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Isolation and Characterization of Fructans from *Polygonatum odoratum* var. *japonicum* Rhizomes

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Four new fructans have been isolated from the rhizomes of *Polygonatum odoratum* Druce var. *japonicum* Hara. They, named Polygonatum-fructans O-A, O-B, O-C, and O-D, have been shown to be composed of 29 units of fructose and 1 unit of glucose, composed of 26 units of fructose and 1 unit of glucose, composed of 18 units of fructose and 1 unit of glucose, and composed of 10 units of fructose and 1 unit of glucose.

Periodate oxidation and methylation studies showed that each fructan possesses non-reducing linear structure made up of 2→1 linked β-D-fructofuranose residues having one D-glucopyranose residue linked as in *neo*-kestose in the middle of the molecule.

As the constituents of the rhizome of *Polygonatum odoratum* Druce var. *japonicum* Hara (=*Polygonatum odoratum* Druce var. *pluriflorum* Ohwi), one of us has already reported the presences of a mucous polysaccharide, "odoratan," D-fructose, D-glucose, and polysaccharides and oligosaccharides composed of fructose and glucose.[2] The characterization of odoratan was described in that paper. Now we report the isolation and characterization of four new fructans from the neutral polysaccharide fraction which was obtained in good yield, 17.7% from dehydrated weight of the material.

After extraction of the fresh rhizomes with hot methanol, the residue was extracted with hot water. The mucilage was precipitated from the water extract by addition of ethanol, and the supernatant was concentrated, then applied to a charcoal column. Five fractions were obtained by elution with water and stepwise increments of ethanol.

The fraction eluted with water contains fructose and glucose. The fraction eluted with 6% ethanol is sucrose, and the fraction eluted with 15% ethanol contains raffinose and oligosaccharides composed of fructose and glucose. But these fractions were obtained in low yields, and the major part of the whole eluate was occupied by the fractions eluted with 20% and 25% ethanol.

The last two fractions were combined and applied to a column of Sephadex G-25. The repeated gel chromatography gave new four non-reducing polysaccharides which showed respectively single spot on double ascending cellulose thin-layer chromatography (TLC). The names "Polygonatum-fructan O-A, O-B, O-C and O-D" are proposed for the polysaccharides in order of molecular weight. They were obtained as white powder and easily soluble in water. Specific rotations of them were as follows: O-A, $[\alpha]_D^{20}$ −42.6° (H_2O, c=1), O-B, $[\alpha]_D^{20}$ −40.0° (H_2O, c=1), O-C, $[\alpha]_D^{20}$ −36.6° (H_2O, c=1) and O-D, $[\alpha]_D^{20}$ −33.3° (H_2O, c=1).

TLC of the hydrolysates of the polysaccharides and gas-liquid chromatography (GLC) of trimethylsilylated derivatives of the methanolysates revealed that the component sugars of them are fructose and glucose. The values of molecular weight obtained by the use of a vapor pressure osmometer and the results of quantitative determination of the component sugars of the polysaccharides are shown in Table I.

Owing to these results, it is able to conclude that "O-A" is composed of twenty-nine fructose units and one glucose unit, "O-B" is composed of twenty-six fructose units and one

1) Location: *1-5-30, Shibakoen, Minato-ku, Tokyo, 105, Japan.*

2) M. Tomoda, Y. Yoshida, H. Tanaka, and M. Uno, *Chem. Pharm. Bull.* (Tokyo), **19**, 2173 (1971).

Table I. Molecular Weights and Sugar Compositions

	Molecular weight	Fructose (%)	Glucose (%)
Polygonatum-fructan O–A	4940	97.5	2.6
Polygonatum-fructan O–B	4320	96.2	3.8
Polygonatum-fructan O–C	3120	95.3	4.8
Polygonatum-fructan O–D	1750	90.4	9.6

glucose unit, "O-C" is composed of eighteen fructose units and one glucose unit, and "O-D" is composed of ten fructose units and one glucose unit.

