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STRUCTURAL IDENTIFICATION OF PRINCIPAL POLYSACCHARIDE COMPONENT IN WATER EXTRACT OF *PORPHYRA YEZOENSIS* UEDA

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Abstract

This work provided polysaccharide from *Porphyra yezoensis* Ueda for structural identification by biotechnological methods. Firstly, principal polysaccharide component was separated from *P. yezoensis* by water extraction and alcohol precipitation followed by chromatography. The component was identified as homogeneous polysaccharide by high performance liquid chromatography, optical rotation and ultraviolet absorption spectrophotometry. Then, structural identification was conducted by chemical methods, including periodate oxidation, reductive hydrolysis, gelatin nephelometry, methylation and desulfating analysis, and spectroscopic methods. Analysis showed that the polysaccharide component was composed of galactose (92%), as well as 3,6-inner ether galactose (3%) and 6-methyl galactose (5%) with substituents. The molecular weight was 246 kDa, and acidophobe percentage composition of 9%. It can be inferred that the polysaccharide was composed of precursor [(1→3)-β-D-galactose-(1→4)-6-OSO_3-α-L-galactose] and repetitive units of agarobiose [(1→3)-β-D-galactose-(1→4)-3,6-inner ether-2-OCH_3-α-L-galactose] and [(1→3)-β-D-galactose-(1→4)-α-L-galactose] in appropriate ratio of 9 : 9 : 1. Moreover, there were natural methoxy groups on the 2nd position of galactose residue with 1, 4 connection and the 6th position of galactose residue with 1, 3 connection.

Introduction

Polysaccharide is a macromolecule active substance widely existing in flora and fauna. At present, the ocean functional polysaccharide has become a new research hotspot. *Porphyra yezoensis* Ueda (Fam.: Bangiaceae) is mainly distributed in Liaodong Peninsula to coastal Fujian, China. Polysaccharide in *P. yezoensis* contains sulphate (Yoshizawa *et al.* 1995) which has functions of enhancing body immunity (Yoshizawa *et al.* 1995), antioxidation (Isaka *et al.* 2015), reducing blood fat (Qian *et al.* 2014), antineoplastic (Yu *et al.* 2015a), anti-inflammatory (Isaka *et al.* 2015), anti-radiation and anti-aging activities. Moreover, *P. yezoensis* can inhibit myofibrillar protein degeneration (Jiang 2014), reduce liver injury induced by carbon tetrachloride (Guo *et al.* 2007), adjust intestinal flora (Kawadu *et al.* 1995), promote lipid metabolism (Tsuge *et al.* 2004) and glucose metabolism in diabetics (Kitano *et al.* 2012). It also can be used as genetic vector of nanoparticles of positive ion (Yu *et al.* 2015b).

In this work, the principal polysaccharide component was separated from *P. yezoensis* by water extraction and alcohol precipitation to identify its primary structure.

Materials and Methods

Porphyra yezoensis collected from Lvsi Marine Area, Jiangsu Province, China, was dried and triturated after washing with purified water. After 3 hrs of backflow in alcohol, *P. yezoensis* was dried in shade. Then, water was added in solid-liquid ratio of 1 : 50 (g/m), and filtration was conducted after heat at 100ºC for 3 hrs. After being concentrated, the filtrate was centrifuged at

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4,000 rpm for 10 min, and sediment was discarded. After deproteinization was conducted by Sevage method (Staub 1965), supernatant was precipitated with ethanol and separated by centrifugation. Crude polysaccharide was obtained after freeze drying

By chromatography with DEAE-52 column (2.6 cm × 25 cm, Whatman product) at flow rate of 0.5 ml/min, the solution of crude polysaccharide was eluted with distilled water and 3 mol/l NaCl solution. Then, the eluted solution, collected in 5 ml tube, was detected by sulfuric acid method (Dubois *et al.* 1956). After dialysis for desalting, the polysaccharide component was freeze-dried. By chromatography with Sepharose CL-6B (2.6 cm × 60 cm) column at 0.1 ml/min, the solution was collected, detected and combined after being eluted with 0.02 mol/l NaCl. Two flocculent polysaccharide components were obtained by freeze-dried. As the principal component was higher in content, the first component was marked as PY-G1 and stored at 4ºC.

