide, showed hyaluronidase-

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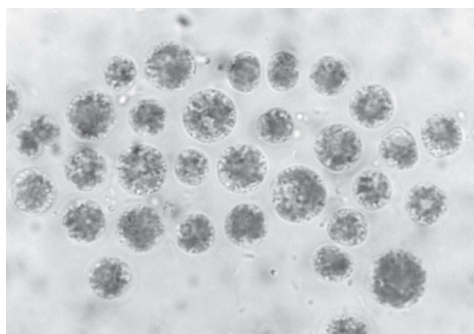


Fig.1. Photomicrograph of *Porphyridium purpureum*.

inhibitory activity. Alginic acid, which exists in the extracellular matrix and the cell membrane of brown algae, was also reported to possess this activity (Asada *et al.*, 1997). Consequently, such hyaluronidase-inhibiting polysaccharides are expected as material functional foods with anti-allergens. The microalga *Porphyridium purpureum* (Fig. 1) is reported to produce hyaluronidase-inhibitory polysaccharides which can be extracted with hot water (Fujitani *et al.*, 2001).

In this study, we attempted to purify the polysaccharide and investigated the relationship between its structure and inhibition mechanism.

Materials and Methods

Materials Freeze-dried *P. purpureum* was supplied by MicroAlgae Co. (Gifu, Japan). Hyaluronidase (Type IV-S), hyaluronic acid potassium salt, and toluidine blue O were purchased from Sigma-Aldrich Japan (Tokyo). Hyaluronic acid for use as a dalton marker was purchased from Funakoshi Co. Ltd. (Tokyo). All other chemicals were of analytical grade.

Determination of inhibitory activity of activated hyaluronidase The inhibitory effect of *P. purpureum* polysaccharides on activated hyaluronidase was determined by a modified method described by Fujitani *et al.* (2001). Hyaluronidase (1.88 mg) was dissolved in 5 mL of 0.1 M acetate buffer (pH 4.0). A mixture of 0.1 mL hyaluronidase solution with 0.2 mL polysaccharide solution was incubated at 37 °C for 20 min, after which 0.2 mL of Compound 48/80 solution was added, and the final mixture was kept at 37 °C for 20 min. Hyaluronic acid solution (0.5 mL) was added to the mixture, which was then incubated for 40 min. Sodium hydroxide solution (0.4 M, 0.2 mL) was added to terminate the reaction. The inhibitory effect was determined by the modified Morgan-Elson method (Maeda *et al.*, 1987). The polysaccharide (inhibitor) was replaced by 0.1 M acetate buffer for the control, and the enzyme solution was replaced by buffer solution for the blank. Percent inhibition was calculated as follows:

$$\text{Inhibition(\%)} = [(A - B) - (C - D)] / (A - B) \times 100$$

Here, A is the control, B is the control blank, C is the control with inhibitor and enzyme, and D is blank with inhibitor but without enzyme. The specific activity of the inhibitor was defined as the amount of polysaccharide that inhibited 50 % hyaluronidase activity (IC₅₀; mg polysaccharide/mL)

under the above conditions.

Acid mucopolysaccharide assay An acidic mucopolysaccharide assay kit (Primary Cell Co., Ltd, Sapporo, Japan) was used to assay acidic mucopolysaccharides.

Sulfate Determination Determination of sulfate was performed according to method C of Dodgson (1961). A calibration curve was prepared using K₂SO₄ solution containing between 20 and 200 µg SO₄²⁻ ions.

Protein Assay Protein concentration was measured with a Bio-Rad protein reagent (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as the standard.

