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ISOLATION AND IDENTIFICATION OF 1-KESTOSE AND NEOKESTOSE FROM ONION BULBS

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Previously, the present author and his colleagues¹⁴⁾ studied on the changes of sugar contents in the bulbs and leaves of growing onion plants and reported the experimental results as follows: The contents of tri- and oligo-saccharides increase in the bulbs with onion growth but decrease in the leaves. However, the contents of glucose, fructose, and sucrose increase in both of the organs. These findings suggested that the mechanisms of biosynthesis, transfer, and accumulation of fructo-oligo- and fructo-poly-saccharides may be different between the individual organs of growing onion plants, and that sucrose and trisaccharides are possibly key substances responsible for the biosynthesis of higher fructo-saccharides. A study for identifying some trisaccharides in the onion bulbs had been made as the first step of biogenetical elucidation of the fructo-saccharides in onion plants, and a part of this study was briefly announced in a previous report¹⁵⁾. The present paper is concerned with the details of further studies on the same subject.

Materials and Methods

Materials

Onion (*Allium Cepa* L. cult. var. Sapporo-Yellow) bulbs were obtained from commercial sources.

Quantitative Analysis

Total hexoses, ketoses and reducing sugars were quantitatively determined by anthrone⁷⁾, ROE's¹¹⁾, and SOMOGYI-NELSON's^{9,17,18)} methods, respectively. Glucose was determined with commercial Glucostat reagent (Worthington Biochemical Corp.).

Paper and Thin Layer Chromatography

Solvent systems used for paper chromatography (ascending technique)

are (I) butanol-acetic acid-water (4:1:2) and (II) n-propanol-ethyl acetate-water (7:1:2); those for thin layer chromatography [Silica gel G, acc. to Stahl (type 60) for TLC, gypsum content ca. 13%, E. Merck, Darmstadt] are (III) butanol-isopropanol-water (10:5:4), (IV) butanol saturated with water-methanol (2:1), (V) ether-toluene (2:1), and (VI) acetone-water-conc. NH_4OH (250:3:1.5). As spray reagents for sugar detection, aniline phthalate¹⁰ and anisidine phosphate⁸ were employed.

Isolation of Oligosaccharides

Macerated onion bulbs (10 kg) were extracted with 70% ethanol containing a small amount of calcium carbonate by boiling on a water bath. The treatment was repeated four times. The resulting extract was concentrated *in vacuo* at 30–35°C to 3.5 liters, which, after addition of basic lead acetate, was allowed to stand over night. After a precipitate was removed by filtration, the filtrate was bubbled with hydrogen sulfide gas, and the resulting precipitate was filtered off. The solution was degassed, neutralized with 0.5 N sodium hydroxide, and concentrated *in vacuo* to give a sugar solution. Content of total hexoses in the concentrated sugar solution thus obtained was 410 g.

A portion of the concentrate containing 82 g of sugars as hexose was diluted with water and applied on a carbon-Celite (1:1) column (8×57 cm). The charcoal (Tokusei-Shirasagi Brand, Takeda Chemical Industries, Ltd.) and Celite-535 (Nakarai Chemical Industries, Ltd.) were used after washing with hydrochloric acid and then with water. After a large portion of glucose, fructose, and sucrose was washed out with water, oligosaccharides were eluted with 30% aqueous ethanol. The eluate was concentrated *in vacuo* and lyophilized. White powder (22 g) was obtained. The powder was re-chromatographed on a similar carbon-Celite column (5×80 cm) by successive elutions with water, 10%, 15%, and 30% ethanol to give 8 fractions, whose yields are shown in Table 1 with the results of chromatographic estimation of saccharides.

