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Short Communication

Isolation and characterization of a novel polysaccharide from the mucus of the loach, *Misgurnus anguillicaudatus*

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Abstract

A method for the isolation of polysaccharides from the mucus of the loach, *Misgurnus anguillicaudatus*, has been devised. It involves the extraction of the skin mucus with tap-water (pH 7) at room temperature, followed by centrifugation, vacuum vaporization, Sevag extraction, precipitation with ethanol, vacuum filtration and lyophilization. The sediment preparation was identified to be a carbohydrate compound by several qualitative tests. Using Sephadex G-100 column (1 × 50 cm) gel filtration to treat the sediment preparation, two major constituents, P and O, were yielded. P was identified to be a novel polysaccharide and named *M. anguillicaudatus* polysaccharide (MAP) or misgurnan. Its average molecular weight ($M_w = 1.30 \times 10^5$, $M_n = 1.23 \times 10^5$) was determined by gel permeation chromatography (GPC). It was characterized by UV, IR and NMR spectroscopy. The major structural monomers of MAP were identified to be D-galactose, L-fucose and D-mannose by paper chromatography (PC) and gas chromatography (GC). Smith degradation test showed that the monoses link each other by α-1,3 bonds. This is the first report on the chemical structure of a free neutral hetero-polysaccharide in loach mucus.

Keywords: Loach; *Misgurnus anguillicaudatus*; Polysaccharide; Misgurnan; Isolation; Structural study

1. Introduction

The mucus coat of fish skin contains a variety of secretions from epidermal goblet cells and epithelial cells. These secretions have been implicated in many important biological functions (Whitear, 1984). Some vertebrate lectins were purified from the skin mucus or egg of the loach (*Misgurnus anguillicaudatus*) and it was found that they could induce release of cytoxin from fresh murine bone narrow cells or macrophages and lyse tumor cells but not normal spleen cells (Goto-Nance & Watanabe, 1995). A novel antimicrobial peptide named misgurin, which consists of 21 amino acids from the loach, *M. anguillicaudatus*, had been isolated and identified (Park, Lee, Park, Kim & Kim, 1997). The carbohydrate compounds of the skin mucus of several species of fish was examined and it was found that the sialic acids in skin mucus of the loach, *M. anguillicaudatus*, consisted predominantly of 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid (KDN). A deaminated neuraminic acid-containing glycoprotein from the skin mucus of the loach, *M. anguillicaudatus*, was isolated and characterized (Mariko, Yoichiro, Toshhisa & Makio, 1994).

We have devised a method to isolate the carbohydrate compounds from the mucus of the loach, *M. anguillicaudatus* and found a novel free polysaccharide, which has not been reported previously. We named it *M. anguillicaudatus* polysaccharide (MAP) or misgurnan. This report describes: (a) isolation and purification of the MAP from the loach mucus; (b) qualitative tests and mean molecular weight survey of the MAP by gel permeation chromatography (GPC); (c) structural characterization of the MAP by elementary analysis, UV, IR and NMR spectroscopy; and (d) elucidation of the monose component and the bond structure in the MAP from the loach mucus.

2. Materials and methods

2.1. Materials

The loach (*M. anguillicaudatus*) was purchased from the market in Wuhan City, China. The following were bought from commercial sources: Sephadex G-100, Pharmacia

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Biotech Limited (Denmark); L-fucose, L-rhamnose, D-galactose and D-mannose, Sigma Chemical (USA); Pullulan standard series P-112000, P-47300, P-22800, P-11800, P-5900, P-2700, Wako Chemical (Japan); the other analytically pure reagents, Shanghai Chemical Reagent (P.R. China).

