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Hyaluronidase-Inhibiting Polysaccharide Isolated and Purified from Hot Water Extract of Sporophyll of *Undaria pinnatifida*

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Polysaccharide showing inhibitory activity against hyaluronidase, which is known to be related to inflammation and tumor metastasis, was purified from the sporophyll of *Undaria pinnatifida* by fractionated extraction and column chromatography. On the basis of chemical analyses, the purified compound was found to be a kind of sulfated polysaccharide. The molar ratio of sugars and sulfuric acid in the purified compound was estimated to be L-fucose : D-galactose : D-glucuronic acids : sulfuric acid=1.0 : 1.0 : 0.04 : 5.2. The number-average molecular weight of the sulfated polysaccharide was estimated to be 63,000 by high-performance liquid chromatography. This polysaccharide inhibited hyaluronidase activity (IC_{50}=13.0 μg/ml) in a dose-dependent manner.

Keywords: hyaluronidase, *Undaria pinnatifida*, sporophyll, inhibitor, polysaccharide

Hyaluronidase [EC 3.2.1.35] hydrolyzes hyaluronic acid and chondroitin sultate which are the constituents of the amorphous substances of connective tissues. The enzyme was reported to play a key role in the development of inflammatory diseases, since the destruction of the connective extracellular matrix promotes the spreading of chemotactic factors of inflammation (Facino *et al*., 1993). In addition, a considerable amount of hyaluronidase is present in rat peritoneal mast cells, and it has been suggested that hyaluronidase is one of the enzymes directly controlling the degranulation of mast cells (Kakegawa *et al*., 1985). Hyaluronidase has also been reported to be implicated in tumor invasiveness and metastasis. The interaction of hyaluronidase with hyaluronic acid results in the disruption of basement membrane integrity and produces an angiogenic response. Invasive breast adenocarcinoma has a significantly higher level of hyaluronidase expression compared to other breast tissues (Madan *et al*., 1999). It was also reported that hyaluronidase was expressed by metastatic human melanoma, colon carcinoma and glioblastoma cell lines, and further by tumor biopsies from patients with colorectal carcinomas (Liu *et al*., 1996). Moreover, angiogenesis could be blocked by an inhibitor of hyaluronidase (Liu *et al*., 1996).

Perilla leaves have been known to possess anti-inflammatory and anti-allergic effects. Asada *et al*. (1999) reported that the glycoprotein obtained from the hot water extract of perilla leaves showed hyaluronidase-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). In view of the important role of hyaluronidase in inflammation and malignant invasion, it is very useful to find hyaluronidase-inhibitory activities in a natural resource, seaweed. *Undaria pinnatifida*, one of the brown algae, is a marine resource commonly found on the coast of Japan. The algae is now produced by artificially seeded marine culture. In Shimane Prefecture (the site of our laboratory), the annual production of *U. pinnatifida* was reported to be approximately 530 t in 1999. The leaf of this seaweed is used in soup or salad as a health food. Only a very small amount of its sporophyll has been eaten as a local domestic food; most of it has been dumped because it is rarely harvested or prepared. The development of efficient usages of sporophyll of *U. pinnatifida* is required. The sporophyll of *U. pinnatifida* is believed to contain physiologically active substances, not yet identified, so that efficient ways of using it should be developed.

In this investigation, we attempted to isolate a specific polysaccharide from the hot water extract of the sporophyll and to characterize its physiologically active property.

Materials and Methods

Materials Hyaluronidase [EC 3.2.1.35] (from bovine testis), hyaluronic acid potassium salt and toluidine blue O were purchased from Sigma Chemicals Co. (St. Louis, MO). Pullulan was purchased from Showa Denko K. K. (Tokyo). All other chemicals were of analytical grade.

Undaria pinnatifida *U. pinnatifida*, which was commercially cultured off Shimane Peninsula (Kasaura, Mihonoseki, Shimane prefecture, Japan) in May 2000, was used in this study. Its sporophyll was dried by freeze-drying.