Because No. 2 fraction gave only a single spot on paper and thin-layer chromatograms, it was purified by chromatography on a column (5×80 cm; elution with 10% ethanol) packed with acid-washed carbon-Celite (1:1) to give 2.2 g of a trisaccharide. The saccharide was termed saccharide A. From No. 4 fraction (2 g), another trisaccharide (850 mg) was isolated by silica gel column chromatography [Wako gel C-200 (Wako Pure Chemical Industries, Ltd.), 5.5×85 cm column, elution with solvent III] and then by carbon-Celite (1:1) column chromatography (elution with 10% ethanol). The trisaccharide thus obtained was termed saccharide B.

TABLE 1. Re-chromatography of Fructo-oligosaccharides on a Carbon-Celite Column, and Oligosaccharides Chromatographically Detected in Eluates

Fraction No.	Eluent (lit.)	Yield* (g)	Oligosaccharides detected in eluates**)
1	10% Ethanol (3.3)	3.257	Sucrose and small amounts of trisaccharide
2	"	(1.6) 3.010	Trisaccharide (Saccharide A)
3	"	(0.8) 0.368	Small amounts of trisaccharide; and tetrasaccharides
4	"	(4.4) 7.650	Trisaccharide (Saccharide B); tetra- and penta-saccharides
5	15% Ethanol (3.9)	1.133	Tetra-, penta-, hexa-, and hepta-saccharides
6	"	(0.5) 0.374	Small amounts of tetrasaccharides; and hexasaccharides
7	"	(0.7) 2.100	Tetra-, penta-, hexa-, and hepta-saccharides
8	30% Ethanol (2.3)	3.413	Penta- and higher-saccharides

*) On dry basis.

***) Estimated by paper- and thin layer-chromatographic examinations.

Methylation and Methanolysis

Methylation of the isolated oligosaccharides was carried out by HAKOMORI's method⁹. A solution of 250 mg of the isolated oligosaccharides in 12 ml of dimethyl sulfoxide (DMSO) was prepared by stirring under a stream of nitrogen. On the other hand, to prepare a solution containing carbanion, a mixture of 500 mg of sodium hydride and 5 ml of DMSO was stirred in a flask under a nitrogen stream for 1 hr at 50°C. After the solution thus obtained was added to 12 ml of the former saccharide solution and then stirred for 3.5–5 hr at 20°C, 3 ml of methyl iodide was added and stirred for additional 15 hr. Then, the reaction mixture was diluted with water and extracted with chloroform. The chloroform extract was, after washing with water, concentrated *in vacuo* to give methylated products as a sirupy residue. The methylated products were purified by silica gel column chromatography (Wako gel C-200, 3.5 × 70 cm column, elution with solvent V).

The purified methylated saccharide was methanolized by heating with 3% methanolic hydrochloric acid for 30 min at 90°C. The reaction mixture was treated with Amberlite IR-120 and IRA-410 to remove the hydrochloric acid, and then evaporated *in vacuo* to dryness to yield methanolizates.

Gas Chromatography of Methanolizate

The methanolizates were dissolved in a small amount of methanol and gas-chromatographed: a Hitachi K 53 Type Gas-chromatogram apparatus;

column, stainless steel (3 mm × 1 m); column packing, 15% butane-1, 4-diol succinate polyester on acid-washed Celite; flow rate of carrier nitrogen gas, 60 ml/min.

Hydrolysis

(1) Acid Hydrolysis

Ten mg of the isolated oligosaccharide was dissolved in 1 ml of 0.1 N hydrochloric acid solution and completely hydrolyzed by heating for 30 min at 100°C.

Also, 10 mg of the permethylated saccharide was suspended in 1 ml of 1 N sulfuric acid and completely hydrolyzed by heating for 7 hr at 100°C.

(2) Enzymic Hydrolysis

Five mg of the isolated oligosaccharide was hydrolyzed by incubation for 15 hr at 30°C in 0.2 ml of β -fructofuranosidase solution. The enzyme solution had been previously prepared from 2 mg of the enzyme (Sigma VI, yeast β -fructofuranosidase) and 1 ml of McILVAINE buffer (pH 5.5).

