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Water-soluble Carbohydrates of Zizyphi Fructus. II.[1] Isolation of Two Polysaccharides and Structure of an Arabinan

Masashi Tomoda, Mie Takahashi (née Ōnuma) and Satomi Nakatsuka

Kyoritsu College of Pharmacy[2]

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A neutral polysaccharide and an acidic polysaccharide have been isolated from the dried fruits of *Zizyphus vulgaris* Lamarck var. *inermis* Bunge. The former, named Zizyphus-arabinan, possesses one mole of D-galactose per thirty moles of L-arabinose residues. As the results of methylation and periodate oxidation studies, it is able to concluded that the arabinan has a chain of 1→5 linked α-L-arabinofuranose units having a highly branched structure with 1→2 branch point. The component sugars of the acidic polysaccharide were D-galacturonic acid, L-rhamnose, L-arabinose, D-xylose and D-galactose.

The dried fruits of *Zizyphus* genus (Rhamnaceae) have long been used as a crude drug for the purpose of analeptic and palliative. On the constituents of this crude drug, the presences of the much amounts of D-fructose, D-glucose and oligosaccharides composed of fructose and glucose were described in the previous paper.[1] As already reported, some polysaccharides were also found in the material, and the yield of them was higher in Japanese Zizyphi Fructus than in Chinese one. We have now isolated two polysaccharides from Japanese Zizyphi Fructus, that is, the dried fruits of *Zizyphus vulgaris* Lamarck var. *inermis* Bunge, and the structure of a neutral polysaccharide is described in the present paper.

The material was extracted with hot water, and the extract was treated repeatedly on a Sephadex G-25 column. Then the solution of crude polysaccharide fraction was applied to a DEAE-cellulose (acetate form) column, and a neutral polysaccharide and an acidic polysaccharide were obtained respectively from the eluate with water and with potassium acetate solution.

Both polysaccharides gave respectively single spot on glass-fiber paper electrophoresis in alkaline borate buffer. The neutral polysaccharide was homogeneous on gel chromatography with Sephadex G-200 and the acidic polysaccharide also showed one peak on gel chromatography, but the elution volume of the latter was very near to the void volume of the Sephadex G-200 column. Gel chromatography of standard dextran fractions of known molecular weights on Sephadex G-200 has given the calibration curve shown in Fig. 1. The molecular weight of the neutral polysaccharide thus estimated was 22000.

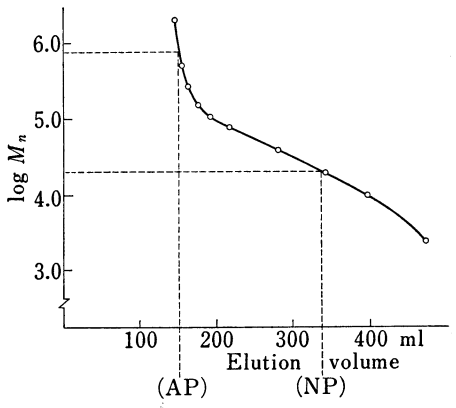

Fig. 1. Plot of Elution Volume against log *M*W for Dextran Fractions on Sephadex G-200 with 0.1 M Ammonium Formate

AP=acidic polysaccharide
NP=neutral polysaccharide (Zizyphus-arabinan)

1) Part I: M. Tomoda, H. Asakura and A. Iida, *Shôyakugaku Zasshi*, **23**, 45 (1969).
2) Location: *1-5-30, Shibakôen, Minato-ku, Tokyo, 105, Japan.*

It was shown that the neutral polysaccharide is almost composed of L-arabinose, but small amount of D-galactose is also contained as its component sugar. The acidic polysaccharide is composed of D-galacturonic acid, L-rhamnose, L-arabinose, D-xylose and D-galactose.

