Phytochemistry, Vol. 23, No. 3, pp. 587–590, 1984.
Printed in Great Britain.

0031–9422/84 $3.00 + 0.00

CHARACTERIZATION OF A WATER-SOLUBLE GLUCAN FROM *ANGELICA ACUTILOBA*

HARUKI YAMADA, HIROAKI KIYOHARA and YASUO OTSUKA

Oriental Medicine Research Center of the Kitasato Institute, Minato-ku, Tokyo 108, Japan

(*Received* 16 *June* 1983)

Key Word Index—*Angelica acutiloba*; Apiaceae; polysaccharide; glucan.

Abstract—A water-soluble glucan, AR-Glucan, from the roots of *Angelica acutiloba* was obtained homogeneous as determined by ultracentrifugal analysis, electrophoresis, and gel filtration. AR-Glucan was composed of D-glucose, and its MW was estimated to be 13 500. Methylation analysis indicated that AR-Glucan contained 4-*O*- and 4,6-di-*O*-substituted glucosyl residues. ^{1}H and ^{13}C NMR data accorded with the results of methylation analysis, and the glycosidic linkages in AR-Glucan were shown to have the α-configuration. The results of β-amylase, α-amylase, and pullulanase treatments of AR-Glucan showed that it contained (1 → 4) linked α-D-glucosyl side chains of long chain length such as amylopectin. Thus, AR-Glucan is a (1 → 4) linked α-D-glucan to which are attached glucosyl side chains at O-6 of the glucosyl residues of the main chain.

INTRODUCTION

Angelica acutiloba Kitagawa (Yamato-Tohki) has been used in a Chinese herbal medicine for gynaecological diseases. Recently, it was reported that the crude water-soluble polysaccharide fraction from *A. acutiloba* showed mitogenic activity to B lymphocytes [1], interferon-producing activity [2], anti-tumour activity to Erlich ascites [1] and anti-complementary activity. It is known that *A. acutiloba* contains a few phthalides [3, 4] and a few coumarins [4] as low molecular components. However few studies on polysaccharides have been undertaken. We have fractionated the polysaccharides from *A. acutiloba* and obtained several polysaccharide fractions, some of which showed interferon-producing activity and anti-complementary activity [unpublished data]. *A. acutiloba* also contained a water-soluble glucan. We have now purified this glucan to elucidate its properties and structural features.

RESULTS AND DISCUSSION

Non-dialysable hot water extract from *Angelica acutiloba* was fractionated by the addition of cetyltrimethylammonium bromide in the presence of borate ion, and the resulting supernatant was further fractionated into an unabsorbed fraction and absorbed fractions by column chromatography on DEAE-Sephadex A-50 (Cl^- form). The unabsorbed fraction was the major polysaccharide and consisted of glucose, whereas the absorbed fractions consisted of arabinose, galactose, glucose and galacturonic acid. The neutral fraction was further purified by gel filtration on Sepharose CL-6B, and the major fraction (AR-Glucan) was used for the structural analysis.

AR-Glucan gave a single peak by gel filtration on Sepharose CL-6B and Pevikon zone electrophoresis, and a single spot on glass-fibre paper electrophoresis. On ultracentrifugal analysis AR-Glucan gave a broad single peak due to a low MW. From these data AR-Glucan was judged to be homogeneous. AR-Glucan showed a positive specific rotation, $[\alpha]_D^{22} + 14.0°$ (*c*1.02, H_2O), and showed characteristic absorption at 840 cm^{-1} due to α-configuration in the IR spectrum. The average MW of AR-Glucan was estimated to be 13 500 from the calibration curve which was made by elution volume of standard dextrans, T-2000, 500, 70, 40 and 10 by gel filtration of Sepharose CL-6B. The hexose content was found to be 90.1% by the phenol–sulphuric acid method with glucose as the standard. AR-Glucan contained no phosphorus, and protein content was negligible. The component sugar of AR-Glucan was determined to be only glucose by the identification on TLC of the acid hydrolysates, and on GC of the alditol acetate derivatives prepared from the hydrolysates. Because the hydrolysates of AR-Glucan were completely oxidized by D-glucose oxidase, it was estimated that AR-Glucan consisted of D-glucose. The iodine reaction of AR-Glucan was negative and periodate oxidation of AR-Glucan showed that 0.94 mol of periodiate per glucosyl residue was consumed.