Results and Discussion

By paper and thin layer chromatography, the purely isolated oligosaccharides A and B were revealed to be homogeneous, respectively.

Several properties of the two saccharides are shown in Table 2. Both of the saccharides did not show any reducing power and the products resulting from the hydrolysis with acid or with yeast β -fructofuranosidase were paper-chromatographically confirmed to be glucose and fructose. The degrees of polymerization, D. P. 3, shown in Table 2 were determined by R_S -values of the isolated saccharides and by molar ratios of reducing sugars to glucose or to ketose in the acid-hydrolyzates of the isolated saccharides. Thus, both of the saccharides A and B were found to be trisaccharides

TABLE 2. Several Properties of Fructo-oligosaccharides Isolated from Onion Bulbs

Saccharide	[α] _D ²⁵	R_S -value*)	Reducing power	Molar ratio in hydrolyzate**)		D. P.***)
				Reducing sugar/Glucose	Reducing sugar/Ketose	
A	+29.6	0.71	Nil	3.05	1.56	3
B	+19.7	0.79	Nil	3.08	1.53	3

*) Relative R_f -values (R_f of sucrose=1) when thin layer chromatography (triple development) was carried out with the solvent system III.

***) Products obtained by the complete acid hydrolysis.

***) Degree of polymerization.

comprised of 1 mole of glucopyranose and 2 moles of β -fructofuranose.

Elemental analyses of the permethylates of the saccharides A and B gave the following values, which agreed with the calculated ones, respectively :

Saccharide A : C, 53.16%, H, 8.08%

Saccharide B : C, 53.02%, H, 7.95%

Calcd. for permethylated trisaccharides, $C_{29}H_{64}O_{16}$: C, 52.88%, H, 8.26%

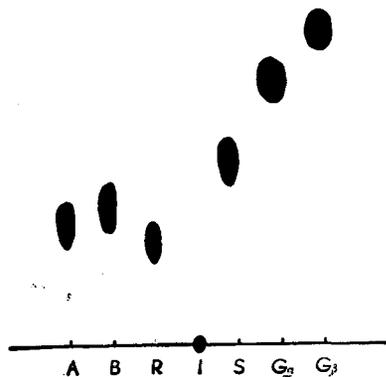


Fig. 1. Thin Layer Chromatogram of Saccharides Permethylated by HAKOMORI's Method.

Thin layer chromatography was carried out with the solvent V.

A, B, R, I, S, G_{α} , and G_{β} are the permethylates of the saccharide A, saccharide B, raffinose, inulin, sucrose, methyl- α -D-glucoside, and methyl- β -D-glucoside, respectively.

Spraying reagent : 10% H_2SO_4 .

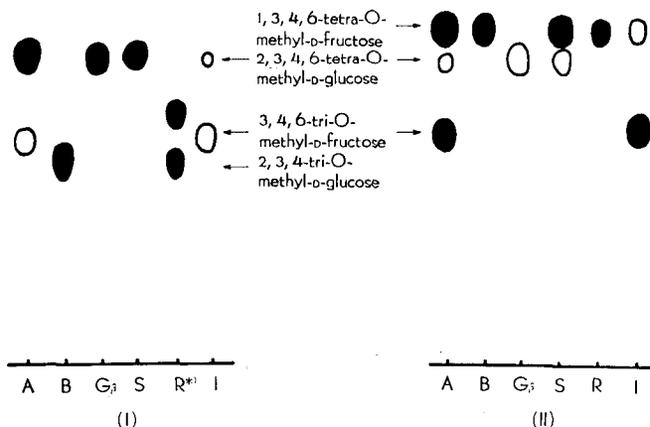


Fig. 2. Examinations of Hydrolyzates of Methylated Saccharides by Thin Layer Chromatography.

Thin layer chromatography was carried out with the solvent system VI.

(I): Sprayed with aniline phthalate.

(II): Sprayed with anisidine phosphate.