The purity of polysaccharide component PY-G1, in concentration of 2 mg/ml, was tested with series of KS-805 and KS-804 chromatographic column in high performance liquid chromatography (HPLC) (HP1100 type, Agilent Co., Ltd.). The mobile phase was distilled water in flow rate of 0.5 ml/min at column temperature of 25ºC. Meanwhile, analysis was conducted with polarimeter and nucleic acid protein detector. The molecular weight of PY-G1 component was detected by HPLC (Alsop and Vlachogiannis 1982). Glucose and glucan standard sample in Dextran series (SIGMA Company) were used as control.

Polysaccharide sample was desulfated by reacting with dimethyl sulfoxide, pyridine, pyromellitic acid and antimonous oxide in sequence at 120ºC, followed by dialysis and lyophilization (Miller and Blunt 1998). The resultant sample was marked as PYTS.

Gelatin nephelometry with barium chloride was conducted to determine the acidophobe content in polysaccharide PY-G1 and acidophobe component PYTS.

In periodate oxidation reaction, sodium periodate and polysaccharide was mixed under 25ºC away from light and with agitation. Sample was taken in 4, 24 and 48 hr to determine optical density at 223 nm until the value was stable (Ahrazem *et al.* 2006).

To make alkali modification of polysaccharide, PY-G1, NaOH and $NABH_4$ was mixed for 3.5 hrs at 100, followed by neutralization and dialyzed (Miller *et al.* 1995). The samples were marked as PYAM.

Monosaccharide composition was analyzed by reductive hydrolysis (Stevenson and Furneaux 1991). The sample was mixed with potassium bromide and compressed into disc to acquire FTIR spectrum in the range of 500 - 4000/cm. The sample was dissolved with D_2O and placed in AVANCE 500 type nuclear magnetic resonance spectrometer (Bruker Company, Swiss) to detect ^{13}CNMR and ^{1}HNMR spectra. In methylation analysis, sample was methylated and detected by infrared spectrum (Needs and Selvendran 1993). Fully methylated polysaccharide was extracted by adding chloroform to analyze monosaccharide composition (Stevenson and Furneaux 1991).

Results and Discussion

For the different charge characteristics of polysaccharide, crude polysaccharide showed 2 eluting peaks in long distance after DEAE-52 column. The later peak had higher polysaccharide content. Then, the later polysaccharide was further separated into two components according to molecular weight purified by Sepharose CL-6B gel column. The component with higher content was marked as PY-G1.

There are four identification methods at present, specific rotation method, ultracentrifugal method, electrophoresis method and gel filtration chromatography. The common method with high accuracy is HPLC. In general, mutual verification by more than two methods is required to ensure

the identity of polysaccharide. In this experiment, the principal component of *P. yezoensis* PY-G1 showed real pure after being detected by sulfuric acid-phenol method, and no absorption peak was seen at 260 and 280 nm UV scanning. HPLC detection showed a single peak with good symmetry, while the specific rotation determination revealed fixed specific rotation: $[\alpha]_D^{20} = -1.121°$(c 1.0 H_2O). The results show that PY-G1 is the polysaccharide with relatively homogeneous molecular weight.

The molecular weight of polysaccharide may directly affect its physicochemical property and biological activity. At present, the common methods to determine the molecular weight of polysaccharide include viscosity, osmostic pressure, light scattering, ultrafiltration and gel chromatography. The last one, gel filtration chromatography, is a method of separation according to the selection and distribution of sample in gel pores. In this experiment, the standard curve of relative molecular weight is calculated by substituting retention volume with retention time. Finally, the molecular weight of PY-G1 was determined as 246,000 Da.

By gelatin nephelometry with barium chloride, the acidophobe percentage content in PY-G1 sample was 9%, while that in PYTS sample was 2.6%. It means that the acidophobe in the latter sample has been almost eliminated.

Periodic acid can selectively oxidize and break at the joint with dihydroxy or trihydroxy to generate polysaccharide aldehyde and methanoic acid. The reaction occurs quantitatively. One molecule of periodic acid will be consumed to break one C-C bond. Therefore, the location, connection method, branch status, polymerization degree and other structural information of glucosidic bond can be determined by detecting the consumption of periodic acid and the production of formic acid. For the glycosyl bonded at 1→4 site, only one molecule of periodic acid was consumed for every glycosyl residue after periodiate oxidation without release of formic acid. On contrary, the glycosyl bonded at 1→3 site is not oxidized by periodic acid. The optical density of sodium periodate in concentration of 0.015 mol/l is about 0.6, and PY-G1 tends to be stable after 72 hrs of periodate oxidation. The optimal density of sample solution is compared with the absorption value of original sodium periodate solution. It can be obtained that 0.51 mol sodium periodate was consumed for every mol of monosaccharide residue. It indicates that the skeleton of polysaccharide is mainly composed of glactose bonded at 1→3 site or 3,6-inner ether-galactose bonded at 1→4 site in mole ratio of 1:1.

