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Note

Analyses of α-linked disaccharides of D-glucose by high-performance liquid chromatography

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Sugars have been analysed by ion-exchange resins[1] and by paper[2], thin-layer[3], and gas-liquid chromatography[4]. Each of these methods has certain advantages for specific problems. However, due to difficulties encountered in qualitative and quantitative determinations of complex sugar mixtures, analyses are time consuming and are generally not suitable for trace analysis. Sugar analysis by h.p.l.c. is a rapid and convenient method, and there are various column packings such as polystyrene-based anion-[5] and cation-[6] exchange resins, as well as aminopropyl-[7], carbamoyl-[8], and cyano-derivatized silica gels[9]. Nonetheless, individual analyses of α-linked disaccharides of D-glucose by h.p.l.c. has not been reported, possibly due to their all having same molecular weight and the same number of D-glucose units, which precludes their separation by straightforward methods.

It is known that various kinds of disaccharides such as nigerose, kojibiose, neotrehalose, etc., are produced from maltose by the intensive action of cyclomaltodextrin glucanotransferase (EC 2.4.1.19)[10]. In the course of this study, it was our goal to develop a method for the determination of α-linked disaccharides of D-glucose. In this work we desired to determine α-linked disaccharides of D-glucose in mixtures composed of these disaccharides, D-glucose, and trisaccharides by h.p.l.c.. We found that a combination of data obtained from three different h.p.l.c. columns could be used to determine the individual amounts of each of the disaccharides.

A column packed of polystyrene-based cation-exchange resins has size-exclusion, ion-exclusion, and ligand-exchange mechanisms[11–13]. Columns packed of carbamoyl- or cyano-silica induce separation of sugars either by partitioning between the water-enriched stationary phase layer and the mobile phase or by adsorption *via* hydrogen bonding between the hydroxyl groups of the carbohydrate molecule and the functional groups of the stationary phase[14–15]. These columns show different separation patterns

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because of the different functional groups bonded to the silica gel support matrix. Here it was reasoned that a separation method using a combination of factors — size-exclusion, ion-exclusion, ligand exchange, liquid-partition and/or adsorption — might result in separation of these sugars.

EXPERIMENTAL

Materials. — Glucose (Kanto Chemicals Co., Ltd., Tokyo, Japan), maltose, isomaltose, trehalose, maltotriose (Seikagaku Kogyo Co., Ltd., Tokyo, Japan) and nigerose (Sigma Chemicals Co., St. Louis, Missouri, U.S.A.) were obtained from the commercial sources indicated. Kojibiose was kindly donated by Dr. Kazuo Matsuda, Nosanriyo Laboratory, Faculty of Agriculture, Tohoku University. H.p.l.c.-grade acetonitrile was obtained from Kanto Chemicals Co., Ltd.

Preparation of sugar alcohols. — Sugar alcohols of various saccharides were prepared according to the procedures of Abdel-Akher *et al.*[16] and Bishop[17], with some modification. To a solution of sugar (5 mg) in water (0.5 mL) was added sodium borohydride (2 mg). After keeping the slightly alkaline reaction mixture at room temperature for 3 h, the mixture was acidified with Amberlite IR-120[H^+] to decompose the excess sodium borohydride. After removing the resin by filtration through filter paper (Toyoroshi no. 2), the mixture was concentrated almost to dryness. Absolute methanol (5 × 2 mL) was added to and evaporated from the residue in order to remove residual boric acid.

