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Isolation and analysis of polysaccharide showing high hyaluronidase inhibitory activity in *Nostochopsis lobatus* MAC0804NAN

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An active substance with high hyaluronidase inhibitory effect was isolated from the edible cyanobacterium *Nostochopsis lobatus* MAC0804NAN strain and characterized. The active component in the hot water extract was purified by anion exchange and gel filtration chromatography and was found to be a polysaccharide. The IC_{50} against hyaluronidase of the purified polysaccharide was 7.18 μg/ml whose inhibitory activity is 14.5 times stronger than that of disodium cromoglycate (DSCG), an anti-allergy medication. The carbohydrate composition which was analyzed by GC–MS and NMR was found to be composed mainly of glucose, glucuronic acid, fucose, 2-*O*-methylfucose, mannose, galactose and xylose.

[**Key words:** Edible cyanobacteria; *Nostochopsis*; Hyaluronidase inhibitor; Polysaccharide; Methylfucose]

Nostochopsis lobatus which is known as an edible cyanobacterium grows abundantly on rocks during the dry season in northern Thailand, specifically along the Nan River in Nan Province (1,2). This cyanobacterium is consumed not only as a food item but is also utilized as a medicine because of its anti-pyretic ingredients. Peerapornpisal et al. reported that an ethanol extract of *N. lobatus* showed various therapeutic activities such as anti-gastric ulcer, anti-inflammatory, anti-oxidant, and hypertensive properties (1). Previously we have also reported tyrosinase inhibitory effect and production of vitamin B_{12} in *N. lobatus* MAC0804NAN (3,4).

Moreover, we have found that the ethanol-insoluble fraction of hot water extract of *N. lobatus* showed high inhibitory effect on hyaluronidase, and the IC_{50} of the fraction contained a quarter of disodium cromoglycate (DSCG) which is used as an anti-allergy medication (5). However, the active component in the extract has not been identified yet.

Hyaluronidases are a class of enzymes which predominantly hydrolyze hyaluronic acid and play an important role in physiological processes such as angiogenesis (6), carcinogenesis (7,8), inflammation (9,10) and type I allergy (11). Therefore, the screening of inhibitory substance in hyaluronidase is important for health and medicine.

In the present study, we isolated an active substance showing hyaluronidase inhibitory effect from *N. lobatus* MAC0804NAN, and characterized this isolated substances.

MATERIALS AND METHODS

Strain and culture conditions *N. lobatus* MAC0804NAN was collected from Nan River in Nan Province in northern Thailand in 2008. These isolated cells were then cultured in nitrogen-free BG11 medium (12) in 1.8×10^5 ml cylindrical acrylic tanks with air-bubbling at temperatures of 20–25°C in continuous illumination of 300 μmol/m^2/s under fluorescent lights. The cultured cells were harvested by filtration and washed with tap water, and immediately dehydrated by lyophilization.

Extraction and purification of polysaccharide The dried alga (10 g) was extracted with 1000 ml of 90°C hot water for 1 h. The extract was filtered by glass filter (GA-100, Advantec, Japan) after centrifugation, and concentrated to a one-third in vacuo. The concentrated extract was added into 4 volumes of ethanol with stirrer and kept at 5°C overnight. The precipitate was then dissolved in deionized water and dialyzed in tap water overnight followed by deionized water for 24 h.

The dialysate was applied to anion-exchange chromatography (DEAE Toyopearl-650M, φ 2 × 50 cm, Tosoh, Japan) and eluted with 5 ml/min of deionized water for 100 min followed by linear gradient of 0–1.5 M NaCl. Every 10 ml of elute was collected l and monitored by phenol–H_2SO_4 method at 470 nm for carbohydrate (13). Three fractions ware obtained. The obtained each collected fraction was applied to gel filtration (Toyopearl HW-75M, φ 2.5 × 95 cm, Exclusion limit: 2×10^7, Tosoh) and eluted with 10 ml/min of 0.1 M NaCl, respectively.

Chemical analysis of purified polysaccharide Protein contents of the purified polysaccharides were determined by a Bradford method (14). The sugar composition of the purified polysaccharide was identified by standard sugar using gas chromatograph-mass spectrometry (GC–MS) (15,16). The purified polysaccharides (10 mg) dissolved in 0.85 ml of water were hydrolyzed with 0.15 ml of trifluoroacetic acid (TFA) at 105°C for 15 h in screw cap tube filled with nitrogen gas. Hydrolyzed samples (0.2 ml) were dehydrated by lyophilization, and 0.1 ml of ethanethiol-TFA (2:1) was added and allowed to stand for 10 min at room temperature. Then the samples were added into 500 μl of pyridine, 0.5 ml of hexamethyldisilazane and 0.15 ml of TFA and allowed to stand again for 1 h at room temperature. The solvent was removed by nitrogen gas and 0.25 ml of water and 2 ml of hexane were added. One microliter of hexane layer was analyzed for sugar composition by GC–MS (GC: Agilent 6890, MS:JEOL GC mate II) using DB-5MS column (30 m × φ 0.25 mm, J&W) at temperature from 165°C to 235°C (2°C/min) after having been kept at 165°C for 2 min. The sugar composition of the polysaccharides was identified by retention time and mass spectrum of GC–MS. Finally, the concentration of sugar was calculated by calibration curve using the concentration of standard sugar.