Extraction and purification of polysaccharide from *P. purpureum* Freeze-dried *P. purpureum* MACO1KM (30 g) was suspended in 1000 mL of distilled water, and the suspension was kept at 90 °C for 180 min (Fujitani *et al.*, 2001). The extract was filtered to remove insolubles and dialyzed against tap water over night. After being concentrated, the extract was freeze-dried. Crude polysaccharide (1.5 g) was dissolved in 1000 mL of distilled water and Na₂SO₄ was dissolved up to 0.03 M. A cetylpyridinium chloride (CPC) solution (20.12 mL; 10 %) was mixed and kept at 5 °C overnight. The resulting polysaccharide precipitate was removed by centrifugation (8000 rpm, 15 min), and the supernatant was freeze-dried. The precipitate was dissolved in 12 % ethanol with 2 M NaCl, and re-precipitated with a three-fold volume of ethanol to remove CPC. The precipitate of crude polysaccharides was washed with ethanol and dissolved in distilled water up to 700 mL. The solution was dialyzed against tap water and 0.05 M phosphate buffer (pH 6.0). The solution was loaded onto a DEAE-Toyopearl (Tosoh Co., Tokyo, Japan) column (25 × 400 mm) equilibrated with 0.05 M phosphate buffer (pH 6.0). After washing the column with the same buffer, the adsorbed fractions were eluted with a 0 to 0.5 M NaCl gradient in 0.05 M phosphate buffer (pH 6.0). The fractions of saccharides detected by the phenol-sulfuric acid method (Hodge and Hofreiter, 1962) were pooled and dialyzed against water before being freeze-dried.

Cellulose acetate membrane electrophoresis Relative quantities of monosaccharide residue-purified preparation (F-II) were analyzed by cellulose acetate membrane electrophoresis. SELECA-V (Toyo Roshi Kaisha, Ltd, Tokyo) was used as the membrane. Electrophoresis was carried out at 6 mA in 0.1 M calcium acetate. After electrophoresis, the membrane was stained with 0.5 % toluidine blue in 3 % acetic acid solution to detect polysaccharides.

Analysis of sugar composition by HPLC Relative quantities of monosaccharide residues of purified polysaccharide were analyzed with high-performance liquid chromatography (HPLC) by ProteNova Co., Ltd. (Takamatsu, Japan). The purified polysaccharide (1.20 mg) was dissolved in 24 µL of 4 M Trifluoroacetic acid and hydrolyzed at 100 °C for 3 h. The supernatant of hydrolysate was separated by centrifugation (6000×g, 10 min). A 10 µL aliquot diluted 100-fold with ultrapure water was mixed with 40 µL reagent mixture (prepared by mixing 1 mmol ethyl p-aminobenzoate (4-ABEE), 7 mg sodium cyanoborohydride, 8 µL glacial acetic acid, and 70 µL methanol), and the mixture was heated at 80 °C for 60 min. After cooling, the aqueous phase was extracted with 5 mL chloroform to remove excess 4-ABEE and the aqueous layer was subjected to HPLC analysis. HPLC conditions were as follows:

- System; BioAssist eZ (Tosoh Co., Tokyo)

- Column; PN-PAKC18 (3 × 75 mm) Mobil phase: borate buffer/acetonitrile
- Flow rate; 0.5mL/min
- Detection; fluorescence spectrometry (e.g., 305 nm; Em: 360 nm)

Analysis of purified polysaccharide molecular mass The purified polysaccharide was dissolved in 0.1 M phosphate buffer (pH 6.0) with 0.1 M NaCl. The solution was loaded onto a Toyopearl HW-65F (Tosoh Co., Tokyo) column (25 × 825 mm) equilibrated with the same buffer. Chromatography was carried out at a flow rate of 18 mL/h with detection of a saccharide by the phenol-sulfonic acid method (Dubois *et al.*, 1956). The average molecular mass of the polysaccharide was calculated based on the retention time of hyaluronic acid HYA-250K-1, HYA-500K-1, and HYA-1000K-1 as dalton makers.