Quantitative determination of the components of the neutral polysaccharide, named Zizyphus-arabinan (I), showed that the molar ratio of arabinose to galactose was about 30: 1. It gave a negative specific rotation ($[\alpha]_D^{20}$—142.2° in H_2O, c=1.15).

After methylation of I with sodium hydride and methyl iodide in dimethyl sulfoxide,[3] the fully methylated I was methanolyzed and the methanolysate was analyzed by gas–liquid chromatography (GLC). Methyl glycosides of 2,3,5-tri-O-methyl-L-arabinofuranose, 2,3-di-O-methyl-L-arabinose and 3-O-methyl-L-arabinose were produced in a molar ratio of 1:4.3:3.6.

The high negative rotation of I coupled with its rapid rate of acid hydrolysis strongly suggests that arabinose units are furanose type and are connected by α-L-glycosidic linkages. From the observation of methylation study, it is able to conclude that I has a chain of 1→5 linked α-L-arabinofuranose units having a branched structure with 1→2 branch point.

As the result of periodate oxidation, 0.6 mole of periodate per one mole of component anhydro sugar unit in I was consumed with the liberation of 0.03 mole of formic acid. Smith degradation[4] of I produced glycerol and arabinose in a molar ratio of 3: 2.

Both the non-reducing terminal arabinose units and the non-branching intermediate arabinose units of I ought to consume one mole of periodate and produce one mole of glycerol as a Smith degradation product per one mole of component sugar. On the contrary, arabinose units at branching positions in I are not attacked with periodate oxidation. Therefore the periodate oxidation study shows the presences of two branching units per five arabinose units of I, and the fact that the production rate of formic acid is equivalent to the galactose content in I suggests that thirty units of L-arabinose residue possess one unit of D-galactose.

It has been reported that arabinans which occur as components of pectic substances possess branched-chain structures composed of 1→5 and 1→3 linked α-L-arabinofuranose residues.[5–9] Thus I has different branching points from those of ordinary arabinans in pectic substances, although it possesses similar properties to them on the high branched structure composed of α-L-arabinofuranose units and on the relatively low molecular weight.

Experimental

Solutions were evaporated at 40° or below with rotary evaporators under reduced pressure. Specific rotation was measured by the use of JASCO model DIP-SL automatic polarimeter. GLC was carried out by the use of Hitachi model 063 gas chromatograph equipped with hydrogen flame ion detector.

Extraction and Isolation of Polysaccharides——This was similar to that described in the previous report.[1] The dried fruits of *Zizyphus vulgaris* LAMARCK var. *inermis* BUNGE (10 g, dry weight 8.7 g), collected in Nagano prefecture, were crushed, then extracted with hot water (100 ml) for 1 hr. After suction filtration, the extraction was similarly repeated twice. The extracts were combined, concentrated to 10 ml, and applied to a column (3×100 cm) of Sephadex G–25 (Pharmacia Co., fine).

The column was eluted with water, and fractions were collected at 20 ml. The carbohydrates in eluates were measured by phenol-sulfuric acid method.[10] A crude polysaccharide fraction was obtained from tubes 14 to 22. This fraction was applied repeatedly to the similar gel chromatography with Sephadex G–25,

3) S. Hakomori, *J. Biochem.*, **55**, 205 (1964).
4) I.J. Goldstein, G.W. Hay, B.A. Lewis and F. Smith, "Methods in Carbohydrate Chemistry," Vol. V, Academic Press, New York and London, 1965, p. 361.
5) E.L. Hirst, J.K.N. Jones and E. Williams, *J. Chem. Soc.*, **1947**, 1062.
6) E.L. Hirst and J.K.N. Jones, *J. Chem. Soc.*, **1947**, 1221.
7) E.L. Hirst and J.K.N. Jones, *J. Chem. Soc.*, **1948**, 2311.
8) L. Hough and D.B. Powell, *J. Chem. Soc.*, **1960**, 16.
9) G.O. Aspinall, "The Carbohydrates, Chemistry and Biochemistry," Vol. IIB, ed. by W. Pigman and D. Horton, Academic Press, New York and London, 1970, p. 517.
10) M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith, *Anal. Chem.*, **28**, 350 (1956).

and the resulted polysaccharide fraction obtained from tubes 16 to 20 was concentrated and lyophilized. Yield, 70 mg.

