[Chem. Pharm. Bull. 22(10)2306—2310(1974)] UDC 547.458.02.05 : 581.192.615.322.011.5

Constituents of the Radix of *Asparagus cochinchinensis*. I. Isolation and Characterization of Oligosaccharides

MASASHI TOMODA and NORIKO SATOH

Kyoritsu College of Pharmacy[1]

(Received March 22, 1974)

Seven oligosaccharides have been isolated from the tuberous roots of *Asparagus cochinchinensis* MERR. Determination of components, periodate oxidation, methylation, and partial degradation studies provided the evidences that they are *neo*-kestose and the other six oligosaccharides which possess non-reducing linear structure made up of 2→1 linked β-D-fructofuranose residues having a *neo*-kestose unit on the end of the molecule.

The tuberous roots of *Asparagus cochinchinensis* MERR. have been used in Chinese crude drug for the purpose of analeptic, diuretic and cough medicine. On the constituents of this material, only a few substances, that is, β-sitosterol, glucose, and fructose[2] have been reported until present time. In this paper, the isolation and characterization of seven oligosaccharides composed of fructose and glucose in addition to sucrose from the fresh roots of this plant are described.

The material roots were extracted with hot methanol, and the extract was applied to a column of Dowex 50 W for the separation into neutral and amino acid fractions. From the latter, nineteen amino acids were found and determined, and the detail will be reported in the following paper.

The neutral fraction was applied to a charcoal column, and six fractions were obtained by elution with water and stepwise increments of ethanol. The fraction eluted with water contains fructose and glucose, and the fractions eluted with 20% and 25% ethanol were obtained in low yields.

The major part of the whole eluate was occupied by the fractions eluted with 5%, 10% and 15% ethanol. The each of these three fractions was applied to a column of Sephadex G-15. The repeated gel chromatography gave seven non-reducing oligosaccharides, which showed respectively single spot on thin-layer chromatography (TLC), in addition to sucrose.

A trisaccharide (I), a tetrasaccharide (II), a pentasaccharide (III), a hexasaccharide (IV), an octasaccharide (V), a nonasaccharide (VI), and a decasaccharide (VII) were obtained, and specific rotations of them were as follows: I, $[\alpha]_D^{24}$ +16.9° (H_2O, c=4); II, $[\alpha]_D^{20}$ −3.4° (H_2O, c=4); III, $[\alpha]_D^{20}$ −8.4° (H_2O, c=4); IV, $[\alpha]_D^{20}$ −15.1° (H_2O, c=4); V, $[\alpha]_D^{20}$ −17.4° (H_2O, c=4); VI, $[\alpha]_D^{20}$ −20.2° (H_2O, c=4); VII, $[\alpha]_D^{20}$ −22.4° (H_2O, c=4). The homogeneity of I was also checked by the gas-liquid chromatography (GLC) of its trimethylsilyl derivative. Table I gives the *Rf* values on TLC and the retention time on GLC in various conditions.

TLC of the hydrolysates and GLC of the trimethylsilyl derivatives of the methanolysates of the oligosaccharides revealed that the component sugars of them are fructose and glucose. The results of quantitative determination of the component sugars and the values of molecular weight obtained by the use of a vapor pressure osmometer are shown in Table II.

In these results, the values of molecular weight of V, VI, and VII were lower than expected values, but both the determination of glucose and the estimation of formic acid liberated by periodate oxidation provided the conclusion that they are an octasaccharide, a nonasac-

1) Location: *1-5-30, Shibakoen, Minato-ku, Tokyo, 105, Japan.*
2) T. Kobayashi, T. Tomimori, T. Nakajima, and N. Yahagi, *Yakugaku Kenkyu*, **30**, 477 (1958).

NII-Electronic Library Service

Table I. *Rf* Values of Oligosaccharides and Retention Time of Trimethylsilyl Derivative of I

	Cellulose TLC (*Rf*)			GLC (t_R)	
	Solvent A	Solvent B	Solvent C	Condition A	Condition B
Oligosaccharide I	0.29	0.51	0.31	58.9	51.3
Oligosaccharide II	0.22	0.46	0.24		
Oligosaccharide III	0.17	0.42	0.19		
Oligosaccharide IV	0.15	0.39	0.17		
Oligosaccharide V	0.12	0.37	0.15		
Oligosaccharide VI	0.10	0.35	0.13		
Oligosaccharide VII	0.07	0.33	0.10		
Fructose[a]	0.51	0.62	0.48	16.8	12.1
Sucrose[a]	0.38	0.56	0.39	40.3	35.2

a) reference substances
See "Experimental" on the solvents and conditions.

