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Regioselective syntheses of trehalose-containing trisaccharides using various glycohydrolases

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ABSTRACT

Two methods were investigated for the enzymic preparation of trehalose-containing trisaccharides. In the first, a solution of saccharides is circulated through an immobilized-glycosidase column and an activated-carbon column connected in series. In the second, two enzymes having different substrate specificities are sequentially used for condensation and subsequent specific hydrolysis. Thus, 3-*O*-*β*-D-, 4-*O*-*β*-D-, and 6-*O*-*β*-D-galactopyranosyl-*α*,*α*-trehalose; 4-*O*-*α*-D- and 6-*O*-*α*-D-glucopyranosyl-*α*,*α*-trehalose; and 4-*O*-*β*-D- and 6-*O*-*β*-D-glucopyranosyl-*α*,*α*-trehalose were synthesized stereo- and regio-selectively.

INTRODUCTION

The major advantage in applying enzymes for the synthesis of organic compounds lies in the stereo- and regio-selectivity of the reaction. The application to carbohydrate compounds is most attractive, because it avoids protection and deprotection of hydroxyl groups.

We have recently reported a new approach to the use of commercially available glycosidases for the syntheses of di- and tri-saccharides[1–5]. The characteristics of this procedure is the use of an activated-carbon column together with an immobilized-glycosidase column[3]. By circulating a solution of sugars through the two columns connected in series, the oligosaccharides formed accumulate continuously in the activated-carbon column.

This system was employed for the synthesis of trehalose-containing trisaccharides and for investigating the conditions controlling the regioselectivity of the reaction. Some of the trehalose-containing trisaccharides formed (**7** and **8**) are known as component oligosaccharides of the surface antigen of mycobacteria[6,7]. Therefore, their preparation by a convenient procedure may be helpful for the immunological study of *Mycobacterium* species[8].

RESULTS AND DISCUSSION

*Synthesis of O-β-*D*-galactopyranosyl-α,α-threhaloses.* — D-Galactose and trehalose were condensed by passage through columns of an immobilized *β*-D-galactosidase from *Aspergillus oryzae* or *Escherichia coli* and an activated carbon for 24 h at room

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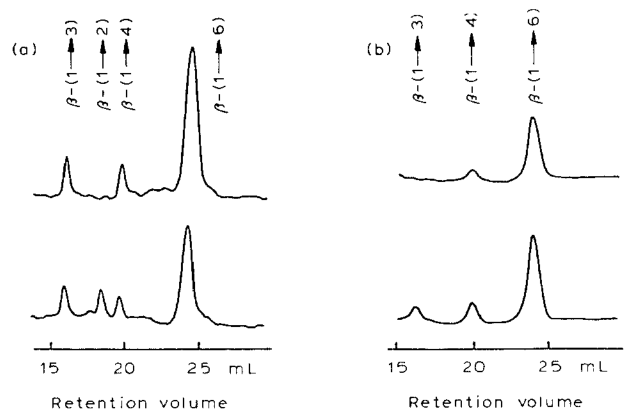


Fig. 1. L.c. of *O*-β-D-galactopyranosyl-α,α-trehaloses produced in the column system (upper traces) and the batch system (lower traces) by use of: (a) β-D-galactosidase (*A. oryzae*)-Eupergit. (b) β-D-Galactosidase (*E. coli*)-Eupergit.

temperature according to our previously published method[3]. The carbon column was washed with water and dilute ethanolic water, and the trisaccharides were eluted with aqueous ethanol and analyzed by l.c. (Fig. 1) and their structures were identified by ^{13}C-n.m.r. spectroscopy (see Table I).

When β-D-galactosidase from *A. oryzae* was used, the sum of the yields of *O*-β-D-galactopyranosyl-(1→3)-(**2**), *O*-β-D-galactogranosyl-(1→4)- (**3**), and *O*-β-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl α-D-glucopyranoside (**4**) was 7%, and the (1→2) isomer **1** was not detected at all (Fig. 1a, upper trace). On the other hand, when D-galactose and trehalose were incubated in the presence of the immobilized β-D-galactosidase from *A. oryzae* by a batch system, **1** was obtained together with the other three trisaccharides (Fig. 1a, bottom trace). The total yield of trisaccharides was 18%.

When the column system, using immobilized β-D-galactosidase from *E. coli* was used, **3** and **4** were obtained in a ratio of ~ 1:4. The sum of the yield of trisaccharides was 4.5%. The batch system reaction using β-D-galactosidase from *E. coli* afforded **2**, **3**, and **4** in 14% yield with the ratio of ~ 2:3:14.

In the batch system, ~2% of β-D-galactosyl-(1→6)-D-galactose was obtained together with the trehalose-containing trisaccharides. However, trisaccharides other than the β-D-galactosyltrehaloses were not detected by l.c. with a refractive-index monitor. In the column system, only β-D-galactopyranosyl-α,α-trehaloses were eluted from the carbon column, and disaccharides, if produced, would have been eluted together with trehalose.