The polysaccharide fraction (480 mg) was dissolved in water (20 ml) and applied to a column (2.6×100 cm) of DEAE-cellulose (Brown Co.). DEAE-cellulose was used as acetate form by the previous treatments with hydroxide and potassium acetate.[11] The column was eluted with water, followed by gradient elution with potassium acetate solution. Fractions of 50 ml were collected and analyzed by phenol-sulfuric acid method. The result is shown in Fig. 2. The eluate with water was lyophilized and an arabinan was obtained as white powder. Yield, 79.2 mg. The eluate with potassium acetate solution was dialyzed for two days, and the solution was passed through a mixed bed resin column (0.5×2 cm) containing Dowex 50W (H^+) and Dowex 2 (OH^-). After lyophilization, 142.1 mg of an acidic polysaccharide was obtained.

Glass-fiber Paper Electrophoresis——Electrophoresis was carried out with Whatman GF 83 glass-fiber paper (12×38 cm long) and with alkaline borate buffer of pH 12 (0.1N NaOH: 0.025M borax, 1:1) at the condition of 570 volt for 1 hr. Samples were applied in line at 13 cm from the anode, and moved toward the cathode. The inside of the apparatus was cooled with dry ice. *p*-Anisidine-sulfuric acid reagent[12] was used for detection. The arabinan gave one spot at a distance of 8.4 cm and the acidic polysaccharide gave one spot at a distance of 9.2 cm from the origin. Standard glucose gave a spot at a distance of 7.1 cm.

Gel Chromatography on a Sephadex Column——A column (2.6×96 cm) of Sephadex G-200 (Pharmacia Co.) was prepared and the elution was carried out with 0.1M ammonium formate as an eluant by ascending method.[13] Fractions were collected at 5 ml and analyzed by phenol-sulfuric acid method.

Rate of Acid Hydrolysis——Polysaccharides (2 mg) were hydrolyzed with 1N sulfuric acid (1 ml) in a sealed tube at 100°. After varying periods of time, the hydrolysates were analyzed for reducing activity by the method of Park and Johnson.[14] The rise of reducing activity is shown in Fig. 3.

Qualitative Analyses of Component Sugars——For hydrolysis, I was heated with 1N sulfuric acid at 100° for 2 hr, and on the other hand, the acidic polysaccharide was treated with 2N sulfuric acid at 100° for 6 hr. After neutralization with barium carbonate, filtrates were passed through a small column of Dowex 50W (H^+) for the removal of barium ion. Thin-layer chromatography (TLC) using Avicel SF cellulose was carried out and following two solvent systems were used: A, AcOEt: pyridine: AcOH: H_2O (5:5:1:3, by vol.); B, BuOH: pyridine: AcOH: H_2O (10:6:1:3, by vol.). The component sugars were revealed with silver nitrate reagent[15] and naphthoresorcinol-phosphoric acid reagent.[16]

For analysis by GLC, I was methanolyzed with 2% methanolic HCl in a sealed tube at 70° for 16 hr, and the acidic polysaccharide was treated with 4% methanolic HCl at 70° for 30 hr. Then the solutions were evaporated and treated three times with small amount of methanol followed by evaporation for the removal of HCl. The methanolysates were trimethylsilylated by the method of Sweeley, *et al.*,[17] and applied to a gas chromatograph. Following two conditions were used.

GLC: A, column, 2% OV 17 on Chromosorb W (AW, DMCS) (80 to 100 mesh) (0.3 cm×2 m long stainless steel), programmed column temperature, increase in 3° per min from 120° to 210°, carrier gas, N_2 (20 ml per min); B, column, 3% SE 52 on Chromosorb W (80 to 100 mesh) (0.3 cm×2 m long stainless steel), programmed column temperature, increase in 3° per min from 130° to 220°, carrier gas, N_2 (20 ml per min).