Isolation by thin-layer chromatography and identification of sugar The following operations were carried out to analyze the sugar which was not identifiable by GC–MS. *Nostochopsis* hot water extract (300 mg), which was treated with 80% ethanol and dialyzed, was dissolved in 30 ml of water and hydrolyzed with 5 ml

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of TFA at 105°C for 15 h. The hydrolysate (253 mg) was applied to silica gel 60F_{254} preparative thin-layer chromatography (TLC) plate (Merck, Germany) and developed with *n*-butanol/acetone/water (4:3:1, v:v:v). After drying, the TLC plates were developed with *n*-butanol/ethyl acetate/isopropanol/water (8:4:7:3, v:v:v:v). Both sides of the preparative TLC plate were cut and visualized with 5% sulfuric acid in methanol and heated to detect sugar. The detected band was identified with standard sugar. The band with similar Rf value as unidentified sugar was collected from untreated preparative TLC and extracted with water (30 ml). The extract was dissolved in D_2O after lyophilization, and then the structure determined by the NMR spectra of COSY, HMQC, and HMBC analysis by using JNM-ECS400 (JEOL, Japan). Sodium 2,2-dimethyl-2-silapentane-5-sulfonate was used as internal standard for NMR analysis. FAB-MS analysis was performed with JMS-700 MStation (JEOL, Japan).

^{1}H NMR (D_2O, 400 MHz) spectrum data of 2-*O*-methylfucose α-Isomer: δ 5.27 (1H, d, J = 3.6 Hz, H-1), 3.71 (1H, dd, J = 10.5 and 3.2 Hz, H-3), 3.62–3.59 (1H, m, H-4), 3.59–3.55 (1H, m, H-5), 3.32–3.28 (1H, m, H-2), 3.30 (3H, s, 2-OMe), 1.07 (3H, d, J = 6.4 Hz, H-6). β-isomer: δ 4.42 (1H, d, J = 8.2 Hz, H-1), 4.04–3.98 (1H, m, H-5), 3.52–3.49 (1H, m, H-3), 3.49–3.45 (1H, m, H-4), 3.43 (3H, s, 2-OMe), 3.00 (1H, dd, J = 10.1 and 8.2 Hz, H-2), 1.05 (3H, d, J = 6.9 Hz, H-6).

Measurement of anti-hyaluronidase activity Hyaluronidase (from bovine testes, type IV-S) and compound 48/80 were purchased from Sigma Chemical Co. (MO, USA). Hyaluronic acid sodium salt (from rooster comb) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Disodium cromoglycate (DSCG) was purchased from Enzo Life Sciences, Inc. (Lausen, Switzerland). The other chemicals used were of special grade.

Hyaluronic acid sodium salt, hyaluronidase, compound 48/80, $CaCl_2$ and NaCl were dissolved in 0.1 M acetate buffer (pH 4.0). Fifty microliters of 4000 unit/ml hyaluronidase, pre-incubated in advance with 50 μl of test sample at 37°C for 20 min, were incubated with 0.1 ml of mixture of 0.5 mg/ml compound 48/80, 12.5 mM $CaCl_2$ and 0.75 N NaCl at 37°C for 20 min. After incubation, 0.25 ml of 0.8 mg/ml hyaluronic acid potassium salt was added and the reaction mixture was further incubated at 37°C for 40 min, and then the reaction was terminated by adding 0.1 ml of 0.4 N NaOH and allowed to stand for 10 min in ice-water. The inhibitory effect was determined by a modified Morgan–Elson method (17). Test samples were replaced by the buffer solution for the control, while the enzyme solution was replaced by buffer solution for the blank.

Percent of inhibition was calculated as follows:

$$\text{Inhibition (\%)} = \{(A-B)-(C-D)\}/(A-B)\times 100 \quad (1)$$

where A is OD585 without sample, B is OD585 without sample and hyaluronidase, C is OD585 sample, and D is OD585 without hyaluronidase sample.

RESULTS AND DISCUSSION

Purification of polysaccharide with anti-hyaluronidase activity of *Nostochopsis lobatus* Yield of hot water extract of *N. lobatus* was 41.6% of dried alga, and yield of the 80% ethanol treated and dialyzed extract was 39.5%.