Results

Extraction and purification of polysaccharide from *P. purpureum* A total of 2.33 g of crude polysaccharides were yielded from 30 g of freeze-dried *P. purpureum* MACO1KM by hot-water extraction. Prior to purification, the crude polysaccharide, a gray, cotton-like powder, was measured for hyaluronidase-inhibitory activity, and the activity was found to be almost the same as that reported by Fujitani *et al.* (2001). The purification scheme is summarized in Fig. 2. Hyaluronidase-inhibitory activity was measured in each step (Table 1). Extracted polysaccharides were dissolved in aqueous CPC solution containing sodium sulfate. The resultant precipitate was obtained as total

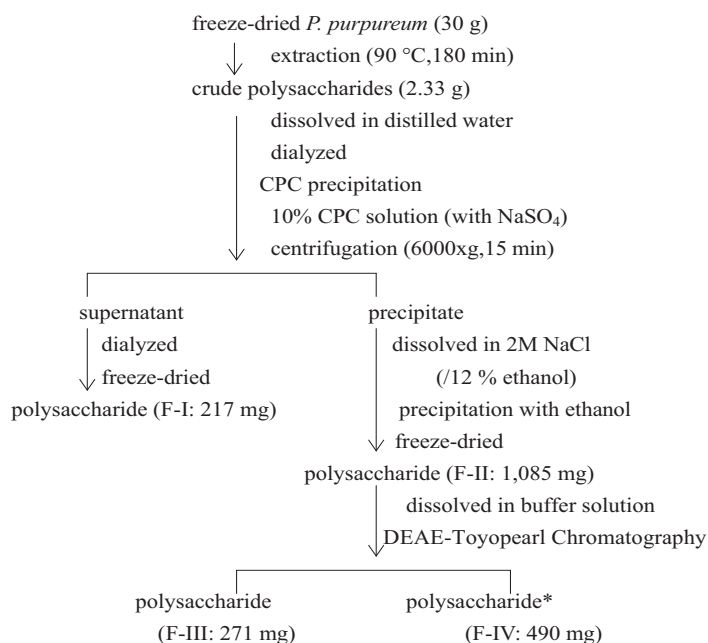


Fig. 2. Purification steps of *P. purpureum* polysaccharide.

* used for further experiments

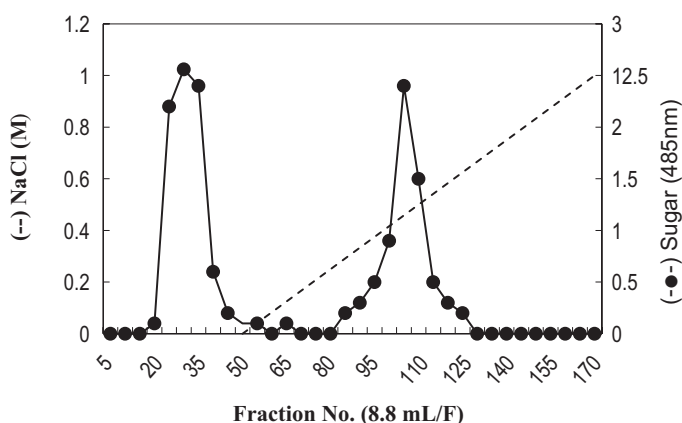


Fig. 3. DEAE-Toyopearl chromatography of polysaccharides.

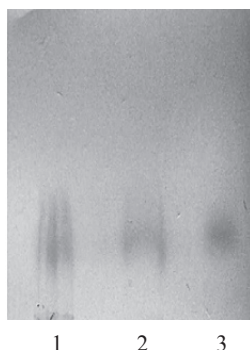


Fig. 4. Cellulose acetate membrane electrophoresis of polysaccharides.

Lane 1, crude polysaccharides; Lane 2, F-II; Lane 3, F-IV (purified polysaccharide)

acidic polysaccharides. The precipitate was loaded onto the DEAE-Toyopearl column, and adsorbed saccharides were eluted with a linear gradient of 0 to 0.5 M NaCl in 0.05 M phosphate buffer (pH 6.0). The results of DEAE-Toyopearl chromatography (Fig. 3) showed two peaks of polysaccharide (F-III and F-IV, with values indicating elution order). F-IV polysaccharide, a colorless cotton-like powder, was used as the final preparation for further experiments.