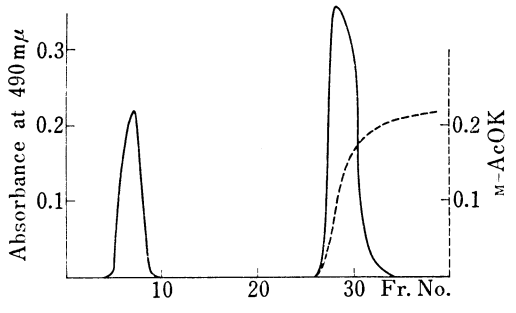

Fig. 2. Chromatogram on DEAE-cellulose

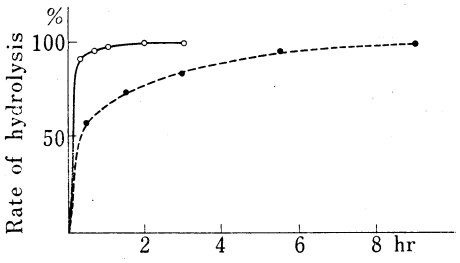

Fig. 3. Rise of Reducing Activity by Acid Hydrolysis

——: zizyphus-arabinan
------: acidic polysaccharide

11) M. Tomoda, Y. Yoshida, H. Tanaka and M. Uno, *Chem. Pharm. Bull.* (Tokyo), **19**, 2173 (1971).
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14) J.T. Park and M.J. Johnson, *J. Biol. Chem.*, **181**, 149 (1949).
15) W.E. Trevelyan, D.P. Procter and J.S. Harrison, *Nature*, **166**, 444 (1950).
16) V. Prey, H. Berbalk and M. Kausz, *Mikrochim. Acta*, **1961**, 968.
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Table I. *Rf* Values of Component Sugars and Retention Times of Their Trimethylsilyl Methyl Glycosides

	TLC		GLC	
	Solvent A	Solvent B	Condition A (2% OV 17)	Condition B (3% SE 52)
Rhamnose	0.69	0.54	10.1	9.7
Xylose	0.57	0.43	12.9	12.0
Arabinose	0.50	0.36	9.6	9.1
Galactose	0.39	0.25	16.4, 17.4, 18.2	17.2
Galacturonic acid	0.14	0.05	17.9, 18.9, 22.4	15.2, 16.3, 18.6
Hydrolysate of I	0.50	0.36		
	0.39	0.25		
Hydrolysate of acidic polysac.	0.69	0.54		
	0.57	0.43		
	0.50	0.36		
	0.39	0.25		
	0.14	0.05		
Methanolysate of I			9.6, 16.4, 17.4, 18.2	9.1 17.2
Methanolysate of acidic polysac.			9.6, 10.1, 12.9, 16.4, 17.4, 17.9, 18.2, 18.9, 22.4	9.1, 9.7, 12.0, 15.2, 16.3, 17.2, 18.6

Table I shows *Rf* values of component sugars in TLC and retention times of their trimethylsilyl methyl glycosides in GLC.

Determination of Component Sugars of I——Arabinose and galactose were estimated by GLC in the condition B after methanolysis followed by trimethylsilylation as described above. Xylitol (t_R of its trimethylsilyl derivative, 13.3) was used as an internal standard. Arabinose was also determined by orcinol method.[18] The results showed that I is composed of 96.1% of arabinose and 3.9% of galactose.