2.2. Extraction of carbohydrate compounds in loach mucus

2500 g of live loaches were first immersed in 2500 ml of clean tap-water for 24 h at room temperature. Then, after being supersonicated for 1 h on an ultrasonic shaker (model SB2200, Shanghai, China), the loaches were removed by filtration. The extractive was centrifugated at 0°C and 8000 rpm for 10 min in a high-speed centrifuge (model RS-2III, Tomy Seiko, Tokyo, Japan), and then concentrated to 1/3 volume under vacuum subsequently. The residue was extracted with 50 ml/time Sevag reagent, $CHCl_3 - {}^{n}BuOH$ (v/v = 4 : 1) three times to deproteinize (Navarini, Gilli & Gombac, 1999). After removing the Sevag reagent, four times the volume of absolute alcohol was added to the water phase and kept at 4°C overnight in a refrigerator to precipitate carbohydrate compounds. After filtering with a Buchner funnel under vacuum and washing with absolute alcohol, the sediment was frozen at −79.5°C overnight in a super low-temperature freezer (Nuaire, Japan), and lyophilized to obtain 7.5 g of white powder preparation, which has a colour reaction with phenols in sulfuric acid or with 3,5-dinitrosalicylic acid in sodium hydroxide, but no color reaction with ninhydrin or biuret reagent. Qualitative tests showed that this sediment preparation might be free neutral reductive polysaccharides.

2.3. Isolation and purification of MAP

The dried sediment preparation(2 g) was homogenized with 100 ml of hot distilled water, and after adding to it 50 ml of anhydrous ethanol, put into a refrigerator and kept at 4°C overnight. The MAP sediment was separated by centrifugation at 10,000 rpm, washed with anhydrous ethanol for three times, frozen and lyophilized to yield fine MAP (300 mg). The MAP was further purified on a Sephadex G-100 gel column (1 × 50 cm), using HAc–NaAc buffer solution (pH 5) as eluent at a flow rate of 0.1 ml/min. The fractions were detected by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956; Staub, 1980).

2.4. General analysis methods of MAP

Specific rotations were determined at 25°C with a WZZ-2A automatic polarimeter (Shanghai Optical Instrument Factory, P. R. China); Ultraviolet spectra were recorded with a Lambda Bio-40 UV/Vis spectrometer (Perkin Elmer, USA), and infrared spectra were measured on an EQUINOX 55 infrared spectrometer (Bruker, Germany and Switzerland) with OMNIC 2.1 software; Contents of C, H, N in the polysaccharide were determined by a LECO CHN 600 elementary analyzer; Gas chromatography was conducted on an Auto System XL gas chromatograph (Perkin Elmer, USA) equipped with a C60-polysiloxane capillary column (13.2 × 0.25 mm) and a flame ionization detector, and detailed experimental conditions were as follows, H_2: 30 ml/min; air: 200 ml/min; carrier: N_2 (20 ml/min); injection temperature: 280°C detector temperature: 250°C sampling volume: 1 μl; column temperature programmed from 170 to 250°C at 3°C/min; Gas chromatography–mass spectrometry was performed on a HP-5988 instrument (Hewlett–Packard, USA) equipped with the same column as and gas chromatography (GC).

2.5. Total sugar content

Total sugar content was determined as anhydroglucose by the modified phenol–sulfuric acid method (Dubois et al., 1956; Staub, 1980) using D-galactose as a reference.

2.6. Homogeneity and molecular weight

The homogeneity and molecular weight of MAP were determined by GPC with a Waters HPLC apparatus (Waters, USA) equipped with a TSK G-3000 SW column (300 × 7.5 mm), a model 410 Refractive index detector and a Millennium-32 Workstation (Shen & Perreault, 1998) was used for the calculation of average molecular weights. The Pullulan standards (P-112000, P-47300, P-22800, P-11800, P-5900, P-2700) were used for the calibration curve. The detailed experimental conditions are column temperature: 21°C (column temperature auto-control system); column pressure: 5 Mpa (model 600 pump); injection volume: 50.00 μl; sampling volume: 20 μl; mobile phase: HAc–NaAc buffer solution (pH 5); mobile rate: 1.0 ml/min; run time: 40 min.