Determination of inhibitory activity on activated hyaluronidase The inhibitory effect of the extract of the sporophyll of *U. pinnatifida* on activated hyaluronidase was determined by the method described by Asada *et al*. (1999). Hyalulonidase (0.15 mg/ml) and hyaluronic acid (0.76 mg/ml) were dissolved in 0.1 M acetate buffer (pH 4.0). Sodium chloride was used as an activator of hyaluronidase. The quantitative analysis of *N*-acetylamino sugar was carried out by the modified Morgan-Elson method (Reissing *et al*., 1955). The percent of inhibition was cal-

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Abbreviations: CPC, cetylpyridinium chloride; DSCG, disodium cromoglycate; DPPH, 1-diphenyl-2-picryhydrazyl

culated as follows:

$$\text{inhibition } (\%) = 100 \times \{1 - (S - B)/(C - B)\},$$

where B is absorbance without enzyme, S is absorbance with inhibitor, and C is absorbance without any inhibitor. Specific activity of the inhibitor was defined as follows: 1 unit is the amount of inhibitor that inhibits 50% of hyaluronidase activity in the above system.

Purification of extracted polysaccharide from sporophyll of Undaria pinnatifida Dried sporophyll of *U. pinnatifida* (5 g) was suspended in 200 ml of 100 mM aqueous calcium chloride, and the suspension was boiled for 20 min. The extract was filtered to remove insoluble materials. Cetylpyridinium chloride (CPC), a precipitant for polysaccharides, was added to bring the filtrate to the final concentration of 0.1%, and then the mixture was let stand at 37°C for 16 h. The obtained precipitate was dissolved in 4 M aqueous sodium chloride and reprecipitated with a three fold volume of ethanol to remove CPC. The precipitate of crude polysaccharides was dissolved in water and dialyzed against water to remove sodium chloride. After the addition of an equal volume of 100 mM borate buffer (pH 7.6), the solution was loaded onto a DEAE-Toyopearl (TOSOH Co., Tokyo) column (25×400 mm) equilibrated with 50 mM borate buffer (pH 7.6). After washing the column with the same buffer, the fractions were eluted with 1 M aqueous sodium chloride in 50 mM borate buffer (pH 7.6). The fractions with inhibitory activity on hyaluronidase were pooled and dialyzed against water.

Cellulose acetate membrane electrophoresis The purified preparation was analyzed by cellulose acetate membrane electrophoresis. SEPARAX-SP (FUJI PHOTO FILM Co., Tokyo) was used as the membrane. Electrophoresis was carried out at 5 mA electric current in 200 mM calcium acetate. The membrane was stained with 0.5% toluidine blue solution to detect polysaccharides.

Analysis of sugar and sulfuric acid components The purified polysaccharide was hydrolyzed with 2 ml of 2 N hydrochloric acid at 100°C for 5 h in a sealed glass tube. After removal of the hydrochloric acid by evaporation, the residue was dissolved in 2 ml of water and then chromatographed with a Carbo Pac PA1 column of a DX-500 liquid chromatograph (DIONEX Co., Osaka) to analyze sugar components. Neutral monosaccharides were eluted with 20 mM sodium hydroxide, and uronic acids were eluted with 100 mM sodium hydroxide and 150 mM sodium acetate. Chromatographic separation was carried out at a flow rate of 1 ml/min using a pulsed amperometric detector. Eluted monosaccharides were identified by comparing the retention time to those of standard monosaccharides. Sulfuric acid in the hydrolysate was also analyzed with an Ion Pac AS11 column of the DX-500 liquid chromatograph using a conductometric detector. The eluent flow was 1 ml/min at 0.5 mM sodium hydroxide for up to 5 min, followed by a linear gradient to 5 mM sodium hydroxide from 5 to 10 min.