A, B, G_{β} , S, R, and I are the hydrolyzates of the permethylates of saccharide A, saccharide B, methyl- β -D-glucoside, sucrose, raffinose, and inulin, respectively.

*) According to STOFFIN et al.¹⁹⁾, the upper and lower spots are due to 2, 3, 4, 6-tetra-O-methyl-D-galactose and 2, 3, 4-tri-O-methyl-D-glucose, respectively.

The permethylated saccharides A and B gave only a single spot on thin layer chromatogram, respectively (Fig. 1). The hydrolyzates of these two permethylates were analyzed by thin layer chromatography, in which the hydrolyzates obtained from permethylates of methyl- β -D-glucoside, sucrose, raffinose, and inulin were also run as reference substances (Fig. 2). The analysis showed that the hydrolyzate of the permethylated saccharide A gave three spots equivalent to 2, 3, 4, 6-tetra-O-methyl-D-glucose, 3, 4, 6-tri-O-methyl-D-fructose, and 1, 3, 4, 6-tetra-O-methyl-D-fructose. In the case of the saccharide B, two spots corresponding to 1, 3, 4, 6-tetra-O-methyl-D-fructose and 2, 3, 4-tri-O-methyl-D-glucose were recognized.

Then, the methanolizates of the permethylated saccharides A and B were analyzed by means of gas liquid chromatography. Relative retention times of the methyl glycosides found in the methanolizates are shown in Table 3. The methanolizate from the saccharide A gave six peaks corresponding to methyl-2, 3, 4, 6-tetra-O-methyl-D-glucoside (relative retention times, 1.02 and 1.40), methyl-3, 4, 6-tri-O-methyl-D-fructoside (2.53 and 3.75), and methyl-1, 3, 4, 6-tetra-O-methyl-D-fructoside (1.02 and 1.25). The methanolizate from the saccharide B afforded four peaks ascribed to methyl-1, 3, 4, 6-tetra-O-methyl-D-fructoside (1.04 and 1.25) and methyl-2, 3, 4-tri-O-methyl-D-glucoside (2.45 and 3.44).

TABLE 3. Gas-liquid Chromatographic Analysis of Methanolizates of Permethylated Saccharides

Methanolizate from permethylated	Relative retention time*)					
	Me-2, 3, 4, 6-tetra-O-Me-D-glucoside		Me-1, 3, 4, 6-tetra-O-Me-D-fructoside	Me-2, 3, 4-tri-O-Me-D-glucoside	Me-3, 4, 6-tri-O-Me-D-fructoside	Me-2, 3, 4, 6-tetra-O-Me-D-galactoside
	β	α				
Saccharide A	1.02	1.40	(1.02 1.25)	—	(2.53 3.75)	—
Saccharide B	—	—	(1.04 1.25)	(2.45 3.44)	—	—
Sucrose	1.02	1.40	(1.02 1.25)	—	—	—
Raffinose	—	—	(1.04 1.24)	(2.42 3.42)	—	1.68
Inulin	1.02	1.40	(1.02 1.25)	—	(2.53 3.75)	—
Me-2, 3, 4, 6-tetra-O-Me- β -D-glucoside**)	1.00	—	—	—	—	—
Me-2, 3, 4, 6-tetra-O-Me- α -D-glucoside**)	—	1.41	—	—	—	—

*) Retention time of methyl-2, 3, 4, 6-tetra-O-methyl- β -D-glucoside=1.

***) Reference glycosides.

From the experimental results mentioned above, it was confirmed that the fructo-oligosaccharides A and B isolated from onion bulbs are 1^F - β -fructofuranosyl sucrose (1-kestose) and 6^{α} - β -fructofuranosyl sucrose (neokestose), respectively.