AR-Glucan was methylated by the method of Hakomori [5], the fully methylated product was hydrolysed with acid, and converted into the alditol acetate. The partially methylated alditol acetates were analysed by GC and GC/MS, and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl glucitol and 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methylglucitol were identified in a molar ratio of 1.0 : 9.1 : 1.1 (Table 1). The results of methylation analysis indicated that AR-Glucan contained mainly (1 → 4) linked glucosyl residues and branching points at O-6 of (1 → 4) linked glucosyl residues.

^{1}H NMR of AR-Glucan in D_2O showed signals of anomeric protons due to α-configuration at δ 4.91 and 5.30, and the spectrum was similar to that of amylopectin from potato. An anomeric proton at δ 4.91 of AR-Glucan showed higher integral intensity than that of the amylopectin, so that it contains more branching points than amylopectin.

^{13}C NMR spectrum of AR-Glucan showed a signal at δ 102.8 (Signal A) in the region of the anomeric carbons.

Table 1. Methylation analysis of AR-glucan

Methylated alditol acetate derivates	T*	Mol %	Major mass spectral fragments (m/z)	Linkage
2,3,4,6-tetra-OMe Glc	1.0	1.0	43, 45, 71, 87, 101, 117 129, 145, 161, 205	Glc $^{1}\rightarrow$
2,3,6-tri-OMe Glc	2.3	9.1	43, 45, 87, 99, 101, 113, 117, 233	$\rightarrow^{4}$ Glc $^{1}\rightarrow$
2,3-di-OMe Glc	4.5	1.1	43, 101, 117, 261	$\rightarrow^{4}$ Glc $^{1}\rightarrow$ $\uparrow^{6}$

*Retention times of the corresponding alditol acetates relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on a OV-225 glass column. Calculated from peak areas and molecular weight of derivatives.

Signal B (δ 80.0) was assigned to C-4 of (1 → 4) linked α-D-glucosyl residues, because it was downfield by 7.5 ppm from the signal for C-4 in methyl-α-D-glucopyranoside. From the comparison with the spectrum of the amylopectin other signals of AR-Glucan at δ 76.4, 74.5, 74.2 and 63.5 were assigned to C-3, C-2, C-5 and C-6, respectively (Signals C, D, E and F, Table 2). The results of NMR studies agreed well with that of methylation analysis. The data suggest that the structure of AR-Glucan is similar to that of amylopectin.

AR-Glucan was treated with β-amylase and the reaction products were fractionated by the column of Sepharose CL-6B (Fig. 1), and fraction I and the fraction of the inner volume, II, were obtained. Glucose and maltose (in the molar ratio of 2.5:1.0) were detected in peak II by TLC and HPLC. β-Amylase is an exo-type enzyme that hydrolyses α (1 → 4) linked glucosyl residue to maltose units, but not to glucose. Therefore, maltose was digested with β-amylase using the same conditions as in the control experiment, and the reaction mixture was analysed by TLC and HPLC. The results showed that maltose was hydrolysed to glucose, but maltose which was incubated without β-amylase under the same conditions, was not hydrolysed to glucose. Because the ratios of glucose to maltose liberated from AR-Glucan or maltose by β-amylase treatment were similar in the two substrates, it was estimated that AR-Glucan liberated glucose with α-glucosidase which was a contaminant in the commercial β-amylase [6]. β-Amylase resistant fraction I was methylated by the above procedure, and the same partially methylated sugars were detected by GC, but the molar ratios of 2,3,4,6-tetra-*O*-methyl-D-glucose and 2,3,6-tri-*O*-methyl-D-glucose changed from 1.0:9.1 to 1.0:3.9 (Table 3). This result shows that AR-Glucan consists of (1 → 4) linked α-D-glucosyl main chain and side chains.

β-Amylase resistant AR-Glucan was further treated with α-amylase and the products were fractionated to the various oligosaccharides by the column of Bio-gel P-4 as shown in Fig. 2. Peak IV showed glucose and maltose by TLC. Peak III contained oligosaccharides that have α-(1 → 4) linked glucosyl residues substituted at O-6, but did not contain α-(1 → 6) linked glucooligosaccharide.

To determine the length of the side chain of AR-Glucan, it was treated with a pullulanase that specifically hydrolyses α(1 → 6)-glucosidic linkage. The reaction products were fractionated by the column of Bio-gel P-4, and various higher oligosaccharides than hexasaccharide were obtained as shown in Fig. 3. This result shows that AR-Glucan has various polymerized side chains.

