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A Novel Oligosaccharide from the Mucus of the Loach

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Abstract: A novel oligosaccharide was isolated and purified from the mucus of the loach, *Misgurnus anguillicaudatus.* It was identified by several qualitative tests and characterized by elementary analysis, UV and IR spectrum. Its average molecular weight (M_w=1539.4) was determined by gel permeation chromatography. The major structural monomers of *Misgurnus anguillicaudatus* oligosaccharide were identified to be D-galactose and L-fucose by paper chromatography and gas chromatography.

Keywords: Loach, *Misgurnus anguillicaudatus* oligosaccharide, isolation, structural study.

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[1]. Mariko Kimura *et al.* had examined the carbohydrate compositions of the skin mucus of several species of fish and found that the sialic acids in skin mucus of the loach, *Misgurnus anguillicaudatus*, consisted predominantly of 2-keto-3-deoxy-D-glcero-D-galacto-nonunic acid (KDN), a deaminated neuraminic acid-containing glycoprotein[2]. C.B. Park, *et al*, had isolated and identified a novel antimicrobial peptide named as misgurin, from the loach, *Misgurnus anguillicaudatus* which consists 21 amino acids[3]. We have devised a method to isolate the carbohydrate compositions from the mucus of the loach, *Misgurnus anguillicaudatus* and found a novel free oligosaccharide, which has not been reported previously. We named it as *misgurnus anguillicaudatus* oligosaccharide (MAO).

Extraction of carbohydrate compounds in loach mucus

2500 g live loaches were firstly immersed in 2500 mL clean tap-water for 24 h at room temperature. Then, after being supersonicated for 1 h on an ultrasonic shaker (SB2200 model, Shanghai, China), the loaches were removed by filtration. The extract was centrifugated (8000 rpm) at 0°C for 10 min., and 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) ×3 to deprotein[4]. After removing the Saveg reagent, 4 times volume of absolute alcohol was added into the water phase and kept at 4°C overnight in a refrigerator to precipitate carbohydrate compounds. Being filtrated under vacuum and

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washed with absolute alcohol, the sediment was frozen at -79.5°C overnight in a superlow-temperature freezer, and lyophilized to obtain 7.5 g white powder.

Isolation and purification of MAO

The dried sediment preparation (2 g) was homogenized with 100 mL hot distilled water, added 50 mL anhydrous ethanol, and kept at 4°C in a refrigerator overnight. The MAO sediment was separated by centrifugation at 10000 rpm, washed with anhydrous ethanol for 3 times, frozen and lyophilized to yield fine MAO (300 mg). The MAO was further purified on a Sephadex G-100 gel column (1×50 cm), using HOAc-NaOAc buffer solution (pH = 5) as eluent at the mobile rate of 0.1 mL/min. The fractions were detected by the phenol-sulfuric acid method.

Equipments and conditions for analysis of MAO

Specific rotations were determined at 25°C with a WZZ-2A automatic polarimeter (Shanghai, China); Ultraviolet spectra were recorded with a Lambda Bio-40 UV/Vis spectrometer (Perkin Elmer Co., U.S.A), and infrared spectra were measured on a EQUINOX 55 infrared spectrometer (Bruker Co., U.S.A); Contents of C, H, N in the polysaccharide were determined by a LECO CHN 600 elementary analyzer; gas chromatography was conducted on a Auto Systerm XL gas chromatography (Perkin Elmer Co., U.S.A) equipped with a C_{60}-polysiloxane capillary column (13.2 m×0.25 mm) and a flame ionization detector, and the detailed experimental conditions are 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: 1μL; column temperature programmed from 170 to 250°C at 3°C/min; Gas chromatography-mass spectrometry was performed on a HP-5988 instrument equipped with the same column as GC.

Total sugar content

Total sugar content was determined by the modified phenol-sulfuric acid method[5] using D-galactose as a reference.

Homogeneity and molecular weight

The homogeneity and molecular weight of MAO were determined by GPC with a Waters HPLC apparatus equipped with a TSK G-3000 SW column (300 mm × 7.5 mm), a 410 model detector and a Millennium-32 Workstation was used for the calculation of molecular weights. The Pullulan standards (P-112000 , P-47300 , P-22800 , P-11800, P-5900 , P-2700) were used for the calibration curve. The experimental conditions are as follows: column temperature 21°C (column temperature auto-control system); column pressure 5 Mpa (600 model pump); injection volume 50.00μL; sampling volume 20μL; mobile phase HOAc-NaOAc buffer solution (pH = 5); mobile rate 1.0 mL/min; run time 40 min.