2.7. Analysis of monose components

MAP (10 mg) was hydrolyzed with 1 mol/l H_2SO_4 (2 ml) in a sealed tube for 15 h at 100°. The hydrolyzate was neutralized with $BaCO_3$ and filtered, the filtrate being concentrated and analyzed by PC on Xinhua No. 2 filter paper with a solvent system: ${}^{n}BtOH$–pyridine–water (v/v = 6 : 4 : 3). After the filtrate was dried, the residue was derivatized to alditol acetates in the usual way (Antoni, Emma & Susana, 1999; Leon de pinto, Martinez & Mendoza, 1996) and subjected to GC analysis. The following sugars were used as references: D-glucose, D-xylose, D-mannose, D-galactose, D-rhamnose, L-fucose, D-arabinose, and L-glucuronic acid lactone.

2.8. Periodate oxidation and smith degradation

A suspension of MAP (50 mg) in 0.015 mol/l sodium metaperiodate (50 ml) was kept at 10°C in the dark while stirring. At intervals, the periodate consumption was determined by the Fleury–Lange method (Fleury & Lange,

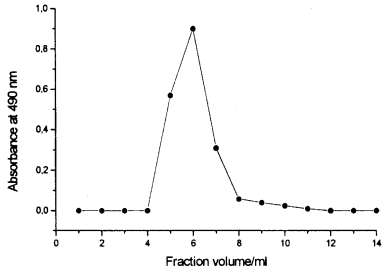

Fig. 1. Elution curve of MAP by Sephadex G-100 gel filtration.

1933). The excess of periodate was reduced with ethylene glycol after the consumption of periodate was kept constant (10 days), and the liberated formic acid was titrated with 0.01 mol/l sodium hydroxide.

After the oxidized product was dialyzed against tap-water (2 days) and distilled water (1 day), the residue in dialysis bag was filtered and re-dissolved in distilled water (20 ml), and reduced with sodium borohydride (0.2 g) for 20 h at 20°C, the excess of sodium borohydride was decomposed by addition of 10% acetic acid to pH 5.5, the reaction mixture was dialyzed against tap-water and distilled water and filtered, a white powder of MAP polyalcohol (MAP-I) was obtained. 10 mg of MAP-I was hydrolyzed with 88% formic acid (2 ml) for 3 h at 100°C and then with 1 mol/l H_2SO_4 for 8 h at 100°C in sealed tubes (formic acid was removed by evaporation prior to treatment with sulfuric acid). The mixture was neutralized with barium carbonate, filtered, and evaporated to dryness. The sugars or alcohols thus obtained were converted into their alditol acetates and subjected to gas chromatography. D-glucose, D-galactose, L-fucose, D-mannose, glycerol and erythritol were used as references.

MAP-I (20 mg) was also subjected to hydrolysis with 0.25 mol/l sulfuric acid (5 ml) for 20 h at room temperature. The acid in mixture was neutralized with 0.5 mol/l sodium hydroxide, the solution was dialyzed against de-ionized water (1.5 l) for 3 days. The fraction in dialysis bag was obtained by filtration and dried in vacuum, yielding 15 mg. The dialyzable fraction was de-ionized with Amberlite IR-118 (H^+) and IRA-410 (OH^-) resins and the eluent was concentrated and analyzed by PC on Xinhua No. 2 filter paper with a solvent system: ethyl acetate–pyridine–water (v/v = 10:4:3). The compounds on the chromatogram were located with aniline-phthalate.