Thus, the structure of AR-Glucan is considered to involve an α-(1 → 4) linked D-glucan, to which some of the residues of which various α-(1 → 4) linked D-glucooligosaccharides longer than a hexasaccharide are attached at position 6. The structure inferred was similar to amylopectin. AR-Glucan was a major glucan in the polysaccharide fraction from *A. acutiloba*. But, AR-Glucan was not shown to have anti-complementary activity, interferon-inducing activity or mitogenic activity [unpublished data]. The purification of the anti-complementary polysaccharide from *A. acutiloba* was difficult, because this glucan was often contaminated in the active fraction [unpublished data]. AR-Glucan is an α-

Table 2. ^{13}C NMR data of AR-Glucan

Sugar	Chemical shift [δ values (ppm)]					
	C-1	C-2	C-3	C-4	C-5	C-6
Methyl-α-D-glucopyranoside	102.2	74.5	76.0	72.5	74.1	63.5
Maltose*	101.0	74.3	74.6	78.5	71.6	62.5
Isomaltose*	99.4	73.3	75.0	71.3	71.3	67.4
Amylopectin	103.2	74.2	76.2	80.9	74.2	63.3
AR-Glucan	102.8	74.5	76.4	80.0	74.2	63.5
	(A)	(D)	(C)	(B)	(E)	(F)

*Values have been assigned by Usui *et al.* [17].

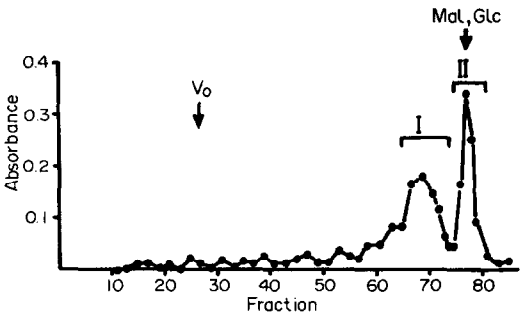

Fig. 1. Gel filtration of β-amylase digested AR-Glucan on Sepharose CL-6B. V_0 void volume; Mal, maltose; Glc, glucose; —●—, carbohydrate, 490 nm.

Table 3. Methylation analysis of AR-Glucan and β-amylase resistant AR-Glucan

Methylated alditol acetate derivatives	AR-Glucan	β-amylase resistant AR-Glucan
2,3,4,6-tetra-OMe Glc	1.0	1.0
2,3,6-tri-OMe Glc	9.1	3.9
2,3-di-OMe Glc	1.1	0.8

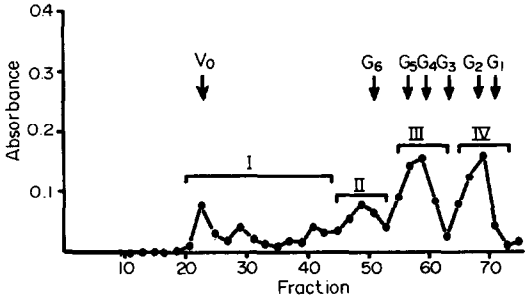

Fig. 2. Gel filtration of α-amylase digest of β-amylase resistant AR-Glucan on Bio-gel P-4. The eluate was collected in 3.8 ml fractions. V_0, void volume; G_6, glucohexaose; G_5, glucopentaose; G_4, glucotetraose; G_3, glucotriose; G_2, glucobiose; G_1, glucose; —●—, carbohydrate, 490 nm.

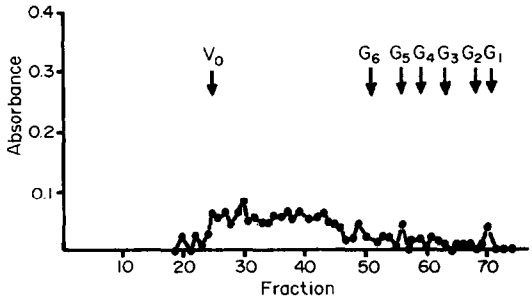

Fig. 3. Gel filtration of pullulanase digested AR-Glucan on Bio-gel P-4. —●—, carbohydrate, 490 nm.

amylase sensitive glucan, so, these enzyme digestions may be useful in the purification of the anti-complementary polysaccharide.

