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Structural Features of a Polysaccharide from *Centella asiatica*

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Abstract: A polysaccharide, isolated from *Centella asiatica*, was a complicated arabinogalactan (AG), which contained a little α-(1→4)-linked Gal*p*A and α-(1→2)-linked Rha*p* residues. Based on composition and methylation analyses, partial acid hydrolysis, periodate oxidation, NMR, ESI-MS experiments, it was shown to have a backbone of β-(1→3)-linked D-Gal*p* residues, with heavily branched side chains. Ara*f* residues were linked to *O*-3 of (1→3, 6)-linked Gal*p* residues.

Keywords: Arabinogalactan, polysaccharide, *Centella asiatica*, structure.

Centella asiatica has been used as remedy for sodation, stabilization and against lepra, anabrosis[1] in the oriental countries. The low-molecular-weight constituents in *Centella asiatica* have been investigated[1]. However, no polysaccharides have been reported. 1BI was the first polysaccharide isolated from *C. asiatica*, which had immunostimulating activity *in vitro*. In present study, we report the structural features of 1BI.

Experimental

Extraction, isolation and purification

Dried *C. asiatica* (19 kg, purchased from Shanghai Medicinal Materials Cooperation, code: 000201), previously defatted with 95% alcohol, was extracted with hot water for 4 h. The solution was extensively dialyzed against water (MWCO 5000 Da). The retentate was concentrated, precipitated with EtOH, washed by acetone and vacuum-dried at 40 °C to yield a black powder (JXCA, yield 2.1 %). A portion (8.0 g) of JXCA was fractionated on DEAE-cellulose column (Cl^- type, Φ 5.0× 50 cm), eluted with H_2O, 0.1, 0.3, 0.5, 1.0 mol/L NaCl gradually. From the eluate of 0.1 mol/L NaCl, two fractions, named S1A and S1B (5.1 %, from JXCA), were obtained in turn. S1B was further isolated on DEAE-cellulose column, eluted with 0-0.3 mol/L gradient NaCl to give two fractions (1B-1 and 1B-2). Fraction 1B-1 was purified on a Sephacryl S-300 column (Φ 2.6× 90 cm) to give 1BI, which was homogeneous in HPGPC (Waters HPLC instrument system). The HPLC columns (Ultrahydrogel™ 2000 and 500 columns) were pre-calibrated using standard T-dextran (T-4~T-700) and eluted with 0.003 mol/L NaOAc (0.5 mL/min) at 30±0.1°C.

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Sugar composition and methylation analysis

Sugars were analyzed by GC after conversion of the hydrolysate into alditol acetates as described[2] previously. Methylation was performed according to the Needs method[4], and the product was analyzed by GC-MS (Shimadzu QP-5050A, db-1 column), as described[2].

Reduction and partial acid hydrolysis

Reduction was carried out with 1-cyclohexyl-3- (2-morpholinoethyl) carbodiimide metho-*p*- toluenesulphonate (CMC) and $NaBH_4$ as described by Taylor and Conrad[3]. 1BI (70 mg) was dissolved in 0.05 mol/L TFA (20 mL) and kept at 80°C for 30 min. The resulting product was dialyzed and lyophilized to give a degraded polymer (1BI-P1, 62 mg). The dialysate (1BI-O1) was fractionated on a Sephadex G-10 column (Φ 1.6× 90 cm). 1BI-P1 (60 mg) was further hydrolyzed at 100 °C in 0.05 mol/L TFA (20 mL) for 20 min. 41.5 mg 1BI-P2 was obtained. 1BI-O2 was the dialysate.

Periodate oxidation and Smith degradation

1BI-P2 was dissolved in 0.02 mol/L $NaIO_4$ and kept at 5°C in the dark for 7 days. Consumption of $NaIO_4$ was measured by spectrophotometric method[5]. The resulting mixture was neutralized, reduced, hydrolyzed with 0.2 mol/L TFA at 40 °C for 24 h and dialyzed to give a degraded polymer (1BI-P2S), as described[2] previously.

Table 1 Composition analysis of 1BI and its derivatives[b] (molar ratio)

	1BI	1BI-R[a]	1BI-P1	1BI-P1R[a]	1BI-P2	1BI-P2R[a]
Ara	1.0	1.0	1.0	1.0	—	—
Rha	0.3	0.3	0.4	0.4	1.0	1.0
Gal	2.1	2.4	2.9	3.3	7.3	8.2

[a] presence of carboxyl-reduced derivative, [b]recorded on a Shimadzu-GC-14B apparatus with a 5 % OV225/AW-DMCS-Chromosorb W column (Φ 3 mm × 2.5 m).