Analysis of molecular mass of purified polysaccharide The purified polysaccharide was dissolved in water (10 mg/ml) and applied to high-performance liquid chromatography using a TSK guard column PW_{XL} connected with TSKgel $G3000PW_{XL}$ and TSKgel $G6000PW_{XL}$ (TOSOH Co.). Chromatography was carried out using 0.5 M acetate buffer (pH 4.6) at a flow rate of 0.8 ml/min at 40°C with a refractive index detector. The average molecular weight of the polysaccharide was calculated based on the retention time of standard pullulans as markers with the GPC Program (Shimadzu Co., Kyoto).

Results

Purification of a polysaccharide from sporophyll of Undaria pinnatifida An active substance showing inhibitory activity against hyaluronidase was purified. The scheme of purification is shown in Fig. 1. Hyaluronidase-inhibitory activity was measured in each step and results are also shown in Fig. 1. Dried sporophyll of *U. pinnatifida* was suspended in aqueous calcium chloride, and extraction was performed in boiling water. The extract was precipitated with CPC to obtain total acidic polysaccharides. The precipitate was loaded onto a DEAE-Toyopearl column, and the substance possessing hyaluronidase-inhibitory activity was eluted with 1 M aqueous sodium chloride. From 5 g of dried and powdered sporophyll of *U. pinnatifida*, 185 mg of purified polysaccharide was obtained. When the purified preparation was analyzed on cellulose acetate membrane electrophoresis, it gave a single band as shown in Fig. 2.

Characterisrics of the purified inhibitor Sugar analyses of the purified preparation were carried out by phenol-sulfuric acid reaction (Dubois *et al.*, 1956; method for detecting total carbohydrate) and carbazole-sulfuric acid reaction (Bitter & Muir, 1962; method for detecting uronic acids). Protein was measured by a

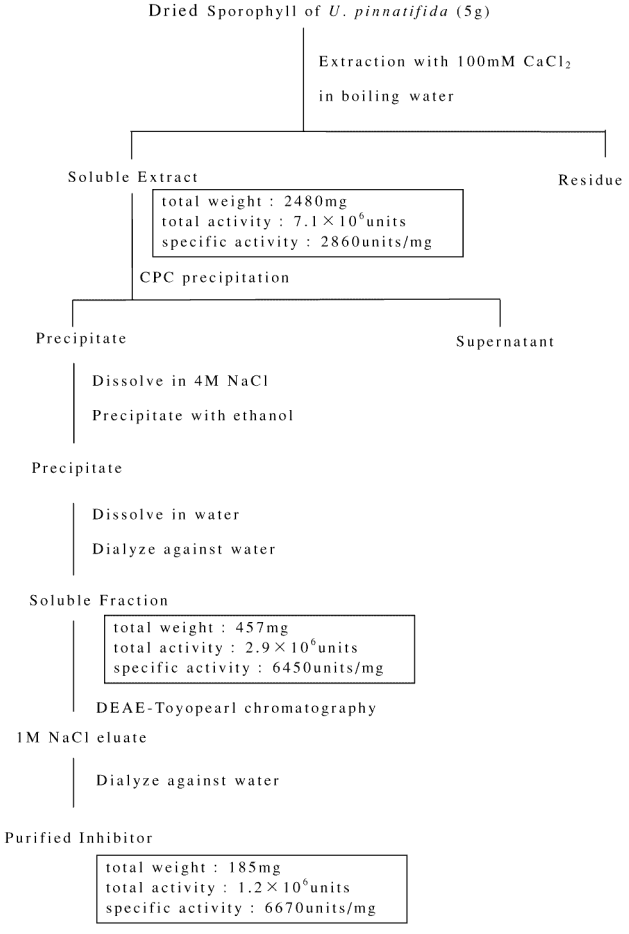

Fig. 1. Scheme for extraction and isolation of a hyaluronidase inhibitor from sporophyll of *U. pinnatifida*.