Table II. Sugar Compositions and Molecular Weights of Oligosaccharides

	Fructose (%)	Glucose (%)	Molecular weight[a]
Oligosaccharide I	67.8	32.2	525
Oligosaccharide II	77.4	22.6	632
Oligosaccharide III	81.3	18.7	839
Oligosaccharide IV	84.9	15.1	992
Oligosaccharide V	87.3	12.5	1137
Oligosaccharide VI	89.1	10.9	1152
Oligosaccharide VII	90.4	9.6	1176

a) values obtained by a vapor pressure osmometer

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

	Periodate consumption	Formic acid liberation
Oligosaccharide I	1.26	0.38
Oligosaccharide II	1.20	0.27
Oligosaccharide III	1.04	0.20
Oligosaccharide IV	0.92	0.16
Oligosaccharide V	0.89	0.12
Oligosaccharide VI	0.90	0.11
Oligosaccharide VII	0.94	0.10

charide, and a decasaccharide. 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 oligosaccharides are given in Table III.

The oligosaccharides were methylated with methyl iodide and silver oxide in dimethylformamide.[3] After mild hydrolysis and methanolysis of the methylated products, the methanolysates were analyzed by GLC. In all cases but I, 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. It was revealed that the methanolysate obtained from methylated I is composed of the glycosides of 1,3,4,6-tetramethyl D-fructofuranose and 2,3,4-trimethyl D-glucopyranose.

3) R. Kuhn, H. Trischmann, and I. Löw, *Angew. Chem.*, **67**, 32 (1955).

NII-Electronic Library Service

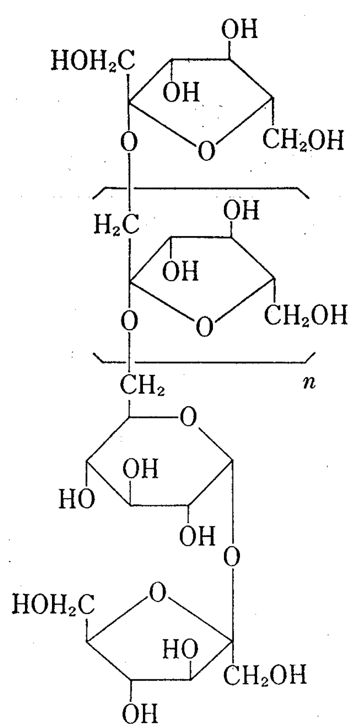

Chart 1

From these results, it is able to conclude that each oligosaccharide contains a D-glucopyranose residue, which consumes two moles of periodate with release of one mole of formic acid, in the middle of the molecule. And in addition to this aldohexose, there are two fructose units in I, three fructose units in II, four fructose units in III, five fructose units in IV, seven fructose units in V, eight fructose units in VI, and nine fructose units in VII. The values of specific rotation and the rapid rates of enzymic hydrolysis with β-fructofuranosidase strongly suggest that fructofuranose units are connected by β-D-glycosidic linkages. Thus I must be O-β-D-fructofuranosyl-(2→6)-O-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside, namely *neo*-kestose, and the other oligosaccharides possess the linear structures in which chains of 2→1 linked β-D-fructofuranose residues are joined with a *neo*-kestose unit.

Controlled acid hydrolysis of the oligosaccharides except I liberated fructose and yielded a trisaccharide which was identified with *neo*-kestose (I) by TLC and GLC. Sucrose was also found in the products, but neither iso-kestose nor glucose were able to be detected. Owing to these results, the structure illustrated in Chart 1 could be proposed to Asparagus-oligosaccharides. Thus the chain of 2→1 linked D-fructofuranose residues is combined with position 6 of D-glucopyranose in sucrose unit on the end of the molecule.

Although it may have been a mixture of two substances, the presence of a tetrasaccharide corresponding to II in the tubers of *Leucojum vernum* and *Leucojum aestivum* was reported.[4] But none of the other oligosaccharides or polysaccharides composed of a linear structure containing *neo*-kestose unit in the molecule was found in these plants. However, four new fructans having such a structural type have been obtained from the rhizomes of *Polygonatum odoratum* var. *japonicum* in our laboratory.[5] Thus Asparagus-oligosaccharides described in this report occupy the positions in the region between Polygonatum-fructans and *neo*-kestose.