EXPERIMENTAL

Materials. Angelica acutiloba Kitagawa (Yamato-Tohki), which was produced in Nara Prefecture in 1981, was purchased from Uchida Wakanyaku Co. Ltd., Tokyo, Japan. Sweet potato β-amylase, *Enterobacter aerogenes* pullulanase and *Aspergillus niger* D-glucose oxidase were purchased from Sigma, and *Bacillus subtilis* α-amylase (Liquefying type) from Seikagaku Kogyo Co. Ltd., Tokyo, Japan. Standards of dextrans were purchased from Pharmacia.

General methods. TLC of mono- and oligosaccharides was performed on cellulose-coated plastic sheets (Merck, 5577) in EtOAc–pyridine–HOAc–H_2O (5:5:1:3), and sugars were detected by using alkaline silver nitrate [7]. GC (Simadzu GC-6A) was equipped with FID and a glass column (0.3 × 200 cm) of 3% ECNSS-M or 1% Silicone OV-225 on Uniport Hp at 190° or 180°, respectively. Nitrogen was used as a carrier gas at flow rate of 60 ml/min. Sugars and methylated sugars were converted into corresponding alditol acetates by heating with a mixture of pyridine and Ac_2O (1:1) at 100° for 10 min [8]. HPLC was conducted by using a Waters Model ALC/GPC 244 equipped with a column (0.4 × 30 cm) of μ Bondapak/carbohydrate (Waters Assoc. Co. Ltd.) and developed with MeCN–H_2O (7:3). Glass fibre paper electrophoresis was performed in 0.026 M borate buffer (pH 9.2) at a constant current of 5 mA/cm for 2 hr, and the polysaccharide was detected with α-naphthol–H_2SO_4 reagent [9]. Zone electrophoresis was carried out using Pevikon C-870 (polyvinyl resin, M and S instruments Inc., Japan) as the supporting medium, which was equilibrated with 0.026 M borate buffer (pH 9.2). A block (1.5 × 3 × 40 cm) was prepared and a soln of the sample in the borate buffer was placed 20 cm from the cathode and allowed to migrate at a constant current of 15 mA/cm^2 for 15 hr. After migration, the Pevikon block was cut into 40 segments, each segment was extracted with 10 ml of H_2O, and the carbohydrate content was determined by the PhOH–H_2SO_4 method [10]. The sedimentation pattern was observed at 60000 rpm by using a Hitachi Model 282 analytical ultracentrifuge equipped with a schlieren optical system. The total carbohydrate and uronic acid contents were determined by the PhOH–H_2SO_4 method [10] and the *m*-hydroxydiphenyl method [11] with glucose and galacturonic acid as the respective standards. Protein was assayed by the method of Lowry *et al.* [12] with bovine serum albumin as the standard. Phosphorus content was measured as described by Chen *et al.* [13] with Pi as the standard.

Isolation of water-soluble polysaccharides. Angelica acutiloba (300 g) was decocted with 6 l. H_2O to half vol. and the residual material was further decocted with 3 l. H_2O as above. The extracts were combined and lyophilized to give water-soluble extract (AR-0, yield 122 g). AR-0 was refluxed with 1.2 l. of MeOH for 1 hr and centrifuged to give a MeOH-insoluble ppt. The ppt. was dissolved in H_2O and then 3 vols of EtOH were added to this soln. The resulting ppt. was obtained by centrifugation. This ppt. was redissolved in H_2O and dialysed against running H_2O for 3 days. The non-dialysable portion was centrifuged to remove H_2O-insoluble material and the supernatant was lyophilized (AR-1, yield 15.8 g). AR-1 was dissolved in 0.79 l. of H_2O and half the vol. of 8% cetyltrimethylammonium bromide added to separate the polysaccharides by the method of Yamada *et al.* [14].

After standing at 20° for 24 hr, the soln was centrifuged to remove the ppt. (AR-2). The supernatant was added to an equal

vol. of 1% H_3BO_3 and the pH was adjusted to 8.8 by the addition of 1 M NaOH, followed stirring for 24 hr. The soln was centrifuged to remove ppt. (AR-3), and the resulting supernatant was acidified by HOAc and 3 vols of EtOH were added together with KOAc, and then the ppt. was obtained by centrifugation. This ppt. was dissolved in H_2O and dialysed against distilled H_2O for 2 days. The non-dialysable portion was lyophilized to obtain a water-soluble polysaccharide (AR-4, yield 1.24 g).