The elution profile of the *N. lobatus* extract (284 mg) from anion exchange chromatography is shown in Fig. 1. We obtained fractions 1 (21.5 mg), 2 (22.0 mg) and 3 (84.0 mg) by anion exchange chromatography. These fractions (fractions 1, 2 and 3) eluted from gel filtration (Toyopearl HW-75M, φ 2.5 × 95 cm) showed a single peak (fractions I, II, and III), respectively. These fractions had similar elution time and were eluted almost without retention (data not shown). The molecular weight of polysaccharides of these fractions were estimated more than 2×10^7. Yields of fractions I, II and III were 2.6%, 2.7%, and 17.9%, respectively, for dried alga, and the IC_{50}s against hyaluronidase were 22.0, 10.9 and 7.18 μg/ml, respectively. Fraction III obtained the highest yield and highest inhibitory activity against hyaluronidase than the other fractions. The inhibitory activities of this fraction was 14.5 times higher than DSCG (IC_{50}: 104.4 μg/ml) which is used as a medicine.

Sugar composition of purified polysaccharide The sugar composition of each fraction was analyzed by using GC–MS. 15 h was necessary to completely hydrolyze each fractions, because of the difficulty hydrolyzing uronic acid than neutral sugar. As shown in Fig. 2, we identified xylose, fucose, glucose, glucuronic acid, mannose and galactose in each fraction using retention time and mass spectra of standard sugar, and there was a large amount of unidentified peak (peak 1 in Fig. 2) at 18.38 min. The unnumbered peaks were not sugar because they did not have

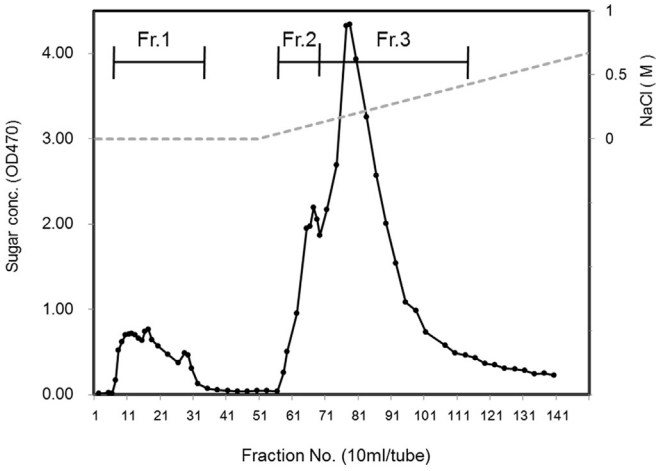

FIG. 1. Elution profile from anion-exchange chromatography of hot water extract of *Nostochopsis lobatus*.

typical mass spectra of sugar. The mass spectrum of the unidentified peak was similar to the fragment pattern of fucose (Fig. 3).

Identification of infrequent sugar In order to analyze the structure of the unidentified peak, *Nostochopsis* hot water extract, which was treated by 80% ethanol and dialyzed, was hydrolyzed with TFA, and the hydrolysate was applied preparative TLC plate. Fig. 4 shows analytical TLC of hydrolysate and standard sugars. Fucose, glucose, mannose and glucuronic acid was identified using standard sugar. The unknown band was extracted and isolated.

The ^{1}H NMR spectra suggested that the sugar was a mixture of equal amounts of α- and β-isomers, and showed the presence of a methoxy group. The COSY resulted in the complete assignment of the sugar protons, and their respective carbons were confirmed by the HMQC. The cross peaks between the 3H singlets observed at δ_H 3.30, 3.43 and δ_C 77.4, 81.5 in HMBC spectra revealed that the methoxy groups were located at the C2-position. A molecular ion peak of the isolated sugar was confirmed to be m/z 201 $[M+Na]^+$ in FABMS. Consequently, the structure of the unidentified sugar was determined as 2-*O*-methylfucose. It is the first report that 2-*O*-

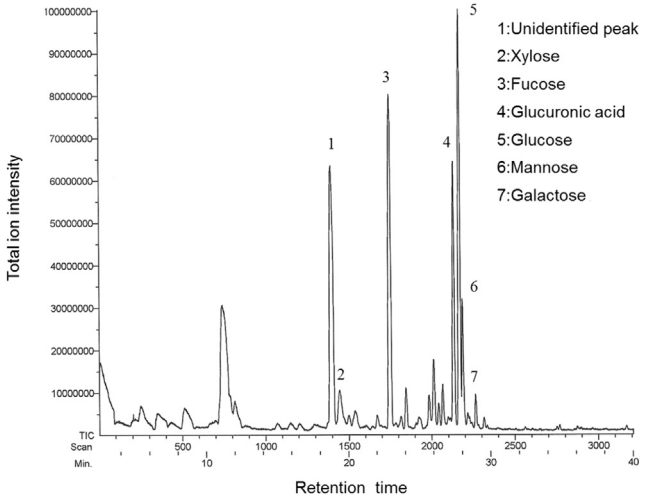

FIG. 2. TIC chromatogram of fraction III by GC–MS.