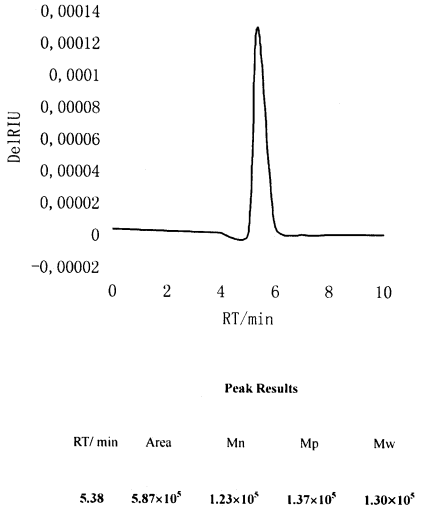

Fig. 2. GPC chromatogram of MAP on a Waters HPLC apparatus. The detailed experimental conditions are described in the text.

2.9. Permethylation analysis

80 mg of MAP was methylated three times by the method of Hakomori (Duenas-Chasco, Rudriguez-Carvajal & Tejero-Mateo, 1998; Hakomoris, 1964; Harris, Henry, Blakeney & Stone, 1984). The reaction mixture was dialyzed against tap water and distilled water, the fraction in dialysis bag was filtered, re-dissolved in $CHCl_3$ and the solution dried with anhydrous potassium carbonate, filtered, with the filtrate evaporated to dryness. The product showed no absorption for free hydroxyl group in its IR spectrum. The permethylated MAP was then hydrolyzed with 88% formic acid and 0.5 mol/l sulfuric acid as described above. The acid in hydrolyzed mixture was neutralized with barium carbonate, filtered, and the neutral hydrolyzate was evaporated to dryness. The sugars thus obtained were converted into their alditol acetates as described above for GC and GC-MS analysis.

2.10. Complex-formation of MAP with Congo Red

The complexation of MAP with Congo Red was evaluated from the shift in the maximum absorption of the dye induced by the polysaccharides in sodium hydroxide solutions at concentrations between 0 and 0.3 mol/l, according to the method of Ogawa et al. (Ogawa, Tsurigi & Watanabe, 1973; Poveda, Santamaria & Bernabe, 1997).

2.11. ^{13}C NMR spectroscopy

The spectra were recorded with a Varian-200 spectrometer operating at 500 MHz for MAP (30 mg) in D_2O (1 ml) at 25°C with external Me_2SO-d_6 and 33,920 scans (Leon de pinto, Martinez & Beltran, 2000).

3. Results and discussion

A water-soluble polysaccharide (MAP) was obtained from the skin mucus of the loach, *M. anguillicaudatus* by extraction with tap-water three times at room temperature, yielding 0.02% fresh live loach. Total sugar content was 95.7%.

MAP is a white powder, soluble in hot water and in Me_2SO with a characteristic absorption of polysaccharide at 190 nm, and no absorption at 280 and 260 nm for protein and nucleic acid. MAP is composed of C 42.21%, H 6.87%

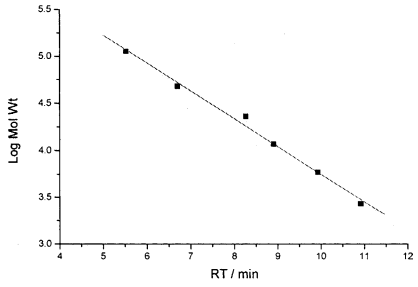

Fig. 3. The GPC calibration curve of Pullulan standards (P-112000, P-47300, P-22800, P-11800, P-5900, P-2700). The detailed experimental conditions are described in the text.

and N 0.03%. The specific rotation of MAP (*c* 0.2, distilled water) is +115°.

Sephadex G-100 gel filtration and GPC of MAP gave a single peak (Figs. 1 and 2). With the Pullulan standards (P-112000, P-47300, P-22800, P-11800, P-5900, P-2700) being used for the calibration curve (see Fig. 3), the average molecular weight of the MAP from the loach mucus is 1.30×10^5 Da.