Chromatographic method. — H.p.l.c. analyses of the sugars and sugar alcohols were performed with a model 576 h.p.l.c. pump (Gaskuro Kogyo Co., Ltd., Tokyo, Japan) equipped with a model 7125 Rheodyne loop injector, a column oven (Nippon Bio-Rad Laboratories Co., Ltd., Tokyo, Japan), an SE-31 r.i. monitor (Showa Denko Co., Ltd., Tokyo, Japan), and an SIC chromatocorder-12 (System Instruments Co., Ltd., Tokyo, Japan). The packed columns used were an SCR-101N (7.9 × 300 mm, Shimadzu Seisakusho Co., Ltd., Kyoto, Japan), a TSKgel Amide-80 (4.6 × 250 mm, Tosoh Co., Ltd., Japan) and a LiChrospher Si 60 (4.0 × 250 mm, E. Merck, Darmstadt, Germany). Chromatographic conditions were as follows: eluent, water; flow-rate, 0.5 mL/min; column temperature, 60°; column pressure, <50 kg/cm^2 on an SCR-101N column and eluent, 70:30 acetonitrile–water; flow-rate, 0.8 mL/min; column temperature, 20°; column pressure, <100 kg/cm^2 on a TSKgel Amide-80 or a LiChrospher Si 60 column. All eluents were degassed by a DG-3310 Degasser (Japan Spectroscopic Co., Ltd., Tokyo, Japan).

RESULTS AND DISCUSSION

Separation of sugars and sugar alcohols on an SCR-101N column. — Table I shows the individual retention times of standard sugars and their sugar alcohols on an SCR-101N column. Retention times of disaccharides were extremely close; therefore, all the disaccharides were not amenable to separation by the column system. The

TABLE I

Retention times of saccharides and their respective alcohols on an SCR-101N liquid chromatography column[a]

Sugar (alcohol)	*Retention time, min*[b]
Glucose (D-Glucitol)	15.0 (17.3)
Maltose (maltitol)	11.9 (12.9)
Isomaltose (isomaltitol)	11.7 (12.8)
Nigerose (nigeritol)	11.7 (11.8)
Kojibiose (kojibiitol)	11.8 (12.6)
Trehalose	11.7
Maltotriose (maltotriitol)	10.6 (11.0)

[a] Column size: 7.9 × 300 mm; eluent: water; flow rate: 0.5 mL/min; temperature: 60° sample injection: 10 μL (0.1 mg). [b] Values in parentheses are for the retention time of the sugar alcohols.

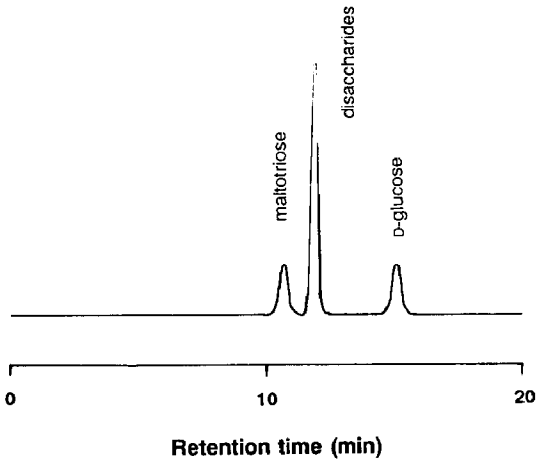

Fig. 1. Separation of sugars on an SCR 101N liquid chromatography column (size: 7.9 × 300 mm). Eluent: water; flow-rate: 0.5 mL/min; temperature: 60°; sample injection: 20 μL (0.1 mg).

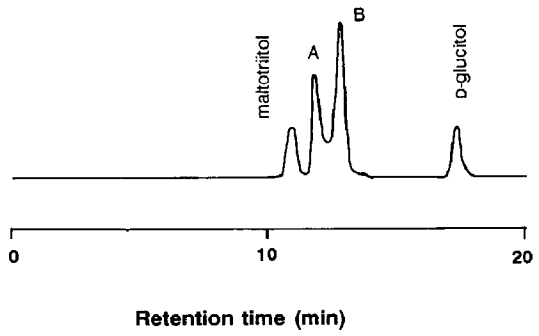

Fig. 2. Separation of sugar alcohols and trehalose on an SCR 101N liquid chromatography column (size: 7.9 × 300 mm). Eluent: water; flow-rate: 0.5 mL/min; temperature: 60°; sample injection: 20 μL (0.1 mg). A = nigeritol and trehalose; B = maltitol, isomaltitol and, kojibiitol.

