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ショ糖からコウジカビの酵素作用による数種オリゴ糖の生成と成績体の構造研究

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Production of Several Oligosaccharides from Sucrose by the Action of an *Aspergillus* Enzyme Preparation and Structural Studies of the Products

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Fructosyltransferase and glycoside hydrolase actions of an *Aspergillus* enzyme preparation on sucrose have been studied from the viewpoint of quantitative alternation of the products. It was found that three higher oligosaccharides than sucrose were produced in the early period of the enzymic reaction. Determination of components, periodate oxidation and methylation studies provided the evidences that they are the oligosaccharides which possess non-reducing linear structure made up of 2→1 linked β-D-fructofuranose residues having a sucrose unit on the end of the molecule. The production of O-β-D-fructofuranosyl-(2→1)-D-fructofuranose was also found in the process of enzymic production and hydrolysis of the higher oligosaccharides.

The enzyme preparation made from *Aspergillus oryzae*, "Takadiastase", has the action of fructosyltransferase and it produces a series of oligosaccharides from sucrose.[1)-6)] Pazur,[1)] Bealing and Bacon[2)] found the production of a trisaccharide and a tetrasaccharide from sucrose by paper partition chromatography. Bacon and Bell[3)] reported the isolation of a trisaccharide as a product of this enzymic reaction, and they determined its chemical structure as O-α-D-glucopyranosyl-(1-2)-O-β-D-fructofuranosyl-(1-2)-β-D-fructofuranoside, namely *iso*-kestose. Kurasawa, *et al.*[5)] also confirmed the structure of this trisaccharide, and moreover, they isolated a tetrasaccharide and determined its structure as inulotriosyl D-glucoside. Edelman[6)]

1) J. H. Pazur, *J. Biol. Chem.* **199**, 217 (1952).
2) F. J. Bealing and J. S. D. Bacon, *Biochem. J.*, **53**, 277 (1953)
3) J. S. D. Bacon and D. J. Bell, *J. Chem. Soc.*, **1953**, 2528.
4) F. Kurasawa, S. Saito, N. Honma and Y. Yamamoto, *Nippon Nōgeikagaku Kaishi*, **28**, 818, 822 (1954) ; *idem*, *ibid.*, **29**, 332 (1955).
5) F. Kurasawa, Y. Yamamoto, I. Igaue and Y. Nakamura, *Nippon Nōgeikagaku Kaishi*, **30**, 624, 696 (1956).
6) J. Edelman, *Biochem. J.*, **57**, 22 (1954).

described the production of a disaccharide composed of D-fructose, but its structure has not been determined.

We have now studied this reaction from the viewpoint of quantitative alternation of the products and found several oligosaccharides as the reaction products in relatively short time, and the identification and the structural confirmation of them are described in this paper.

The reaction mixture was kept at 30°, and aliquots were removed at 0.5, 1, 2, 4, 8, 16 and 24 hr periods and analyzed for the products by cellulose thin-layer chromatography (TLC). Table I gives the *Rf* values on TLC.

Quantitative determinations of the products were carried out by high performance liquid chromatography (HPLC). The results are shown in Fig. 1 and Table II.

Table I. *Rf* Values of Products with Solvent A*

reaction times / compounds	0.5 hr	1 hr	2 hr	4 hr	8 hr	16 hr	24 hr
Fructose	0.52	0.52	0.52	0.52	0.52	0.52	0.52
Glucose	0.46	0.46	0.46	0.46	0.46	0.46	0.46
Sucrose	0.38	0.38	0.38	0.38	0.38	—	—
Fructosylfructose	—	—	0.41	0.41	0.41	0.41	—
Oligosaccharide A	0.27	0.27	0.27	0.27	0.27	—	—
Oligosaccharide B	0.21	0.21	0.21	0.21	0.21	—	—
Oligosaccharide C	—	0.16	0.16	—	—	—	—

*See "Experimental" on the solvent.