Analysis of monose constituents

MAO (10 mg) was hydrolyzed with 1mol/L H_2SO_4 (2 mL) in a sealed tube for 15 h at 100°C. The hydrolyzate was neutralized with $BaCO_3$ and filtered, the filtrate was 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[6] 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.

Periodate oxidation and Smith degradation

A suspension of MAO (50 mg) in 0.015 mol/L sodium metaperiodate (50 mL) was kept at 10°C in the dark with stirring. At intervals, the periodate consumption was determined by the Fleury-Lange method[7]. The excess of periodate was reduced with ethylene glycol after the consumption of periodate kept constant (10 d), and the formic acid liberated was titrated with 0.01 mol/L sodium hydroxide.

The oxidized product was dialyzed against running tap-water (2 d) and distilled water (1 d), the non-dialyzable residue 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 running tap-water and distilled water and filtered , a white powder of MAO polyalcohol (MAO-I) was obtained. 10 mg of MAO-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 before treatment with sulfuric acid). The sulfuric acid was neutralized with barium carbonate, and evaporated to dryness. The sugars or alcohols thus obtained were converted into their alditol acetates and subjected to gas chromatography. Glucose, galactose, fucose, mannnose, glycerol and erythritol were used as references.

MAO-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 was neutralized with 0.5 mol/L sodium hydroxide, and the solution was dialyzed against de-ionized water (1.5 L) for 3 d. The nondialyzable fraction was obtained by filtration and dried in vacuum, yielded 15 mg. The dialyzable fraction was de-ionized with Amberlite IR-118 (H^+) and IRA-410 (OH^-) resins, 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.

Results and Discussion

A water-soluble oligosaccharide (MAO) was obtained from the skin mucus of the loach , *Misgurnus anguillicaudatus* by extraction with tap-water at room temperature, yielded 0.15% , on the basis of the fresh loach. Total sugar content was 96.8%.

MAO is a white powder, soluble in hot water and in Me_2SO with a characteristic ultraviolet absorption of polysaccharide at 190 nm, and no absorption at 280 nm and 260 nm for protein and nucleic acid. MAO is composed of C 42.21%, H 6.17% and N 0.01%. The specific rotation of MAO $[\alpha]_D^{20}$ is +45°(c 0.2, distilled water).

Sephadex G-100 gel filtration and GPC of MAO gave a single peak at 14th fraction tube. The average molecular weight (M_w) of the MAO from the loach mucus is 1.54 kDa.

MAO showed IR (KBr) absorption at 756 cm^{-1}(α-configuration), 1120 cm^{-1}, 1130 cm^{-1}, 1090 cm^{-1} (pyranoside), 1640 cm^{-1}(νc=o), 2920 cm^{-1} (νc-H), 3300 cm^{-1} (νO-H), and no absorption at 890 cm^{-1} for the β-configuration. These results show that MAP consists of α- pyranoside.

MAO was completely hydrolyzed with 1 mol/L H_2SO_4 at 100°C for 15 h, PC analysis shows that MAO is mainly composed of two monoses: D-galactose and L-fucose. The resulted sugar was converted to the alditol acetate, gas chromatography analysis (with 7 monosaccharides as references) indicated that the content ratio of the two monoses D-gal: L-fuc =5:4. The oxidation of MAO 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 per mol of monose residue, respectively. The oxidized MAO was reduced with sodium borohydride , to yield the corresponding polyalcohol (MAO-I). A portion of the resulting MAO-I was completely hydrolyzed with acid. The hydrolyzed products were converted into their alditol acetates, GC analysis indicated without the presence of glycerol and D-glucose. Smith degradation of MAO-I afforded a soluble product. PC analysis showed that it contains monoses only. The nonhydrolyzed fraction, recovered in 97.5% yield (on MAO-I), was resistant to further oxidation. These results indicated that MAO contains a α-(1→3)-linked backbone, and perhaps without a side-chain.

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