Results and Discussion

1BI was homogeneous in HPGPC [MW=1.9×10^5, $[\alpha]_D^{20}$ +18.6 (*c* 0.296, H_2O)]. No absorbance at 280 nm and a negative response to the Lowry experiment indicated 1BI contained no protein. Sugar composition analysis showed 1BI was composed of Ara, Rha, Gal and GalA in a molar ratio of 1.0:0.3:2.1:0.3 (**Table 1**). The *m*-hydroxylbiphenyl method[6] showed 1BI contained 9.2 % galacturonic acid. In the ^{13}C NMR spectrum, the signals were listed in **Table 3,** referring to the previous reports[7-8]. The residues molar ratio of 1BI and 1BI-R in **Table 1** suggested 1BI was an AG that contained Rha*p* & Gal*p*A residues. With acid hydrolysis of 1BI, 32 % Ara*f* residues were removed and 96 % Gal*p* residues were kept in 1BI-P1 (MW=1.8×10^5). 1BI-O1 contained 80 % (molar %) Ara. 1BI-P2 (MW=1.64×10^5) was obtained with the further acid hydrolysis of 1BI-P1. In the hydrolysis, Ara*f* residues were removed completely, while 84 % Gal*p* residues were kept. 1BI-O2 contained 68 % Ara and 30 % Gal. All the results suggested 1BI, 1BI-P1and 1BI-P2 had the same backbone consisting of Gal*p* residues. Methylation analysis (**Table 2**)

showed the ratios of (1→3, 6)-linked Gal*p* residues decreased and (1→6)-linked Gal*p* residues increased after hydrolysis, indicating arabinosyl chains were linked to *O*-3 of (1→3, 6)-linked Gal*p* residues in 1BI.

Table 2 Methylation analyses of the derivatives of original and degraded polysaccharides

Glycosyl residue	Deduced glycosyl linkage	Molar ratio		
		1BI	1BI-R	1BI-P2R
Ara*f*	t	3.0	3.0	—
	1,3	0.6	0.6	—
	1,5	1.6	1.6	—
	1,3,5	2.3	2.3	—
Rha*p*	t	0.5	0.6	0.2
	1,2	0.9	0.8	0.9
	1,2,4	1.0	1.0	1.0
Gal*p*	t	4.2	4.3	4.1
	1,3	5.0	5.0	4.9
	1,6	2.7	2.7	3.3
	1,3,6	3.7	3.7	3.1
	1,4	—	2.5	2.0

The disaccharide and trisaccharide fractions were obtained from 1BI-O1. The disaccharide fraction only contained Ara. The trisaccharide fraction was identified to mainly contain α-arabinotriose and α-Ara*f*-(1→5)-α-Ara*f*-(1→3)-β-Gal*p* with ESI-MS and GC-MS experiments. 1BI contained a large amount of terminal residues and (1→3, 6)-linked Gal*p* residues (**Table 2**), indicating 1BI had a heavily branched side chains. 1BI-P2S still had (1→3, 6)-linked Gal*p* residues in methylation analysis (not be listed), indicating the following sequence existed in the branched chain of 1BI.

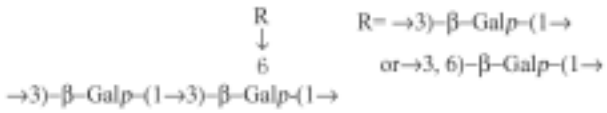

Table 3 ^{13}C NMR spectral data of acid polysaccharide 1BI in D_2O (CH_3OH in δ 49.5 ppm)*

	C-1	C-2	C-3	C-4	C-5	C-6
t-α-L-Ara*f*	109.8	80.8	77.1	84.4	61.5	—
α-(1→3)-L-Ara*f*	107.9	80.8	82.6	84.4	61.5	—
α-(1→3,5)-L-Ara*f*	107.9	80.8	82.6	84.4	69.9	—
α-(1→5)-L-Ara*f*	107.9	80.8	77.1	84.4	69.9	—
t-α-L- Rha*p*	101.3	70.7	70.7	72.3	68.4	17.1
α-(1→2)-L-Rha*p*	101.3	77.8	70.7	72.3	68.4	17.1
α-(1→2,4)-L-Rha*p*	101.3	77.8	70.7	77.3	68.4	17.3
t-β-D-Gal*p*	103.2	71.3	73.3	68.9	74.8	61.5
β-(1→3)-D-Gal*p*	104.8	71.3	81.8	68.9	75.2	61.5
β-(1→3,6)-D-Gal*p*	104.0	71.3	81.8	69.1	75.6	69.9
β-(1→6)-D-Gal*p*	104.5	71.3	73.8	69.1	75.6	69.9
α-(1→4)-D-Gal*p*A	99.1	70.7	70.6	79.8	75.6	176.1

*Assignment was made up by 2D ^1H-^1H COSY, HMQC and HMBC experiments with a Brüker AM-400 NMR spectrometer

1BI-P2S showed three main peaks: MW=4.6×10^4 (54 %, area %), 3.0×10^4 (27 %) and 2.1×10^4 (15 %) in HPGPC, which confirmed 1BI had heavily branched side chains. The ^{13}C NMR spectrum of 1BI-P2 was simpler than the spectra of 1BI and 1BI-P1: the signals of Ara*f* residues were lacking and the signals of (1→6)-Gal*p* residues increased. Those results confirmed Ara*f* was linked to *O*-3 of Gal*p* residues. In the ^{1}H NMR spectrum of 1BI, the anomeric signals of α-L-Ara*f* and β-D-Gal*p* occurred at 5.2 and 4.5 ppm, respectively.

In conclusion, 1BI had a backbone consisting of (1→3)-Gal*p* residues and (1→3, 6)-Gal*p* residues and contained a small amount of (1→4)-Gal*p*A and (1→2)-Rha*p* residues. Its side chains contained arabinosyl chains which were linked to *O*-3 of (1→3, 6)-Gal*p* residues, and galactosyl chains. The structure of 1BI was similar to that of the AG reported[9], but the difference was the ratio of the side chains. 1BI and 1BI-P2 had immuno-activity *in vitro.*

References and Notes

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