After hydrolysis, PY-G1 was acetylated for GC analysis. The chromatographic peaks and retention time of PY-G1 were compared with those in standard monosaccharide GC map. It can be seen that PY-G1 is composed of 92% galactose, 3% 3,6-inner ether-galactose and 5% 6-methyl-galactose. Sample PYTS was obtained after desulfating PY-G1. The monosaccharide was similar to that of PY-G1. Its composition included 91.4% galactose, 4% 3,6-inner ether-galactose and 3% 6-methyl galactose. Sample PYAM was obtained after modifying PY-G1 with alkali. The monosaccharide of PYAM was composed of 74.5% galactose, 19.5% 3,6-inner ether-galactose and 4.3% 6-methyl-galactose. The content of 3,6-inner ether-galactose increased significantly, indicating that there were a certain amount of sulfuric acid groups in PY-G1.

In IR spectrum of PY-G1, the broad peak at 1253.5/cm shows that there are sulphate groups. Moreover, absorption peaks at 931.5, 892.9 and 820/cm show that there are 3,6-inner ether-galactose, 1,3 bonded galactose, and acidophobe at 6 site, respectively. The peak near 896/cm is the absorption peak with β-glucopyranosidic bond characteristics, the absorption peak at 874.8/cm shows that the polysaccharide is galactopyranosyl, the absorption at 773.3/cm shows the symmetrical stretching vibration of pyranoid ring. Compared with IR spectrum of PYTS, the broad absorption peak decreases at 1253.5/cm significantly, while disappears at 820/cm. It means that the desulfating is complete. Compared with IR spectrum of PYAM, the absorption peak disappears

near 820/cm, while enhances at 931.5/cm slightly. It means that 3,6-inner ether-galactose generates after alkali treatment, and there is sulfuric acid group at 6 sites in polysaccharide.

In ^{1}H NMR of PY-G1, the signals at δ5.15-5.28 ppm, 5.28 ppm and 5.15 ppm are the anomeric proton signals of (1→4)-α-L-galactose, agarose precursor 6-OSO_3-α-L-galactose residue and 3,6-inner ether-α-L-galactose, respectively. The signal at 3.40 ppm is the methyl signal at site 6 of 6-O-methyl-β-D-galactose, and that at 3.51 ppm is the methyl at site 2 of 2-O-methyl-3 and 6-anhydro-α-L-galactose.

In ^{13}CNMR of PY-G1 and PYTS, the signals at δ105-103 are anomeric carbons in β-configuration, while those at δ100-99 are anomic carbon in α-configuration and anomeric carbon at reducing end in β-configuration. In the spectrum, there is no signal at δ97-92, indicating that the anomeric carbon at reducing end of polysaccharide PY-G1 is only in β-configuration. Signals at δ80-78 are carbon signals at site 4 of 1,4 bonded galactose, while those at δ85-80 are carbon signals at site 3 of 1,3 bonded galactose. Signals at δ74.3, 72.7 and 59.5 may generate for the methyl at site 6 of 6-O-methyl-β-D-galactose. Signals at δ82.9, 78.8 and 59.5 may be corresponding to the methyl at site 2 of 2-O-methyl-3,6-anhydro-α-L-galactose. No signal was found at δ69-68 in PYTS, indicating that there is no 6-OSO_3-α-L-galactose (Zhang *et al.* 2004 and 2005) (Tables 1, 2).

Table 1. Chemical shift assignments for the ^{13}C-NMR spectrum of PY-G1.

Residue		^{13}C chemical shift in ppm						
		C-1	C-2	C-3	C-4	C-5	C-6	Me
(G-A) unit	G	103.1	70.8	82.9	69.8	76.2	62.1	
A		99.1	70.8	80.7	78.1	76.6	70.3	
G		104.3	70.4	81.7	69.8	76.6	62.4	
L6S		102.1	69.5	71.7	79.6	70.8	68.5	
G6M		103.1	70.8	82.9	69.8	74.3	72.7	59.5
	A	99.1	70.8	80.7	78.1	76.6	70.1	
(G-A2M) unit	G	103.1	70.8	82.9	69.8	76.2	62.1	
	A2M	99.1	78.8	79.3	78.1	76.6	70.1	59.5

Table 2. Chemical shift assignments for the ^{13}C-HMR spectrum of PY-TS recorded in solvent stucture.