Table II. Weight Percentages of Products

reaction times / compounds	0.5 hr	1 hr	2 hr	4 hr	8 hr	16 hr	24 hr
Fructose	10.6	15.4	17.4	28.1	34.4	47.8	49.6
Glucose	34.3	43.7	42.6	49.5	50.7	50.7	50.4
Sucrose	33.1	14.5	7.3	3.5	1.6	0	0
Fructosylfructose	0	0	5.7	5.4	5.8	1.5	0
Oligosaccharide A	18.9	19.2	17.7	8.5	4.2	0	0
Oligosaccharide B	3.1	6.5	8.8	5.0	3.3	0	0
Oligosaccharide C	0	0.7	0.5	0	0	0	0

Fructose, glucose, sucrose and fructosylfructose (=O-β-D-fructofuranosyl-(2—1)-D-fructofuranose) were identified by comparing with authentic samples in TLC, HPLC and gas-liquid chromatography (GLC).

On the other hand, the reaction mixture was applied to a charcoal column after

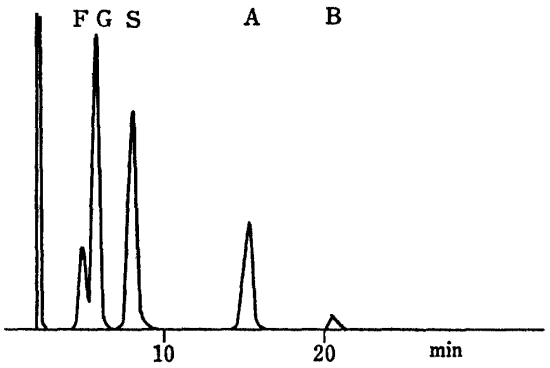

Fig. 1-a. 0.5 hr incubation

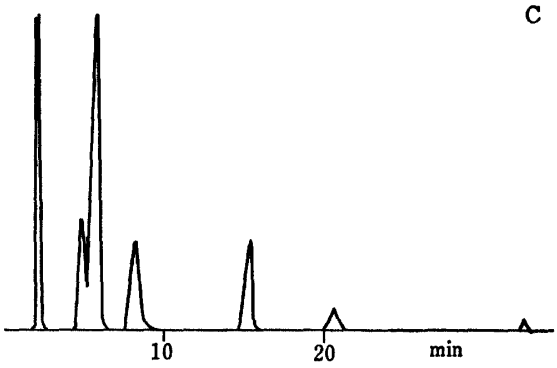

Fig. 1-b. 1 hr incubation

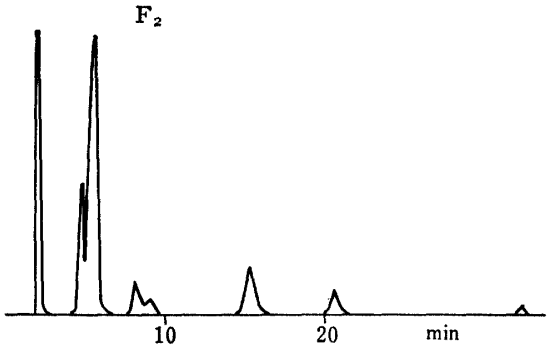

Fig. 1-c. 2 hr incubation

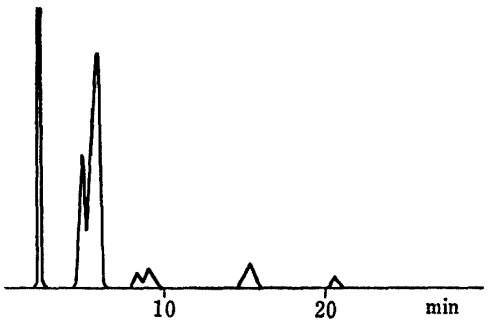

Fig. 1-d. 4 hr incubation

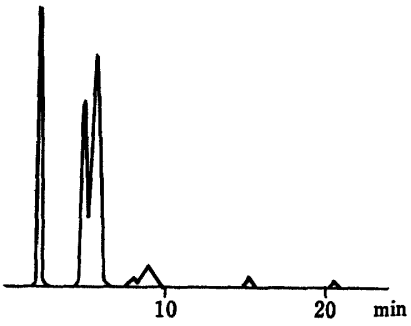

Fig. 1-e. 8 hr incubation

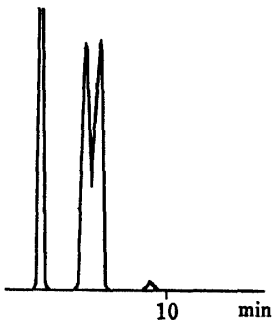

Fig. 1-f. 16 hr incubation

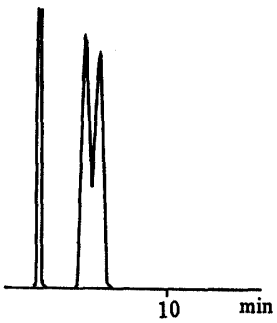

Fig. 1-g. 24 hr incubation

Fig 1. Chromatograms on HPLC

abbreviations : F, fructose : G, glucose ; S, sucrose ; F_2, fructosyl-fructose ; A, oligosaccharide A ; B, oligosaccharide B ; C, oligosaccharide C.

1 hr incubation followed by inactivation of the enzyme. Four fractions were obtained by elution with water and stepwise increments of ethanol. The fraction eluted with water contained fructose and glucose, and the fraction eluted with 5 % ethanol was sucrose. The fractions eluted with 10% ethanol contained the other oligosaccharides, and they were applied to a column of Sephadex G-15. The repeated gel

chromatography gave three non-reducing oligosaccharides (A, B, and C) which showed respectively one spot on TLC and one peak on HPLC. Specific rotations of them were as follows : A, $[\alpha]_D^{26}$ +27.5° (H_2O, c=1.5) ; B, $[\alpha]_D^{26}$ +10.2° (H_2O, c=1.3) ; C, $[\alpha]_D^{26}$ −2.3° (H_2O, c=0.4).