Experimental

Solutions were concentrated 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.

Isolation of Oligosaccharides——The material was obtained in October of 1972 from the plants cultivated in Saitama prefecture. The fresh roots (500 g), which contain 80.5% of water, were crushed, then extracted with hot methanol (2000 ml) for 30 min. After suction filtration, the extraction was similarly repeated. The extracts were combined and concentrated to 200 ml, then applied to a column (4×15 cm) of Dowex 50W–X8 (H^+, 50 to 100 mesh). The eluates were immediately neutralized with 1N NaOH. After washing with water (800 ml), the eluates and washings were collected and concentrated, followed by lyophilization. The yield of the neutral fraction was 35.0 g. The amino acid fraction was obtained by the elution with 1N NH_4OH (1600 ml) from the column of Dowex 50W. After removal of the solvent from the eluate by repeated evaporation, the yield was 16.0 g.

The neutral fraction was dissolved in water, then applied to a column (5×40 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 (2300 ml), 5% ethanol (2100 ml), 10% ethanol (2100 ml), 15% ethanol (2100 ml), 20% ethanol (1300 ml), and 25%

4) H. Hammer, *Acta Chem. Scand.*, **24**, 1294 (1970).
5) M. Tomoda, N. Satoh, and A. Sugiyama, *Chem. Pharm. Bull.* (Tokyo), **21**, 1806 (1973).

NII-Electronic Library Service

ethanol (950 ml). Fractions were collected at 50 ml and analyzed by phenol-sulfuric acid method.[6] The eluates obtained from the column were divided into six groups: Frac. 1, tubes 14 to 42; Frac. 2, tubes 47 to 88; Frac. 3, tubes 89 to 130; Frac. 4, tubes 131 to 172; Frac. 5, tubes 173 to 198; Frac. 6, tubes 199 to 217. The yields were 7.2 g in Frac. 1, 5.1 g in Frac. 2, 9.0 g in Frac. 3, 10.3 g in Frac. 4, 2.5 g in Frac. 5, and 0.17 g in Frac. 6.

Each of Frac. 3 and 4 (0.5 g each) was dissolved in water, then applied to a column (5×77 cm) of Sephadex G-15 (Pharmacia Co., fine) followed by elution with water and fractions were collected at 20 ml. Each fraction was analyzed by cellulose TLC as described later, and following six fractions were obtained from the eluates: Frac. a, tube 32; Frac. b, tube 33; Frac. c, tube 34; Frac. d, tube 35; Frac. e, tubes 36 to 37; Frac. f, tubes 38 to 39. The water solution of Frac. 2 (0.5 g) was applied to the gel chromatography with Sephadex G-15 in the same manner as described above, and following three fractions were obtained from the eluates: Frac. f, tubes 38 to 39; Frac. g, tubes 41 to 43; Frac. h, tubes 44 to 48. After re-chromatography by the use of the similar column of Sephadex G-15, I (106 mg) was obtained from Frac. g, II (208 mg) from Frac. f, III (187 mg) from Frac. e, IV (123 mg) from Frac. d, V (116 mg) from Frac. c, VI (105 mg) from Frac. b, VII (85 mg) from Frac. a, and sucrose from Frac. h. The eluates were concentrated and lyophilized separately.

Analysis by TLC——TLC using Avicel SF cellulose was carried out in the usual way, and following three solvent systems were used: A, BuOH: pyridine: H_2O (6: 4: 3); B, BuOH: pyridine: H_2O (1: 1: 1); C, AcOEt: pyridine: AcOH: H_2O (5: 5: 1: 3). Hydrolysis and analysis of components were carried out as described in the previous paper.[5]

Analysis by GLC——Each sample was dissolved in pyridine and trimethylsilylated with hexamethyldisilazane and trimethylchlorosilane,[7] then applied to a gas chromatograph under two conditions.

GLC: Condition A, column, 3% SE 52 on Chromosorb W (80 to 100 mesh) ($0.3\ \text{cm}\times2$ m long spiral stainless steel); programmed column temperature, increase in 3° per min from 130 to 280°; carrier gas, N_2 (20 ml per min); condition B, column, 2% OV 17 on Chromosorb W (80 to 100 mesh) ($0.3\ \text{cm}\times2$ m long spiral stainless steel); programmed column temperature and carrier gas are the same as condition A. Mild methanolysis and analysis of components were performed as described previously.[5]

Determination of Component Sugars——Fructose was determined by resorcinol method.[8] Glucose was estimated by GLC using *myo*-inositol as an internal standard after methanolysis followed by trimethylsilylation as described previously.[5]

Determination of Molecular Weight——Molecular weights were measured at 60° by the use of Knauer Vapor Pressure Osmometer. Samples were dissolved in water, and 8, 6, 4, and 2% solutions were used. Raffinose was used as a standard.