As the results of periodate oxidation, the values of periodate consumption and formic acid liberation per one mole of the component anhydrosugar unit of the polysaccharides are given in Table II. The periodate-oxidized samples were reduced with sodium borohydride[3] and the analysis of the mild hydrolysates of the products showed the presence of glycerol and no appearance of component hexose.

Table II. Mole Values of Periodate Consumption and Formic Acid Liberation per One Mole of Component Sugar Unit

	Periodate consumption	Formic acid liberation
Polygonatum-fructan O–A	1.01	0.030
Polygonatum-fructan O–B	1.04	0.039
Polygonatum-fructan O–C	1.01	0·051
Polygonatum-fructan O–D	1.05	0.064

Methylations of the polysaccharides were performed with methyl iodide and silver oxide in dimethylformamide.[4] After mild hydrolysis and methanolysis of the methylated products, the methanolysates were analyzed by TLC and GLC. In all cases, methyl 1,3,4,6-tetramethyl D-fructofuranoside, methyl 3,4,6-trimethyl D-fructofuranoside and methyl 2,3,4-trimethyl D-glucopyranoside were identified. The results of methylation study support the presumption that each fructose residue consumed one mole of periodate, while glucose residue consumed two moles of periodate with release of one mole of formic acid. By means of the digestion with β-fructofuranosidase, almost all glycosidic linkages of the polysaccharides were easily cleft. From these results, the structure illustrated in Chart 1 could be proposed to the four polysaccharides.

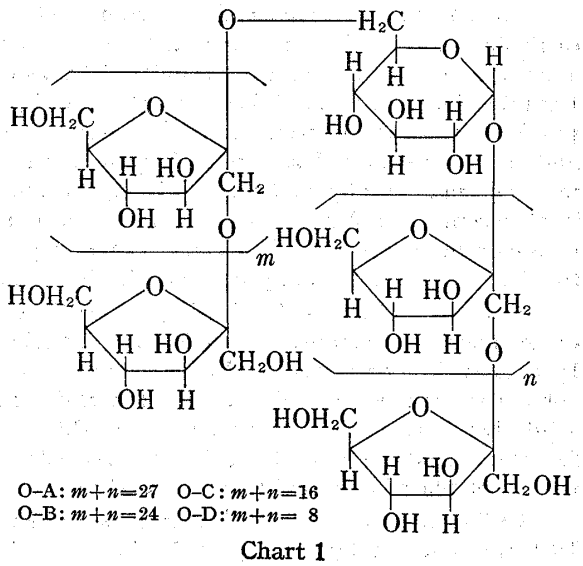

Chart 1

Fructans from various plants are divided into three groups.[5] The first is so-called inulin group which is characterised by chains of 2→1 linked β-D-fructofuranose residues. The second is phlean or levan group which has the main chain of 2→6 linked β-D-fructofuranose residues. The

3) I.J. Goldstein, G.W. Hay, B.A. Lewis, and F. Smith, "Methods in Carbohydrate Chemistry," V, Academic Press, New York, 1965, p. 361.
4) R. Kuhn, H. Trischmann, and I. Löw, *Angew. Chem.*, **67**, 32 (1955).
5) E.L. Hirst, *Proc. Chem. Soc.*, **1957**, 193.

third group is made up of the fructans having branched structure in which both β-2→1 and β-2→6 linkages are present.

The fructans from *Polygonatum odoratum* var. *japonicum* rhizomes are linear chain non-reducing polysaccharides which are mostly composed of 2→1 linked β-D-fructofuranose residues, but they differ from typical inulin-type fructan in containing a D-glucopyranose residue in the middle of the molecule. They have not terminal non-reducing D-glucopyranose residues. The sole example of fructan having such a manner of glucose linkage in the molecule is the polysaccharide from the tubers of *Cordyline terminalis*.[6] But the latter is a highly branched fructan which contains both 2→1 and 2→6 linkages. Thus the four Polygonatum fructans belong in a new class of natural fructan. O-β-D-Fructofuranosyl-(2—6)-O-α-D-glucopyranosyl-(1—2)-β-D-fructofuranoside (=*neo*-kestose) was found in the tubers of *Leucojum vernum* and *Leucojum aestivum*.[7] Polygonatum-fructans are structurally related to this trisaccharide, and the formation of the fructans may be interpreted as a transfructosylation starting from *neo*-kestose.