Before the present author's study, occurrence of 1-kestose and neokestose in the onion plants had been already reported by other workers: BACON¹⁾ confirmed these two trisaccharides by optical rotatory, infrared, and paper-chromatographic data and by analytical results of sugars in acid-hydrolyzates. TAKEICHI *et al.*²⁰⁾ used gas liquid chromatography of O-trifluoroacetyl derivatives to detect the both saccharides. In this study, however, some other techniques were employed for obtaining more precise evidences. The techniques are thin layer chromatography of methyl derivatives and of their acid-hydrolyzates and gas liquid chromatography of methanolzates of the methyl derivatives.

Until now, three naturally occurring β -fructofuranosyl sucroses (trisaccharides) are known in herbaceous plants. They are 1^F - β -fructofuranosyl sucrose (1-kestose), 6^{α} - β -fructofuranosyl sucrose (neokestose), and 6^F - β -fructofuranosyl sucrose (6-kestose). In Liliaceae plants, both 1-kestose and neokestose occur in *Allium Cepa*^{1,15,20)}, *A. Porrum*¹⁾, *A. victorialis*²⁰⁾, *A. tuberosum*²⁰⁾, *A. fistulosum*²⁰⁾, *Tulipa silvestris*⁴⁾, and *Ornithogalum nutans*⁴⁾; and 1-kestose alone is found in *Tulipa Clusiana*⁴⁾. Neokestose is present as the sole trisaccharide in *Asparagus cochinchinensis*²¹⁾. Although 6-kestose is found together with 1-kestose and neokestose only in Gramineae plants (*Avena sativa*¹⁸⁾, *Lolium multiflorum*¹⁾, and *Arrhenatherum elatius*¹⁾), its occurrence in Liliaceae plants is not yet reported. Consequently, 6-kestose in the onion bulbs was carefully examined in this study but any evidences for its existence were never obtained. This negative finding is compatible with the other worker's results.

Recently, several studies on the more highly polymerized fructo-oligosaccharides estimating to be derived from 1-kestose or neokestose in herbaceous plants were published: The present author and his coworkers¹⁶⁾ reported that, other than 1-kestose and neokestose, six fructo-oligosaccharides including a new pentasaccharide were identified in the roots of *Asparagus officinalis*. TOMODA *et al.* isolated seven fructo-oligosaccharides (neokestose derivatives) from the tuberous roots of *Asparagus cochinchinensis*²¹⁾ and four fructans from the rhizomes of *Polygonatum odoratum* var. *japonicum*²²⁾. Several 1-kestose- and neokestose-derivatives were also found in Amaryllidaceae^{5,6)} and Gramineae plants¹²⁾. In this study relating to the onion bulbs, a number of tetra- and/or more higher fructo-saccharides were detected in each

of the 10%, 15%, and 30% ethanol eluates in carbon-Celite column chromatography (Table 1). It is easily presumed that, even though some of these saccharides possess the same chain length, the saccharides having different bond-structures may be eluted in separate fractions according to ethanol concentrations in the eluents. Some of the differently bonded fructo-oligosaccharides in the onion bulbs will be characterized in further experiments.

Because biosyntheses of the fructo-oligosaccharides deriving from 1-kestose or neokestose have been scarcely studied, except the studies by conducted EDELMAN *et al.*²⁾ and so on, a further investigation using onion plants should be preferable and beneficial in elucidation of the chemical structures, biosyntheses, and biochemical behaviors of these saccharides in the plant kingdom.

Summary

Two non-reducing trisaccharides were isolated from 70% ethanol extract of the bulbs of *Allium Cepa* L. (Liliaceae) by a series of carbon-Celite (1:1) and silica gel chromatography. These two were identified as 1^F- β -fructofuranosyl sucrose (1-kestose) and 6^G- β -fructofuranosyl sucrose (neokestose), respectively, by acid- and yeast β -fructofuranosidase-catalyzed hydrolyses, thin layer chromatography of methyl derivatives and their acid-hydrolyzates, and gas-liquid-chromatographic examinations of methanolzates of the methyl derivatives.

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