When F-IV was analyzed by cellulose acetate membrane electrophoresis, it gave a single band as shown in Fig. 4.

Sugar composition of the purified polysaccharide Sugar composition of F-IV was analyzed by HPLC (Table 1). The molar ratio in the polysaccharide (F-IV) was estimated to be glucuronic acid, galactose, glucose, ribose, and xylose (0.7 : 2.3 : 1.0 : 0.2 : 3.2), respectively.

Determination of sulfate The amount of sulfate ions for crude and purified polysaccharide was 5.60 % and 4.50 %, respectively.

Inhibitory activity against hyaluronidase Table 2 shows hyaluronidase-inhibitory activity with

Table 1. Composition of saccharides of the purified polysaccharide.

Sugar	Polysaccharide		
	from <i>P. purpureum</i> (n mol/mg)	from <i>P. cruentum</i> * (%, w/w)	from <i>P. aerugineum</i> (%, w/w)
Glucuronic acid	178.9	NT	NT
Galacturonic acid	ND	NT	NT
Galactose	607.4	30.0	27
Mannose	ND	NT	NT
Glucose	255.2	12.4	20
Arabinose	8.4	NT	NT
Ribose	63.5	NT	NT
N-acetyl-mannosamine	ND	NT	NT
Xylose	840.1	32.8	29.0
N-acetyl-glucosamine	ND	NT	NT
Fucose	ND	NT	NT
Rhamnose	ND	NT	NT
N-acetyl-galactosamine	ND	NT	NT

* Percival, E. and Foyle, J. A. R. (1979). The extracellular polysaccharides of *Porphyridium cruentum* and *Porphyridium aerugineum*. *Carbohydrate Research*, **72**, 165–176.

NT; not test, ND; not detect.

Table 2. IC₅₀ and yield of polysaccharide by purification step.

Polysaccharide	Weight (mg)	Yield (%)	IC ₅₀ (mg/mL)
Crude	2,330	100	0.264
F-I	217	9.3	0.370
F-II	1,085	46.5	0.158
F-III	271	11.6	0.420
F-IV	490	21.0	0.210

the yield of polysaccharides at each purification steps. The purified polysaccharide yield was 490 mg from 30 g of freeze-dried *P. purpureum*. The improvement in the inhibitory activity between crude (IC₅₀ = 0.264 mg/mL) and purified (IC₅₀ = 0.210 mg/mL) saccharide was low (Table 2).

Discussion

Hyaluronic acid is a macromolecular polysaccharide composed of alternately-conjugated β-1,4-D-N-acetylglucosamine and β-1,3-D-glucuronic acid and is an important component of the extracellular matrix. Mammalian hyaluronidase has been reported to play many important roles in physiological processes. In addition, hyaluronidase has also been reported to be an enzyme mediating inflammatory reactions (Sakamoto *et al.*, 1980). Therefore, a hyaluronidase inhibitor could be a useful pharmacological tool. There are many reports regarding hyaluronidase inhibitors such as L-ascorbic acid, L-ascorbic acid 6-hexadecanoate (Botzki *et al.*, 2004), and numerous

acidic polysaccharides (Toida *et al.*, 2003).

Fujitani *et al.* (2001) reported the hyaluronidase-inhibitory effects of crude polysaccharides extracted by hot water from *P. purpureum*. In this study, we purified the crude polysaccharide from *P. purpureum* and attempted to study the relationship between its inhibitory effect and structural features. According to Fujitani's method, we extracted polysaccharides from freeze-dried *P. purpureum*. The inhibitory concentration (IC_{50}) of the extracted polysaccharides was almost the same as that reported by Fujitani *et al.* (2001). We purified the extracted polysaccharide to homogeneous with a CPC precipitation, DEAE Toyopearl chromatography. Two fractions of polysaccharide were eluted by DEAE-Toyopearl chromatography, but we employed another acidic polysaccharide named F-IV for further study. F-IV was defined as a single band on cellulose acetate membrane electrophoresis. The purified polysaccharide inhibited hyaluronidase with a slightly higher activity ($IC_{50} = 0.210$ mg/mL) compared with crude polysaccharides ($IC_{50} = 0.264$ mg/mL).