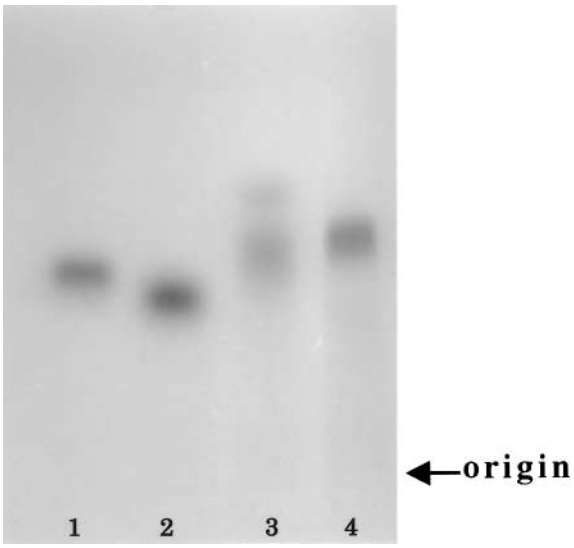

Fig. 2. Cellulose acetate membrane electrophoresis of the purified inhibitor from sporophyll of *U. pinnatifida*. Lane 1, chondroitin sulfate A (Sigma Chemicals Co); lane 2, heparin (Sigma Chemicals Co); lane 3, fucoidan from *Fucus vesiculosus* (Sigma Chemicals Co); lane 4, the purified inhibitor.

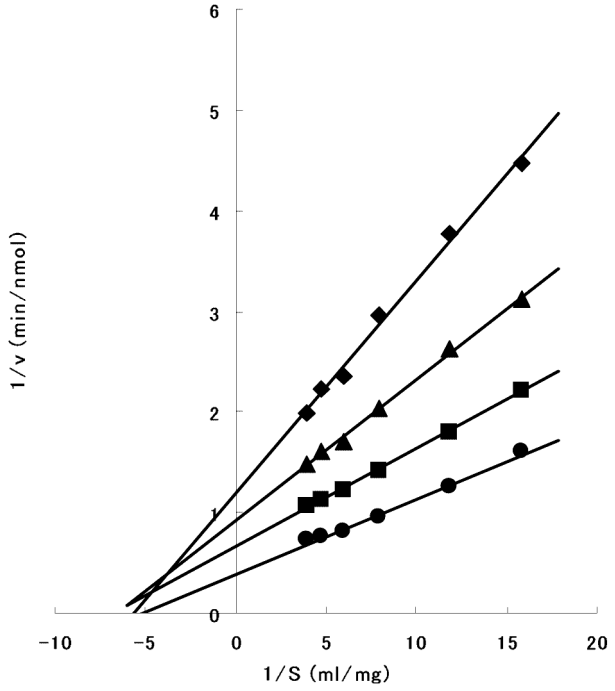

Fig. 3. Lineweaver-Burk plots for hydrolysis of hyaluronic acid in the presence of the purified polysaccharide. The enzyme reaction was done at the substrate concentration from 0.063 mg/ml to 0.25 mg/ml in the absence or presence of the purified polysaccharide, and the resulting *N*-acetylamino sugar was determined by the modified Morgan-Elson method using *N*-acetylglucosamine as a standard. Symbols: 0 μg/ml of the polysaccharide (●); 20 μg/ml (■); 40 μg/ml (▲); 60 μg/ml (◆).