Experimental

Solutions were evaporated at or below 40° 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.

Isolations of Fructans——The extraction of the crushed fresh rhizomes with hot methanol followed by extraction of the residue with hot water were carried out in the same manner as described in the reported on odoratan.[2] The extraction with hot water was done twice, and combined extract was poured into two volumes of ethanol, then centrifuged. The supernatant was concentrated and lyophilized (Frac. C). Yield, 24.9% from dry material.

The water solution of Frac. C (10 g) was applied to a column (4×20 cm) of active charcoal (for chromatographic use, Wako-Junyaku Co.). Active charcoal was previously treated with hot 15% acetic acid followed by washing with hot water. The column was successively eluted with water (800 ml), 6% ethanol (1200 ml), 15% ethanol (2000 ml), 20% ethanol (2400 ml) and 25% ethanol (1600 ml). Fractions were collected at 50 ml and analyzed by phenol-sulfuric acid method.[8] The eluates obtained from the column were divided into five groups: Frac. 1, tubes 6 to 14; Fra . 2, tubes 21 to 40; Frac. 3, tubes 41 to 80; Frac. 4, tubes 81 to 128; Frac. 5, tubes 129 to 160. The yields were 0.65 g in Frac. 1, 0.09 g in Frac. 2, 0.35 g in Frac. 3, 5.01 g in Frac. 4 and 2.08 g in Frac. 5.

Frac. 4 and 5 (300 mg each) were combined and dissolved in water, then applied to a column (5×83 cm) of Sephadex G–25 (Pharmacia Co., fine) followed by elution with water and fractions collected at 20 ml. Each fraction was analyzed by cellulose TLC as described later, and following four fractions were obtained from the eluates: Frac. a, tubes 33 to 34; Frac. b, tube 35; Frac. c, tube 37; Frac. d, tubes 39 to 40. Further purifications of these fractions were respectively performed with a column (2.6×88 cm) of Sephadex G–25 and fractions collected at 5 ml. Polygonatum-fructan O–A (117 mg) was obtained from tubes 39 to 41 in re-chromatography of Frac. a. Polygonatum-fructan O–B (84 mg) was obtained from tubes 42 to 43 in re-chromatography of Frac. b. Polygonatum-fructan O–C (66 mg) was obtained from tubes 44 to 45 in re-chromatography of Frac. c. And Polygonatum-fructan O–D (78 mg) was obtained from tubes 46 to 49 in re-chromatography of Frac. d. The eluates were concentrated and lyophilized separately.

Analysis by TLC——TLC using Avicel SF cellulose was carried out in the usual way. For checking on the purity of fructans, solvent A, BuOH: pyridine: H_2O (1: 1: 1), was used, and the development followed by dryness in air was repeated once again. For the purpose of analysis of the hydrolysates, following two solvent systems were used: B, AcOEt: pyridine: AcOH: H_2O (5: 5: 1: 3); C, C_6H_5OH: 1% NH_4OH (2: 1). Hydrolysis was carried out under two conditions; heating with 0.5N sulfuric acid at 60° for 2 hr and with 2N sulfuric acid at 100° for 3 hr, then neutralized with barium carbonate. Sugars were revealed with naphtho-resorcinol–phosphoric acid reagent[9] and benzidine reagent.[10] Table III shows *Rf* values in TLC at 20°.

GLC of Methanolysate——Samples were methanolyzed with 2% methanolic HCl at 60° for 2 hr, then HCl was removed by the repeated addition and evaporation of methanol. The residue was dissolved in

6) L.A. Boggs and F. Smith, *J. Am. Chem. Soc.*, **78**, 1880 (1956).
7) H. Hammer, *Acta Chem. Scand.*, **24**, 1294 (1970).
8) M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
9) V. Prey, H. Berbalk, and M. Kausz, *Mikrochim. Acta*, **1961**, 968.
10) J.S.D. Bacon and J. Edelman, *Biochem. J.*, **48**, 114 (1951).