Residue		^{13}C chemical shift in ppm						
		C-1	C-2	C-3	C-4	C-5	C-6	Me
(G-A) unit	G	103.1	71.0	82.9	69.5	76.2	62.2	
	A	99.2	71.0	80.8	78.1	76.3	70.1	
(G6M-A) unit	G6M	104.3	71.0	82.9	69.5	74.2	73.0	59.7
	A	101.8	70.5	80.8	78.1	76.3	69.9	
(G-A2M) unit	G	103.1	71.0	82.9	69.5	76.2	62.2	
	A2M	99.2	79.3	79.3	78.1	76.3	70.1	59.7

Chloroform extraction after 7 times of methylation, the evaporated PY-G1 was conventionally hydrolyzed. GC-MS spectrum of methylated PY-G1 was obtained after acetylation. The spectrum shows that there is 1,4 bonded 3,6-inner ether-galactose, 1,4,6 bonded galactose, 1,4 bonded galactose and 1,3 bonded galactose in PY-G1 in ratio of 8.9 : 12 : 23.3 : 9.3, respectively according to peak area (Table 3). PY-G1 has bad solubility in DMSO, so it was difficult to be methylated.

Therefore, some 1,4 bonded galactose may have degraded after several times of methylation. Methylation analysis shows that the ratio of two residues, 1,3 bonded galactose and 1,4 bonded galactose is about 1:1 in PYTS (Table 4). It means that the fundamental repetitive units of this polysaccharide are similar with those of agar polysaccharide in other alga. However, the content of 1,4,6 bonded galactose residue reduced. Compared with the result of PY-G1 methylation, it can be inferred that the substitute of acidophobe is located at site 6 of 1,4,6 bonded galactose. Moreover, the residue bonded with the end group is galactose. In addition, there is natural methyoxy group at sites 2 and 6 (Chizhov *et al.* 1971).

Table 3. GC-MS spectral analysis of PY-G1.

Methylated sugar (as alditol acetates)	Type of linkage	Molar ratios	Mass fragments (m/z)
3,6-anhydro-2-O-Me-galactose	1,4→)Gal	8.9	43, 69, 85, 117, 153, 171, 187, 221
2,3,6-tri-O-Me-galactose	1,4→)Gal	1.2	43, 71, 85, 99, 101, 113, 117, 129, 173
2,4,6-tri-O-Me-galactose	1,3→)Gal	23.3	43, 45, 87, 101, 117, 129, 161, 173, 233, 245, 277
2,3- Di-O-Me-galactose	1,4,6→)Gal	9.3	43, 85, 117, 127, 161, 201, 207, 261, 281

Table 4. GC-MS spectrum analysis of PYTS.

Methylated sugar (as alditol acetates)	Type of linkage	Molar ratios	Mass fragments (m/z)
3,6-anhydro-2-O-Me-galactose	1,4→)Gal	8.0	43, 69, 85, 117, 153, 171, 219, 246
2,3,4,6-Tetra-O-Me-galactose	1→)Gal	1.4	43, 57, 71, 101, 117, 129, 145, 161, 171, 205, 219
2,3,6-Tri-O-Me-galactose	1,4→)Gal	13.5	43, 45, 85, 87, 101, 113, 117, 129, 161, 173, 203, 233
2,4,6-Tri-O-Me-galactose	1,3→)Gal	21.9	43, 45, 85, 101, 117, 129, 161, 173, 233, 245, 277
2,4-Di-O-Me-galactose	1,3,6→)Gal	1.9	43, 85, 87, 117, 129, 159, 189, 201, 233, 245, 305

The results of this experiment show that there are 1,3 and 1,4 alternately bonded repetitive units of galactose and galactan with molecular weight of 246 kDa in principal component of *P. yezoensis*. There is acidophobe at site 6 of 1,4-bonded galactose residue, methoxy group at sites 2 and 6 of 1,3-bonded galactose residue, and inner ether ring at site 3 and 6 of some 1,4-bonded galactose residue. The configuration of anomeric carbon at reducing end is β configuration. Therefore, PY-G1 is mainly composed of precursor [(1→3)-β-D-galactose-(1→4)-6-OSO_3-α-L-galactose]. Moreover, there are repetitive units of agarobiose [(1→3)-β-D-galactose-(1→4)-3,6-inner-ether-2-OCH_3-α-L-galactose] and [(1→3)]- β-D-galactose-(1→4)-α-L-galactose. The ratio of these three components is 9 : 9 : 1.

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