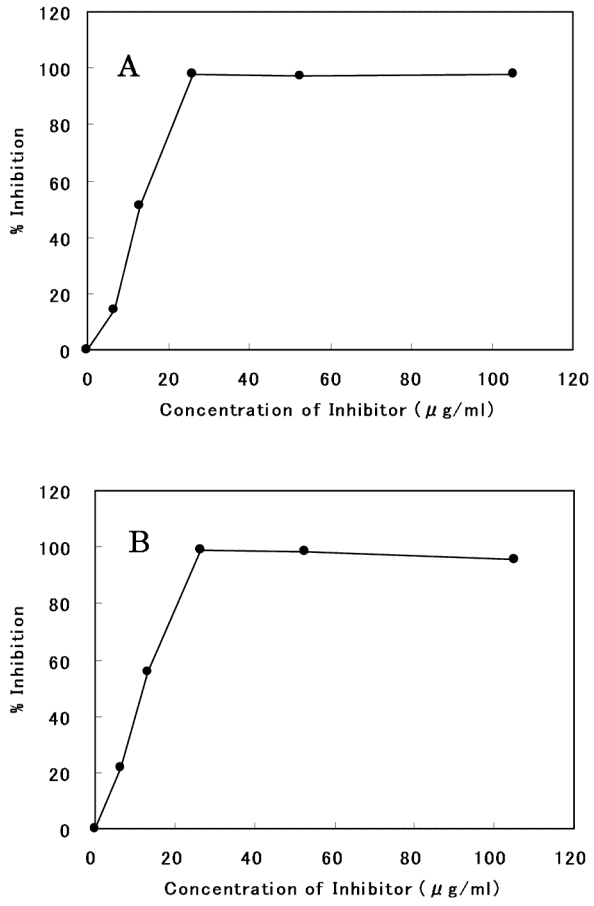

Fig. 4. Inhibitory effects of the purified polysaccharide on the activity of hyaluronidase. Various concentrations of the purified polysaccharide were added to hyaluronidase solution. Detailed experimental procedures are as follows: After incubating hyaluronidase and sodium chloride at 37°C for 20 min, the purified polysaccharide was added and the solution was allowed to stand for 20 min, and then hyaluronic acid was added (A). After incubating hyaluronidase and the purified polysaccharide at 37°C for 20 min, sodium chloride was added, the solution was allowed to stand for 20 min, and then hyaluronic acid was added (B).

DC Protein Assay kit (BIO-RAD Co, Tokyo), but none was detected. The molar ratio of sugars and sulfuric acid in the polysaccharide was estimated to be L-fucose : D-galactose : D-glucuronic acids : sulfuric acid = 1.0 : 1.0 : 0.04 : 5.2. The number-average molecular weight, the weight-average molecular weight and the Z-average molecular weight of the inhibitor were estimated to be 63,000, 178,000 and 348,000, respectively, by high-performance liquid chromatography.

Inhibitory effects against hyarulonidase The effect of substrate concentration on the velocity of hydrolysis in the presence of the purified inhibitor was investigated. Lineweaver-Burk plots indicated a mixed type of uncompetitive and noncompetitive inhibition as shown in Fig. 3. Measurement of the inhibitory effect of the purified inhibitor on activated hyaluronidase showed the purified polysaccharide inhibited hyaluronidase activity in a dose-dependent manner (IC_{50} = 13.0 μg/ml) (Fig. 4A). The degree of inhibitory effect against the activation of inactive hyaluronidase by metal salt was almost the same (IC_{50} = 12.1 μg/ml) as shown in Fig. 4B.

Discussion

Fucoxanthin extracted from the sporophyll of *Undaria pinnatifida* was recently reported to possess a 1-diphenyl-2-picryhydrazyl (DPPH) radical scavenging activity (Yan *et al*., 1999) and also an apoptosis-inducing effect on human leukemia cell line HL-60 (Hosokawa *et al*., 1999). Furthermore, *n*-3 polyunsaturated fatty acids from the sporophyll of *U. pinnatifida* were re-

ported to inhibit icosanoid production in MC/9 mouse mast cells (Ishikawa *et al*., 1998). On the other hand, water-soluble substances such as polysaccharides are known to exist in seaweeds and to possess some physiological activities. Seeking a new seaweed substance, we wanted to isolate the water-soluble and physiologically active substances of sporophyll of *U. pinnatifida*, and did isolate a polysaccharide with inhibitory activity on hyaluronidase.