Table III. *Rf* Values of Polysaccharides and Component Sugars

	Solvent A	Solvent B	Solvent C
Polygonatum-fructan O–A	0.12		
Polygonatum-fructan O–B	0.21		
Polygonatum-fructan O–C	0.30		
Polygonatum-fructan O–D	0.39		
Hydrolysates with 0.5N H_2SO_4		0.54	0.46
Hydrolysates with 2N H_2SO_4		0.48, 0.54	0.35, 0.46
Glucose	0.68	0.48	0.35
Fructosd	0.70	0.54	0.46

pyridine and trimethylsilylated with hexamethyldisilazane and trimethylchlorosilane,[11] then applied to a gas chromatograph by the use of a column (0.3 cm inner diameter ×2 m long spiral stainless steel) packed with 3% SE 52 on Chromosorb W (80 to 100 mesh) and with a flow of 20 ml per min of nitrogen. The programmed temperature was increased 3° per min from 130° to 200°; t_R, trimethylsilylated methyl fructoside, 16.6; trimethylsilylated methyl glucoside. 18.6, 19.1.

Determination of Component Sugars——Fructose was determined by resorcinol method.[12] For the quantitative analysis of glucose, each sample was methanolyzed with 4% methanolic HCl at 75° for 16 hr, then evaporated repeatedly for the removal of HCl, and trimethylsilylated with pyridine, hexamethyldisilazane and trimethylchlorosilane. The product was applied to gas chromatograph by the use of a column (0.3 cm inner diameter ×2 m long spiral stainless steel) packed with 2% OV 17 on Chromosorb W (80 to 100 mesh) and with a flow of 20 ml per min of nitrogen. The programmed temperature was increased 3° per min from 120° to 200°. Myo-inositol was used as an internal standard. (t_R, trimethylsilylated methyl glucoside, 19.6; trimethylsilylated myo-inositol, 22.8).

Determination of Molecular Weight——Molecular weights were measured at 70° by the use of Knauer Vapor Pressure Osmometer. Samples were dissolved in water, and 10, 7.5, 5, and 2.5% solutions were used. Raffinose was used as an standard.

Periodate Oxidation and Smith Degradation——Each sample (4 mg) was oxidized with 0.05M sodium metaperiodate (2 ml) at room temperature in a dark place. The periodate consumption was measured by an arsenite method.[13] The oxidation was completed after two days, then the half of the solution was used for the measurement of formic acid liberation by a titration with 0.01N NaOH after addition of one drop of ethyleneglycol. The residuary half of the reaction mixture was reduced with sodium borohydride (10 mg) at 5° for 16 hr, then acetic acid was added up to pH 5. The solution was passed through a column (0.8×10 cm) of Dowex 50W (H^+) and a column (0.8×7 cm) of Dowex 44 (OH^-) successively. The eluate and washing were combined and evaporated to dryness. The residue was hydrolyzed with 0.2N hydrochloric acid at 100° for 6 hr or with 0.5N hydrochloric acid at 60° for 2 hr. After removal of HCl by addition of methanol and evaporation, the hydrolysate was trimethylsilylated and applied to a gas chromatograph using a column of 5% SE 30 in the same way as described in a former report.[14]

Methylation——Each sample (5 mg) was dissolved in dimethylformamide (2.5 ml), then methyl iodide (1 ml) and silver oxide (0.2 g) were added successively under stirring. The reaction mixture was stirred at room temperature for 2 days in a dark place. After suction filtration, methyl iodide (1 ml) and silver oxide (0.2 g) were added again into the filtrate, then the mixture was stirred at room temperature for one day in a dark place. After re-filtration, the filtrate was diluted with water (8 ml) and 10% potassium cyanide (5 ml), then extracted with chloroform (15 ml) four times. The extract was washed with water and dried with sodium sulfate, and the filtrate was evaporated to dryness. The infrared spectra of the final products had no absorption near 3400 cm^{-1}.