The molecular mass was determined as 500 ± 100 kDa by Toyopearl-HW 65 gel chromatography. Percival and Foyle (1979) reported that species of genus *Porphyridium* (red algae) produce polysaccharide, and *P. cruentum* and *P. aerugineum* were found to produce polysaccharides with a molecular mass of 5,000–6,000 kDa. This molecular mass is large compared with that of the present study, and this difference could be due to the different extraction conditions. The content of sulfate is also different. Other studies of crude polysaccharides from *P. cruentum* or *P. aerugineum* have revealed a sulfate content of 10 %, compared with 5.6 % in the present study.

The sugar composition of polysaccharides from *P. purpureum*, *P. cruentum*, and *P. aerugineum* is compared in Table 1. The molar ratio of glucuronic acid, galactose, glucose, ribose, and xylose in the purified polysaccharide was estimated to be 0.7 : 2.3 : 1.0 : 0.2 : 3.2. Galacturonic acid, N-acetylhexosamine, and fucose were not detected, and N-acetylneuraminic acid constituted only 9.2 pmol/mg polysaccharide (data not shown). These results show that the purified polysaccharide is different from fucose-containing polysaccharides from brown algae such as Ecklonia and Hizikia (Nishino and Nagumo, 1987). Mucopolysaccharides from green algae containing rhamnose or other mucopolysaccharides such as chondroitin, hyaluronic acid, heparin, and keratan sulfate are also different from the purified polysaccharide. On the other hand, the reported *P. cruentum* and *P. aerugineum* polysaccharides were similar in sugar constitution to the purified polysaccharide (Table 1). Of these algae, the sugar constituent of *P. purpureum* is closer to that of *P. cruentum* than to *P. aerugineum*, which may explain why *P. cruentum* and *P. purpureum* are marine red alga, but *P. aerugineum* is a freshwater red alga. The galactose content of the purified polysaccharide was approximately 9 % in monosaccharide form, but no galacturonic acid was detected. According to some reports regarding the inhibitory effect of hyaluronidase, uronic acid content in polysaccharides is important for hyaluronidase inhibition (Asada *et al.*, 1997), but D-galacturonic acid-like pectic substances showed no inhibition (Sawabe *et al.*, 1992).

The purified polysaccharide contained 4.6 % sulfate ions, meaning it is a sulfated polysaccharide. It is also known that sulfated polysaccharides show various biological functions which depend on the presence and spatial positioning of sulfo groups. Seaweed such as fucoidan and carrageenan contain sulfated polysaccharides, and hyaluronidase-inhibiting activity correlates with the degree

of O-sulfonation (Toida *et al.*, 1999). Girish and Kemparaju (2005) reported that the *Naja naja* hyaluronidase inhibitory activity of chitosan is dependent on chain length. These reports indicate that the purified polysaccharide from *P. purpureum* is a sulfated acidic polysaccharide and that its hyaluronidase-inhibitory activity seems to contribute to the high content of glucuronic acid and sulfo groups.

Toida *et al.* (2003) reported the IC₅₀ toward the hyaluronidase-inhibitory activity of sulfated xylan, sulfate amylose, and sulfate cellulose as 3.08, 3.44, and 4.0 (μg/mL), respectively. These values are fairly strong compared with those of purified polysaccharide (210 μg/mL).

The inhibitory activity of the purified polysaccharide against α-glucosidase and sucrase derived from rat intestine was also investigated, but no activity was detected (data not shown).

Acknowledgments

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