Fucose-containing polysaccharides are known to be the main polysaccharide constituents of brown algae such as *Ecklonia kurome*, *Ecklonia cava* and *Hizikia fusiforme* (Nishino & Nagumo, 1987). But their chemical compositions differ among species and vary according to the different extraction methods used (Nishino & Nagumo, 1987). Fucose was the main component in the fucose-containing polysaccharide from *E. kurome* and *E. cava* (Nishino & Nagumo, 1987). The galactose content of the polysaccharide from *H. fusiforme* (Nishino & Nagumo, 1987) was approximately half of fucose. Moreover, all constituents contained D-xylose and D-mannose as minor components. In the sporophyll of *U. pinnatifida*, two fucogalactan sulfates were reported earlier, and the major one of these possessed fucose and galactose in the molar ratio of 1.0 : 1.3 (Igarashi *et al*., 1971). The minor fucogalactan sulfate possessed fucose and galactose in the molar ratio of 1.0 : 1.5.

The sugar components of the new purified preparation with the hyaluronidase-inhibitory activity were L-fucose, D-galactose and D-glucuronic acids (1.0 : 1.0 : 0.04). Hence, in the molecular ratio of fucose/galactose, our preparation differed from the above two fucogalactan sulfates (Igarashi *et al*., 1971). Moreover, our preparation contained a small amount of glucuronic acid, while no glucuronic acid was contained in the fucogalactan sulfates reported by Igarashi and his colleagues (1971).

Fucose-containing sulfated polysaccharide is known to possess the following physiological activities: anti-coagulant (Nishino *et al*., 1991), anti-thrombin (Nishino & Nagumo, 1995), anti-tumor (Zhuang *et al*., 1995), and anti-HIV (McClure *et al*., 1992). These activities of the polysaccharide depend on its structure.

In this study, we found our preparation to show an another activity, the inhibition of hyaluronidase. There has been no previous report that sulfated polysaccharide had hyaluronidase-inhibiting activity. The hyaluronidase was reported to be an enzyme involved in inflammatory reaction (Sakamoto *et al*., 1980). Hyaluronic acid is a macromolecular polysaccharide composed of alternately-conjugated β-1,4-D-*N*-acetylglucosamine and β-1,3-D-glucuronic acids, and exists in abundance in the connective tissues of mammals. It was reported that the concentration and the molecular-weight of hyaluronic acid decreased in articular chronic rheumatism (Gotoh *et al*., 1988). In addition, hyaluronidase has been reported to play an important role in tumor metastasis (Madan *et al*., 1999). From these reports, it is suggested that the purified preparation from sporophyll of *U. pinnatifida* suppresses inflammation and inhibits tumor cell-invasion by inhibiting hyaluronidase activity.

Pectic substances (Sawabe *et al*., 1992) and alginic acid (Asada *et al*., 1997) have been found to be inhibitors of hyaluronidase. Asada *et al*. (1997) reported the amount of uronic acid in the polysaccharide is important for its inhibitory activity on hyaluronidase, and the inhibition of hyaluronidase by alginic acid was the result of substrate competition. The purified preparation from sporophyll of *U. pinnatifida* had glucuronic acid as an uronic acid, but the amount was small (2.0%). Furthermore, the inhibiting manner of the purified preparation was not substrate competition from Lineweaver-Burk plots. Thus, the mechanism of inhibition was different between our preparation and pectic substances or alginic acid.

The hyaluronidase of mammals usually exists in an inactive form, and the enzyme in inactive form is activated by the sodium or calcium ion. Anti-inflammatory agents such as disodium cromoglycate (DSCG) are known to inhibit hyaluronidase activity. DSCG showed stronger inhibitory effect on hyaluronidase when it was added to the enzyme solution prior to a metal salt (Kakegawa *et al*., 1985). There was approximately twenty times difference of IC_{50} values between them. On the other hand, as shown in Fig. 4, the purified polysaccharide inhibited hyaluronidase activity to the same degree when it was added to the enzyme solution both after (Fig. 4A) and before (Fig. 4B) the addition of the metal salt. This result suggests different mechanisms of action between the purified polysaccharide and DSCG.

The isolated and purified sulfated polysaccharide had a different composition of molecular residues from those of other reports. In addition, the hyaluronidase-inhibitory activity was found in the sulfated polysaccharide of *U. pinnatifida*.

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