Ion exchange chromatography of AR-4. A sample of AR-4 (500 mg) was applied to a column (2.4 × 20 cm) of DEAE-Sephadex A-50 (Cl^- form) equilibrated with H_2O. Material was eluted first with H_2O until sugar was no longer detected, and then absorbed polysaccharide fractions were recovered by a linear gradient elution of 0 to 1 M NaCl (500 ml). The unabsorbed fraction was lyophilized to obtain crude glucan (yield 231 mg).

Gel-filtration chromatography. The crude glucan (41 mg) was applied to a column (2.6 × 100 cm) of Sepharose CL-6B, equilibrated with H_2O, and carbohydrates were eluted with H_2O. The eluates of tube No. 57 to 79 were combined and lyophilized to obtain AR-Glucan (total yield 147 mg).

Methylation analysis. AR-Glucan (2 mg) was methylated × 3 by the Hakomori procedure [5] and completeness of methylation was checked using triphenylmethane [15]. The fully methylated AR-Glucan was heated with 90% HCO_2H at 100° for 5 hr, and then with 1 M TFA at 100° for 4 hr. The hydrolysate was converted into alditol acetate. The alditol acetates were analysed by GC/MS. GC/MS was performed on a Shimadzu LKB-9000 instrument equipped with a glass column packed with 3% of Silicone OV-210 on Chromosorb W at 170°, and operated at an ionization voltage of 70 eV, a trap current 60 μA and an ion source at 310°.

NMR studies. ^1H NMR spectra were obtained for a 0.6% soln in D_2O at 90 MHz and 80°, using Varian EM-390. Chemical shifts were expressed relative to that of sodium 3-(trimethylsilyl) propane-1-sulfonate (TSP). ^{13}C NMR spectra were obtained at 25 MHz and room temp. using JEOL PS-100/EC-100 Fourier transform spectrometer with complete proton decoupling. Chemical shifts were expressed as δ values (ppm) from the signal of TSP.

Periodate oxidation. AR-Glucan (6 mg) was dissolved in 50 mM acetate buffer (pH 5.2) and the vol. was made up to 10 ml. To this solution 5.5 mg of $NaIO_4$ was added and the mixture was stirred in the dark at 4°. The consumption of IO_4^- was determined by the method of Avigad [16].

Enzymic hydrolysis. AR-Glucan (20 mg) was dissolved in 10 ml of 0.1 M acetate buffer (pH 4.0) and β-amylase added (1140 units), and then incubated at 25° for 68 hr in the presence of one drop of toluene. The incubation mixture was heated at 100° for 5 min and centrifuged to remove the insoluble residue. The supernatant was lyophilized and lyophilisate was applied to a column (2.6 × 100 cm) of Sepharose CL-6B. The fraction eluted in the inner vol. was analysed for carbohydrate structures by TLC and HPLC. The fraction eluted in the void vol. was further applied to a column (2 × 110 cm) of Bio-gel P-4 (400 mesh) and eluted with H_2O at 55° at 0.41 ml/min. The fraction eluted in the void vol. was combined and lyophilized. The lyophilisate (3.7 mg) was dissolved in 2.5 ml of 50 mM acetate buffer (pH 6.0) and α-amylase (900 units) in 1 ml of 2 mM $Ca(OAc)_2$ was added, and then the reaction mixture was incubated in the presence of one drop of toluene at 40° for 3 days. The incubation mixture was treated as in the above procedure, and the lyophilisate was applied to a column of Bio-gel P-4. The carbohydrate structure of each carbohydrate fraction was analysed by TLC, HPLC and methylation analysis. Pullulanase digestion of AR-Glucan was performed as follows: AR-Glucan (1.1 mg) was dissolved in 2 ml of 10 mM citrate buffer (pH 5.0), pullulanase (1.6 units) was added, and the mixture was incubated at 35° for 2 days. The incubation mixture was treated as in the above procedure, and the lyophilisate was applied to a column of Bio-gel P-4.

Acknowledgements—A part of this work was supported by a equipment fund from Tsumura-Juntendo Co., Ltd., Tokyo, Japan. The authors are grateful to Dr. J.-C. Cyong of our Institute for helpful discussions, and Dr. M. Nishijima, Tokyo Metropolitan Institute, for GC/MS measurements.

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