By means of digestion with β-fructofuranosidase, glycosidic linkages of the oligosaccharides were easily cleft. TLC and HPLC of the hydrolysates of the oligosaccharides revealed that the component sugars of them are fructose and glucose. From the results of quantitative determination of the component sugars and *Rf* values on TLC, it is able to conclude that oligosaccharide A is composed of two fructose units and one glucose unit, oligosaccharide B is composed of three fructose units and one glucose unit, and oligosaccharide C is composed of four fructose units and one glucose unit.

As the results of periodafe 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.

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

compounds	periodate consumption	formic acid liberation
Oligosaccharide A	1.11	0.34
Oligosaccharide B	1.01	0.25
Oligosaccharide C	0.98	0.20

Methylations of the oligosaccharides were performed with methyl iodide and silver oxide in dimethylformamide.[7] After mild hydrolysis and methanolysis of the methylated products, the methanolysates were analyzed by GLC. In all cases, methyl 2,3,4,6-tetramethyl D-glucopyranoside, methyl 1,3,4,6-tetramethyl D-fructofuranoside and methyl 3,4,6-trimethyl D-fructofuranoside were identified. Therefore it is able to conclude that oligosaccharide A is O-α-D-glucopyranosyl-(1—2)-O-β-D-fructofuranosyl-(1—2)-β-D-fructofuranoside (=*iso*-kestose), oligosaccharide B is O-α-D-glucopyranosyl-(1—2) O-β-D-fructofuranosyl-(1—2)-O-β-D-fructofuranosyl-(1—2)-β-D-fructofuranoside(=inulotriosyl D-glucoside) and oligosaccharide C is O-α-D-glucopyranosyl-(1—2)-O-β-D-fructofuranosyl-(1—2)-O-β-D-fructofuranosyl-(1—2)-O-β-D-fructofuranosyl-(1—2)-β-D-fructofuranoside (=inulotetraosyl D-glucoside). Thus a pentasaccharide was first obtained by us in addition to known trisaccharide and tetrasaccharide, and its structure was confirmed now.