MAP showed IR absorption at 756 cm^{-1} (α-configuration), 1120, 1130, 1090 cm^{-1} (pyranoside),1640 cm^{-1} ($\nu_C = 0$), 2920 cm^{-1} (ν_{C-H}), 3300 cm^{-1} (ν_{O-H}),, and no absorption at 890 cm^{-1} for the β-configuration. This shows that MAP consists of α-pyranoside.

MAP was completely hydrolyzed with 1 mol/l H_2SO_4 at 100°C for 15 h, PC analysis result shows that MAP is mainly composed of three monoses: D-galactose, L-fucose, and D-mannose. The resultins sugars were converted to their alditol acetates, gas chromatography analysis (with 7 monosaccharides as references) indicated that the ratio of composition of the three monoses is D-gal: L-fuc: D-man = 5 : 4 : 1. The oxidation of MAP with 0.015 mol/l sodium periodate at 10°C was completed in 10 days, periodate consumed and formic acid liberated were almost 0 mol/mol of monose residue, respectively. The oxidized MAP was reduced with sodium borohydride, to yield the corresponding polyalcohol (MAP-I). A portion of the resulting MAP-I was completely hydrolyzed with acid. The hydrolyzed products were converted into their alditol acetates; GC analysis indicated the absence of glycerol and monoses. Smith degradation of MAP-I afforded a soluble product; PC analysis showed that it contains monoses only. The nonhydrolyzed fraction, recovered with 98.5% yield (on MAP-I),

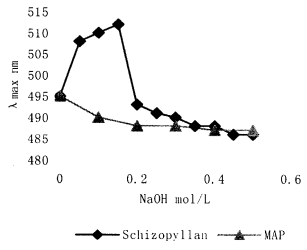

Fig. 4. Change in the absorption maximum of the Congo Red–MAP complex in various concentrations of sodium hydroxide solutions.

was resistant to further oxidation. These results indicated that MAP contains α-(1 → 3)-linked backbone, and perhaps without a side-chain on the main-chain.

Permethylated MAP was obtained by repeating the procedure of Hakomori (Duenas-Chasco et al., 1998; Hakomoris, 1964; Harris et al., 1984) three times. Then, the permethylated product was hydrolyzed with acid, and the derivatized alditol acetates of the hydrolyzate were analyzed with GC and GC-MS. The molar ratios of 2,3,4,6-tetra-, 2,4,6-tri-, and 2,4-di-*O*-methyl-D-galactose were determined to be 1:2.06: 1.21 according to the peak areas. These results indicated that MAP is a (1 → 3)- α-D-galactan, with a branched galactosyl residue at O-6 on every three galactosyl residues of the backbone.

The helical conformation of MAP was confirmed by its complex-forming ability with Congo Red. The absorption maximum of Congo Red was not shifted to longer wave length in aq. sodium hydroxide in the presence of MAP (for details see Fig. 4). This indicates that MAP does not have a helical configuration.

For ^{13}C NMR spectroscopy, solutions in 5% D_2O were used because of the better solubility of the polysaccharides and the absence of highly ordered structures, which resulted in broad signals of low intensity. The α-configuration of the D-galactose or the L-fucose residues was indicated by the C-1 resonance at 102.6 ppm, branching at C-6 by signals for C-6 at 69.4 ppm and for unsubstituted C-6 at 60.3 ppm. The multiple and broad C-3 signal at 85.9 ppm could be attributed to the presence of (1 → 3)-α-linked residues. Signals for other carbons are: 72.8 ppm (C-2), 68.1 ppm (C-4), 75.6 ppm (C-5).

(1 → 3)-α-D-Galactans are said to be potential biomedical drugs against bacterial or viral infections and exhibit antitumor activities (Ho, Lo & Leung, 2000; Lu, Yoshida & Nakashima, 2000; Sang, Chang & Young, 1999). The bioactivities such as antioxidation (Qin, Zhou, Zhao, Huang & Xu, 2001), antiviral infection and antitumor activity of MAP are now being investigated in our laboratory, the results of which will be reported elsewhere.

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