Methylation and Methanolysis——Sodium hydride (50 mg) was mixed with dimethyl sulfoxide (5 ml) and the mixture was stirred at 70° for 1 hr. The arabinan (5 mg) in dimethyl aulfoxide (5 ml) was added into mixture. After 10 min stirring at 70°, methyl iodide (4 ml) was added and the reaction mixture was stirred overnight at room temperature. The procedure were carried out in nitrogen atmosphere. After dilution with water (25 ml), the mixture was extracted with chloroform (20 ml) four times. The extract was dried with Na_2SO_4 and the solvent was evaporated. The residue was methylated again under the same condition. The infrared spectra of the final product showed no absorption near 3400 cm^{-1}. The fully methylated product was heated with 0.5N methanolic HCl in a sealed tube at 70° for 8 hr. After cooling, the solution was treated with Amberlite IR4B (OH^-) to remove HCl, then evaporated to dryness.

Analysis of Methanolysate——Chloroform solution of the methanolysate was applied to a gas chromatograph. Following two conditions were used.

GLC: C, column, 15% Polybutane-1,4-diol succinate on Chromosorb W (80 to 100 mesh) (0.3 cm × 2 m long stainless steel), column temperature, 175°, carrier gas, N_2 (20 ml per min); D, column, 5% Neopentylglycol succinate on Chromosorb G (60 to 80 mesh) (0.3 cm × 2 m long stainless steel), column temperature, 150°, carrier gas, N_2 (20 ml per min). Table II shows relative retention times of the products obtained by methanolysis of the methylated I to methyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside in the two gas chromatographic conditions.

Table II. Relative Retention Times of Methylation Products of I

	Condition C (15% BDS)	Condition D (5% NPGS)
Methyl 2,3,5-tri-O-methyl-L-arabinofuranoside	0.60	0.54
Methyl 2,3-di-O-methyl-L-arabinoside	1.87	1.27, 1.57
Methyl 3-O-methyl-L-arabinoside	3.67	3.27, 7.59

18) M. Tomoda, *Chem. Pharm. Bull.* (Tokyo), **11**, 809 (1963).

Periodate Oxidation and Smith Degradation——The sample (5 mg) was oxidized with 0.05M sodium metaperiodate (3 ml) at 5° to 7° in a dark place. The periodate consumption was measured by a spectrophotometric method.[19] The oxidation was completed after three days, then formic acid liberation was measured by a titration with 0.01N NaOH. The solution was reduced with sodium borohydride (30 mg) at 5° for 16 hr, then acetic acid was added up to pH 5, and the reaction mixture was passed through columns of Dowex 50W (H^+, 2×10 cm) and Dowex 44 (OH^-, 2×5 cm). The eluate and washing were combined and evaporated to dryness. The residue was heated with 0.2N hydrochloric acid at 100° for 6 hr, then evaporated to dryness and further treated three times with methanol followed by evaporation for the removal of acid.

Analysis of Smith Degradation Products——The half amount of the hydrolysate was dissolved in water (0.4 ml) containing hydroxylamine hydrochloride (10 mg), then heated in a sealed tube at 80° for 30 min. After evaporation, the residue was dissolved in pyridine (0.2 ml) containing trimethylolpropane (0.5 mg) as an internal standard, then subjected to trimethylsilylation by addition of hexamethyldisilazane (0.04 ml) and trimethylchlorosilane (0.02 ml). The product was applied to a gas chromatograph and determined. The molar ratio of arabinose to glycerol in the product was 1.00: 1.54.

GLC: column, 5% SE 30 on Chromosorb G (80 to 100 mesh) (0.3 cm×2 m long stainless steel); programmed column temperature, increase in 5° per min from 60° to 260°; carrier gas, N_2 (30 ml per min); t_R, glycerol 19.5; arabinose (oxime) 32.1; trimethylolpropane 23.0.

Acknowledgement The authors thank Misses S. Aoyagi and M. Kawano, for their technical assistance in a part of this work.

19) *a*) J.S. Dixon and D. Lipkin, *Anal. Chem.*, **26**, 1092 (1954); *b*) G.O. Aspinall and R.J. Ferrier, *Chem. Ind.*, **1957**, 1216.