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

As described above, the *Aspergillus* enzyme preparation produces relatively much amount of the inulin-type oligosaccharides in the early period of the reaction, namely within 2 hr. After 16 hr incubation, almost all products were occupied by monosaccharides with the disappearance of oligosaccharides. The latter reaction is presumed to be attributed to hydrolytic enzymes (glycoside hydrolases) in "Takadiastase" rather than to fructosyltransferase. The production of O-β-D-fructofuranosyl-(2—1)-D-fructofuranose was identified by us in the process of this enzymic hydrolysis. Edelman[6] described that the enzyme transfer fructose residues from sucrose to free fructose to give a fructosylfructose. But from the yields of this disaccharide and the higher oligosaccharides, it is conceivable that the fructosylfructose may be a product of hydrolysis of the higher oligosaccharides and the actions of transferase and hydrolase coexist in the course of reaction.

Experimental

Solutions were concentrated at or below 40° with rotary evaporators under reduced pressure. Optical rotations were measured with JASCO model DIP-SL automatic polarimeter. HPLC was carried out by the use of Waters model 244 liquid chromatograph equipped with differential refractometer R401. GLC was performed by the use of Hitachi model 063 gas chromatograph equipped with hydrogen flame ionization detector.

Action of Enzyme on Sucrose——Sucrose (2 g) was dissolved in 0.004 M phosphate buffer (pH 7) (10ml) and "Takadiastase" (0.4 g, Sankyo Research Lab.) was added. The reaction mixture was incubated at 30° under addition of toluene, and aliquots of it were separated after varying periods of time. The enzyme was then inactivated by heating in a boiling water bath for 1 min and the solution was lyophilized or stored at −20°.

TLC of Products——TLC using Avicel SF cellulose was carried out in the usual way with solvent A, BuOH : pyridine : H_2O (6 : 4 : 3) at 30°. Products were revealed with naphthoresorcinol-phosphoric acid reagent[8] and *p*-anisidine reagent[9].

HPLC of Products——The sample solutions were applied to an apparatus by the use of a column (0.4 cm inner diameter × 30 cm long stainless steel) packed with μ-Bondapack/carbohydrate and with a flow of 2.4 to 6 ml per min (during 20 min, curve select 10) of the solvent composed of acetonitrile : water (85 : 15) at 22°.

GLC of Products——Samples were dissolved in pyridine and trimethylsilylated with hexamethyldisilazane and trimethylchlorosilane,[10] then applied to a gas chromatograph by the use of a column (0.3 cm inner diameter × 2m long spiral stainless

8) V. Prey, H. Berbalk and M. Kausz, *Mikrochim. Acta*, **1961**, 968.
9) L. Hough and J. K. N. Jones, *Meth. Carbohyd. Chem.*, **1**, 28 (1962).
10) C. C. Sweeley, R. Bentley, M. Makita and W. W. Wells, *J, Am. Chem. Soc.*, **85**, 2497(1963).

steel) packed with 2 % OV 17 on Chromosorb W (80 to 100 mesh). The programmed temperature was increased 3° per min from 130° to 280° ; t_R, TMS-fructose, 12.2, 14.6 ; TMS-glucose, 15.5, 17.8 ; TMS-sucrose, 35.6 ; TMS-fructosylfructose, 38.9 ; TMS-oligosaccharide A, 51.3.

Isolation of Products after 1 hr Incubation——The reaction mixture obtained from 2 g of sucrose was applied to a column (2.4 × 17cm) of active charcoal (for chromatographic use, Wakô-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 (350 ml), 5 % ethanol (650 ml) and 10 % ethanol (700 ml). Fractions were collected at 50 ml and analyzed by phenol-sulfuric acid method.[11] Following two fractions were obtained from the eluates : Frac. a, tubes 12 to 24 ; Frac. b, tubes 25 to 34.