TABLE II

Retention times of saccharide alcohols and trehalose on a TSK-gel amide-80 liquid chromatography column[a]

Sugar alcohol (sugar)	*Retention time, min*
D-Glucitol	13.6
Maltitol	23.2
Isomaltitol	26.3
Nigeritol	24.6
Kojibiitol	24.3
(Trehalose)	24.4
Maltotriitol	28.9

[a] Column size: 4.6 × 250 mm; eluent: 70:30 acetonitrile–water; flow rate: 0.8 mL/min; temperature: 20°; sample injection: 10 μL (0.1 mg).

injection of the disaccharide mixture gave one peak; however, D-glucose and maltotriose were clearly separated from the disaccharides (Fig. 1). This result demonstrates that each D-glucose oligomer having 1–3 D-glucose units is separated by this column. On the other hand, retention times of their respective sugar alcohols were different from that of their original sugars. A mixture of disaccharide alcohols was separated into two groups as A = nigeritol + trehalose and B = maltitol + isomaltitol + kojibiitol (Fig. 2). The sugar separation mode of this column is by a combination of size-exclusion, ion-exclusion, and ligand-exchange mechanisms[1,2] as this column is packed with polystyrene-based cation-exchange resins.

Separation of sugar alcohols on a TSKgel Amide-80 column. Table II shows the

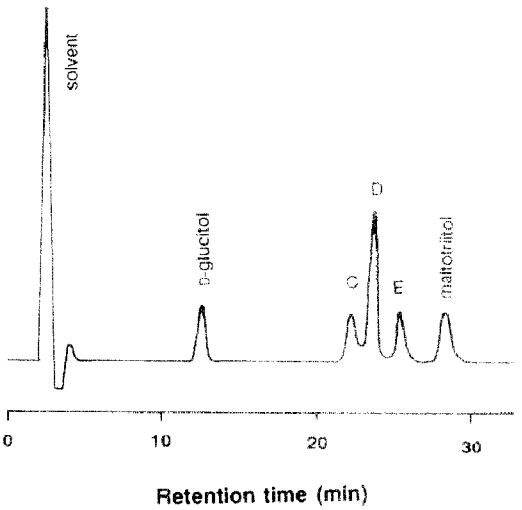

Fig. 3. Separation of sugar alcohols and trehalose on a TSK gel Amide-80 liquid chromatography column (size: 4.6 × 250 mm). Eluent: 70:30 acetonitrile–water flow-rate: 0.8 mL/min; temperature: 20°; sample injection: 20 μL (0.1 mg). C = maltitol; D = nigeritol, kojibiitol and trehalose; E = isomaltitol.

TABLE III

Retention times of saccharide alcohols and trehalose on a LiChrospher Si 60 liquid chromatography column[a]

Sugar Alcohol (Sugar)	*Retention time, min*
D-Glucitol	9.4
Maltitol	12.3
Isomaltitol	12.6
Nigeritol	12.2
Kojibiitol	12.5
(Trehalose)	10.8
Maltotriitol	13.5

[a] Column size: 4.0 × 250 mm; eluent: 70:30 acetonitrile–water; flow rate: 0.8 mL/min temperature: 20°; sample injection: 10 μL (0.1 mg).

retention times of individually injected sugar alcohols and trehalose on a TSKgel Amide-80 column. The retention times of the disaccharide alcohols are quite different from each other. The mixture of disaccharide alcohols and trehalose was separated into three groups as C = maltitol, D = nigeritol + kojibiitol + trehalose and E = isomaltitol. D-Glucitol and maltotriitol were clearly separated from disaccharide alcohols (Fig. 3). It is observed that both the α and β anomeric sugars are separated on silica-type columns under the same conditions[18]. Anomer separation was also observed on a TSKgel Amide-80 column. On the other hand, trehalose, a non-reducing sugar, showed one peak on this column system.