Analysis of Methylation Products——Each product was dissolved in the mixture of methanol (0.9 ml) and 1% oxalic acid (0.3 ml). The solution was heated in a sealed tube at 75° for 16 hr, then neutralized with calcium carbonate and filtered. The filtrate was evaporated to dryness, then dissolved in 0.5% methanolic HCl (1 ml) and left at 40° for 16 hr. HCl was removed by the repeated addition and evaporation of methanol. The methanolysate was dissolved in chloroform and applied to TLC.

TLC was carried out with Wako-gel B5 and with the solvent system of benzene: acetone (4: 1) at 32°. For detection of methylated products, the plate was sprayed with the equal volume mixture of 0.2% resorcinol in ethanol and 0.25N hydrochloric acid, and dried at 100° for a few min. The plate was again sprayed

11) C.C. Sweeley, R. Bentley, M. Makita, and W.W. Wells, *J. Am. Chem. Soc.*, **85**, 2497 (1963).
12) R.G. Kurka, *Biochem. J.*, **63**, 542 (1956).
13) P. Fleury and J. Lange, *J. Pharm. Chim.*, **17**, 107 (1933).
14) M. Tomoda and S. Nakatsuka, *Chem. Pharm. Bull.* (Tokyo), **20**, 2491 (1972).

Table IV. R_G Values and Relative Retention Times of Methylation Products

	TLC	GLC	
		15% BDS	5% NPGS
Methanolysates of Methylated Polygonatum-fructans	0.37	3.57	3.68
	0.48	2.28	2.24
	0.51	2.44	2.48
	0.88	1.05, 1.24	1.09, 1.30
Methyl 2,3,4-tri-O-methyl-D-glucopyranoside	0.34	3.16	3.25
	0.47	2.28	2.25
Methyl 3,4,6-tri-O-methyl-D-fructofuranoside	0.38	3.55	3.67
	0.51	2.44	2.47
Methyl 1,3,4,6-tetra-O-methyl-D-fructofuranoside	0.89	1.05, 1.24	1.07, 1.28

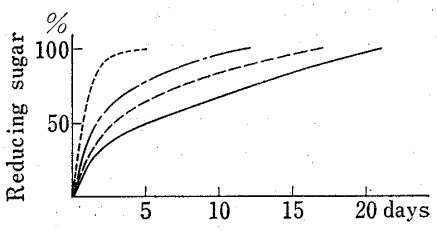

Fig. 1. Rise of Reducing Activities by Enzymic Degradation

———: polygonatum-fructan O-A
———: polygonatum-fructan O-B
———: polygonatum-fructan O-C
----------: polygonatum-fructan O-D

with 2N sulfuric acid and heated at 120° to 150°. Methyl ethers of methyl fructoside give reddish orange spots and methyl ethers of methyl aldohexoside give brownish purple spots on a pale yellow background. Table IV shows R_G values of the products to methyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside.

After separation by preparative TLC, the products eluted with methanol were applied to a gas chromatograph by the use of a column of 15% Poly-butane 1,4-diol succinate at 175° and a column of 5% Neopentylglycol succinate at 150° in the same way as described in a previous paper.[15] Relative retention times of the products to methyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside are also shown in Table IV.

Enzymic Degradation——Each sample (2 mg) was dissolved in water (0.4 ml) and β-fructofuranosidase (0.08 mg, Boehringer Co.) was added. The solution was incubated at 40°, and the hydrolysate was analyzed for reducing activity by the method of Park and Johnson[16] after varying periods of time. The rises of reducing activities are shown in Fig. 1.

15) M. Tomoda, S. Nakatsuka, and E. Minami, *Chem. Pharm. Bull.* (Tokyo), **20**, 953 (1972).
16) J.T. Park and M.J. Johnson, *J. Biol. Chem.*, **181**, 149 (1949).