Each of Frac. a and b was dissolved in water, then applied to a column (5 × 82 cm) of Sephadex G-15 (Pharmacia Co.) followed by elution with water and fractions were collected at 20 ml, Each fraction was analyzed by TLC as described above, and following fractions were obtained from the eluates : Frac. 1, tubes 37 to 39 from Frac. a ; Frac. 2, tubes 40 to 42 from Frac. a ; Frac. 3, tubes 35 to 36 from Frac. b ; Frac. 4, tubes 37 to 39 from Frac. b ; Frac. 5, tubes 40 to 41 from Frac. b. Each of Frac. 1 to 5 was applied again to the similar column of Sephadex G-15. After this re-chromatography, oligosaccharide A (298.1mg) was obtained from tubes 41 to 43 from Frac. 1, tubes 43 to 46 from Frac. 2, and tubes 46 to 53 from Frac. 5. Oligosaccharide B (184.9 mg) was obtained from tube 38 from Frac. 1, tubes 40 to 41 from Frac. 2, tubes 38 to 39 from Frac. 3, and tubes 38 to 39 from Frac. 4. And oligosaccharide C (12.7 mg) was obtained from tubes 34 to 35 from Frac. 3, and tube 35 from Frac. 4.

Enzymic Degradation——Each sample (2 mg) was dissolved in water (0.4 ml) and β-fructofuranosidase (0.1 mg, Boehringer Co.) was added. The solution was incubated at 40° under addition of toluene for four days. The results of TLC and HPLC of the reaction mixture showed that all or almost all glycosidic linkages of the oligosaccharides were cleft. Component sugar compositions were 67.1% of fructose and 32.9 % of glucose in oligosaccharide A, 74.8 % of fructose and 25.2 % of glucose in oligosaccharide B, and 80.7 % of fructose and 19.3 % of glucose in oligosaccharide C.

Periodate Oxidation——Each sample (4 mg) was oxidized with 0.05 M sodium metaperiodate (4 ml) at room temperature in a dark place. The periodate consumption was measured by a spectrophotometric method.[12] The oxidation was com-

11) M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, *Anal. Chem.*, **28**, 350 (1956).

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

pleted after two days, then formic acid liberation was measured by a titration with 0.01 N sodium hydroxide after addition of one drop of ethyleneglycol.

Methylation——Each sample (2 mg) was dissolved in dimethylformamide (2 ml), then methyl iodide (1 ml) and silver oxide (0.1 g) were added successively under stirring. The reaction mixture was stirred at room temperature for two days in a dark place. After suction filtration, methyl iodide (1 ml) and silver oxide (0.1 g) were added again into the filtrate, then the mixture was stirred at room temperature for two days in a dark place. After re-filtration, the filtrate was diluted with water (10 ml) and 10% potassium cyanide (3 ml), then extracted with chloroform (15 ml) four times. The extract was washed with water and dried over sodium sulfate, then filtrated and 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 60° 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 30° for 20 hr. For the analysis of glucose methyl ether, a part of the product was methanolyzed with 4% methanolic HCl at 70° for 16 hr. HCl was removed by the repeated addition and evaporation of methanol. The methanolysate was dissolved in chloroform and 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 report[13] from our laboratory. Table IV shows relative retention times of the products to methyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside.

Table IV. Relative Retention Times of Methylation Products

samples	15% BDS	5% NPGS
Methanolysis products	1.00*, 1.37*, 1.03, 1.24 2.41, 3.55	1.02*, 1.44*, 1.07, 1.30, 2.50, 3.70
Methyl 2, 3, 4, 6-tetra-O-methyl-D-glucopyranoside	1.00, 1.37	1.00, 1.44
Methyl 1, 3, 4, 6-tetra-O-methyl-D-fructofuranoside	1,04, 1.24	1.07, 1.28
Methyl 3, 4, 6-tri-O-methyl-D-fructofuranoside	2.44, 3.55	2.47, 3.67

*These were detected after methanolysis with 4% methanolic HCl at 70° for 16 hr.

13) M. Tomoda, S. Nakatsuka and E. Minami, *Chem, Pharm, Bull.* (Tokyo), **20**, 953 (1972).

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