Separation of sugar alcohols on a LiChrospher Si 60 column.— Table III shows the retention times of individually injected sugar alcohols and trehalose on a LiChrospher

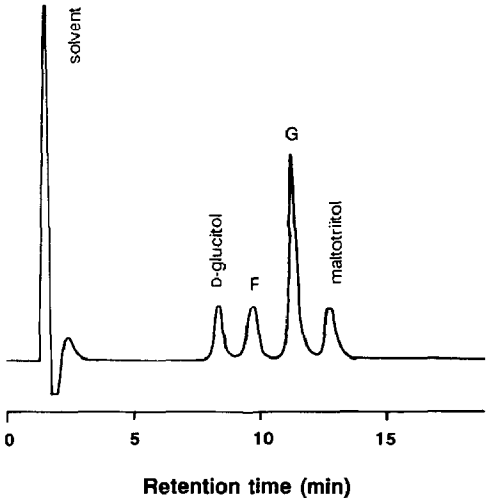

Fig. 4. Separation of sugar alcohols and trehalose on a LiChrospher Si 60 liquid chromatography column (size: 4.0 × 250 mm). Eluent: 70:30 acetonitrile–water flow-rate: 0.8 mL/min; temperature: 20°; sample injection: 20 μL (0.1 mg). F = trehalose; G = maltitol, isomaltitol, nigeritol, and kojibiitol.

TABLE IV

Determination of saccharides content in a mixture containing seven components by a three-column system

Sugar	*Content (%)*[a]
D-Glucose	14.5 ± 0.2
Maltose	14.1 ± 0.5
Isomaltose	14.6 ± 0.6
Nigerose	14.1 ± 0.9
Kojibiose	14.2 ± 0.9
Trehalose	14.5 ± 0.8
Maltotriose	14.0 ± 0.2

[a] Content (%) is expressed as mean value ± s.d. (5 times).

Si 60 column. Using this column system, trehalose was clearly separated from the disaccharide alcohols. The disaccharides were not separable from each other, however, and were observed as a single peak. D-Glucitol and maltotriitol were also separated from trehalose and disaccharide alcohols (Fig. 4).

Calculation of the amounts of individual sugars. — In all the sugar analyses, the peak areas were found to be directly in proportion to the amount (0.1 mg–1.0 mg) of all sugar standards. As a result, we have found that the disaccharides of D-glucose which have α-linkages could be quantitated by the use of the three-column system on analysis of their respective sugar alcohols. By means of the LiChrospher Si 60 column system, the amount of trehalose was determined. The TSKgel Amide-80 column, allowed the quantitation of maltitol and isomaltitol, and by the SCR-101N column, the combined amount of nigeritol and trehalose could be determined. Accordingly, by subtracting the amount of trehalose from the combined amount (obtained from the SCR-101N column), the amount of nigeritol was determined. And furthermore, by the SCR-101N column, the combined amount of maltitol, isomaltitol, and kojibiitol was determined. By subtracting the amounts of maltitol and isomaltitol (obtained from TSK gel Amide-80) from the combined amount, the amount of kojibiitol could be determined.

Analyses of the disaccharides in a mixture containing seven components. — Table IV shows the results of determination of the components by calculation as described above. The amount of each component was the same, and h.p.l.c. data of five runs was taken by use of the mixture. Therefore, the amount (%) of each component should be the same (100/7 %), but the standard deviation (s.d.) was larger for the disaccharides. Nevertheless, the amount of each α-linked disaccharide of D-glucose in the mixture could be roughly estimated by the combination of sugar reduction and the three-column h.p.l.c. system. Direct determination of disaccharides of D-glucose might be possible by use of a mixed-resin column.

Nikolov *et al.* reported the separation of various disaccharides by amino-bonded silica columns[19], but it was difficult to determine the amount of each disaccharide in a mixture by use of a single-column system. In this report, the authors have narrowed their effort to develop a method to determine α-linked saccharides of D-glucose because the samples being analyzed do not contain any other sugar component.

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