Periodate Oxidation——Each sample (4 mg) was oxidized with 0.05M sodium metaperiodate (2 ml) at room temperature in a dark. The periodate consumption was measured by a spectrophotometric method.[9] The oxidation was completed after two days, then formic acid liberation was measured by a titration with 0.01N NaOH after addition of one drop of ethyleneglycol.

Methylation and Methanolysis——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 two days in a dark. 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. After re-filtration, the filtrate was diluted with water (8 ml) and 10% KCN (5 ml), then extracted with chloroform (15 ml) four times. The extract was washed with water and dried over Na_2SO_4, then the filtrate was evaporated. The infrared spectra of the products had no absorption near 3400 cm^{-1}. 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 60° for 16 hr, then neutralized with $CaCO_3$ and filtered. The filtrate was evaporated to dryness, then dissolved in 0.5% methanolic HCl (1 ml) and left at 25° for 20 hr. The solution was evaporated and HCl was removed by the repeated addition and evaporation of methanol.

Analysis of Methylation Products——After separation by preparative TLC with Wako-gel B5 and the solvent system of benzene: acetone (4: 1),[5] the products eluted with methanol were applied to a gas chromatograph under following two conditions: C, a column ($0.3\ \text{cm}\times2$ m long spiral stainless steel) packed with 15% Polybutane 1,4-diol succinate on Chromosorb W (80 to 100 mesh) at 175° with a flow of 20 ml per min of N_2; D, a column ($0.3\ \text{cm}\times2$ m long spiral stainless steel) packed with 5% Neopentylglycol succinate on Chromosorb G (60 to 100 mesh) at 150° with a flow of 20 ml per min of N_2. Table IV shows relative retention times of the products to methyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside in the two conditions.

6) M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
7) C.C. Sweeley, R. Bentley, M. Makita, and W.W. Wells, *J. Am. Chem. Soc.*, **85**, 2497 (1963).
8) R.G. Kurka, *Biochem. J.*, **63**, 542 (1956).
9) *a*) J.S. Dixon and D. Lipkin, *Anal. Chem.*, **26**, 1092 (1954); *b*) G.O. Aspinall and R.J. Ferrier, *Chem. Ind.*, **1957**, 1216.

NII-Electronic Library Service

Table IV. Relative Retention Times[a] of Methylation Products

	Condition C (15% BDS)	Condition D (5% NPGS)
Methanolyzates of methylated oligosaccharides	1.03, 1.24, 2.30, 2.41, 3.18, 3.54	1.07, 1.28, 2.24, 2.48, 3.31, 3.67
Methyl 1,3,4,6-tetra-O-methyl-D-fructofuranoside	1.05, 1.24	1.07, 1.28
Methyl 3,4,6-tri-O-methyl-D-fructofuranoside	2.44, 3.55	2.47, 3.67
Methyl 2,3,4-tri-O-methyl-D-glucopyranoside	2.28, 3.16	2.25, 3.25

a) methyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside=1.00

Enzymic Degradation——The sample (1.8 mg) was dissolved in water (0.4 ml) and β-fructofuranosidase (0.07 mg, Boehringer Co.) was added. The solution was incubated at 40° for 16 hr, and the hydrolysate was analyzed for reducing activity by the method of Park and Johnson.[10] The hydrolysis rates were 100% in II, and 90.2% in VII.

Partial Acid Hydrolysis——The sample (1 mg) was dissolved in 0.1% acetic acid (0.05 ml) and heated in a boiling water bath for 20 min. After removal of acetic acid by repeated evaporation, the hydrolysate was analyzed by TLC and its trimethylsilyl derivative was analyzed by GLC as described above. On the other hand, the sample was dissolved in 0.1% oxalic acid and heated at 60° for 10 min. After neutralization with $CaCO_3$, the filtrate was concentrated and analyzed similarly. The same products were obtained under two conditions of hydrolysis.

10) J.T. Park and M.J. Johnson, *J. Biol. Chem.*, **181**, 149 (1949).

NII-Electronic Library Service