Synthesis of O-*α*-D-*glucopyranosyl-(1→4)-* (**5**),*-(1→6)-*(**6**), O-β-D-glucopyranosyl-*(1→4)-*(**7**), *and-(1→6)-α*-D-*glucopyranosyl* α-D-glucopyranoside (**8**). — A similar column system using α-D-glucosidase from *Saccharomyces* sp., glucoamylase from *Rhizopus niveus*, and β-D-glucosidase from almond was used for the condensation of D-glucose with trehalose. In the reaction using an immobilized α-D-glucosidase column,

TABLE I

^{13}C-n.m.r. chemical shifts of trehalose-containing trisaccharides

Compound	*Residue or group*	*Chemical shifts (δ)*					
		C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
1	β-D-Gal*p*	104.9	72.5	71.9	69.1	75.7	61.6
	α-D-Glc*p*^{1}	93.8	79.7	72.4	70.3	73.2	61.3
	α-D-Glc*p*	93.8	71.8	73.1	70.1	72.7	61.2
2	β-D-Gal*p*	103.8	71.1	71.9	69.2	75.8	61.7
	α-D-Glc*p*^{1}	93.8	72.4	82.3	68.1	73.1	61.6
	α-D-Glc*p*	93.7	71.7	73.2	70.2	72.8	61.2
3	β-D-Gal*p*	103.6	71.3	71.7	69.2	75.9	61.7
	α-D-Glc*p*^{1}	94.0	71.5	71.7	79.1	73.0	61.1
	α-D-Glc*p*	93.7	71.7	73.1	70.3	72.8	61.1
4	β-D-Gal*p*	103.9	71.3	71.7	69.2	75.7	61.6
	α-D-Glc*p*^{1}	93.8	71.4	72.9	69.9	72.9	68.7
	α-D-Glc*p*	93.8	71.3	73.1	70.2	72.7	61.0
5	α-D-Glc*p*^{1}	100.6	72.7	74.0	70.3	73.7	61.5
	α-D-Glc^{1}*p*^{1}	94.4	71.8	73.8	78.0	71.7	61.5
	α-D-Glc*p*	94.2	72.1	73.6	70.7	73.2	61.5
6	α-D-Glc*p*^{1}	98.7	72.5	74.1	70.5	72.8	61.5
	α-D-Glc*p*^{1}	94.4	72.0	73.8	70.7	71.5	66.4
	α-D-Glc*p*	94.4	72.0	73.5	70.7	73.2	61.5
7	β-D-Glc*p*^{11}	103.8	74.4	76.7	70.7	77.2	61.8
	α-D-Glc*p*^{1}	94.6	72.3	73.8	79.7	71.9	61.0
	α-D-Glc*p*	94.3	72.0	73.8	70.9	73.4	61.7
8	β-D-Glc*p*^{11}	103.6	74.1	76.7	70.4	76.9	61.7
	α-D-Glc*p*^{1}	94.3	72.0	73.5	70.7	73.2	69.2
	α-D-Glc*p*	94.3	72.0	73.4	70.6	72.2	61.5

5 was obtained selectively, whereas **5** and **6** were formed in a ratio of ~ 1:10 by the batch system. The structures of both trisaccharides were identified by ^{13}C-n.m.r. spectra (see Table I). The yields of trisaccharides were 2 and 7% for the column system and the batch system reaction, respectively. When glucoamylase was used instead of α-D-glucosidase, the yield of trisaccharides increased from 7 to 14% in the batch system. However, in the column system, the yield was not improved at all.

The reaction using β-D-glucosidase from almond was studied with solutions of 10% of D-glucose and 50% (w/v) of trehalose. In the batch system, **8** was obtained preponderantly in a 5% yield. In contrast, in the column system, **7** was obtained selectively, although the yield was very low (0.9%).

Regioselectivity of the column system reaction and low yield of α-D- *or* β-D-*glucopyranosyl-α,α-trehalose formation.* — In order to clarify the mechanism of the regioselectivity of the systems, the time course of D-glucopyranosyl-α,α-trehaloses formation in the batch system was measured by l.c. In the reaction using α-D-glucosidase, **5** was formed at first, and then **6** increased gradually (Fig. 2a) for a final ratio of ~ 1:10 for **5** vs. **6**, indicating that the reaction is thermodynamically controlled. In the presence of β-D-glucosidase (Fig. 2b), **7** was also formed as a major product in the early stage of the reaction, and then **8** became preponderant. Thus, the regioselectivity of the column system reaction may be explained as follows. In the immobilized α-D-glucosidase column, **5** is formed at first, and the product is immediately adsorbed by the carbon column before **6** might increase, resulting in a continuous accumulation of **5**. The formation of β-D-glucopyranosyl-α,α-trehalose may be explained similary.

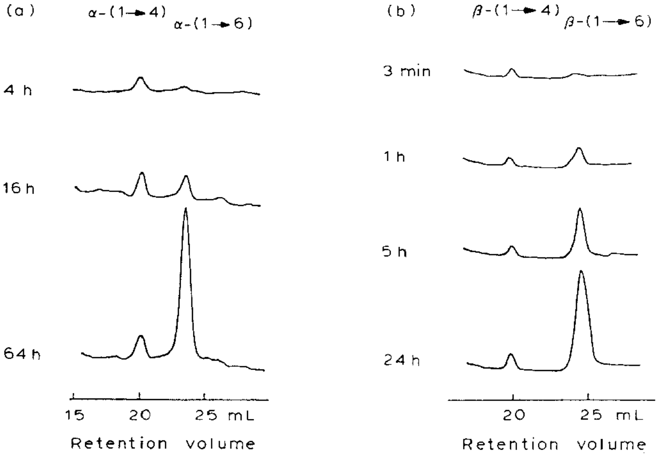


Fig. 2. Time course, (determined by l.c.) of the formation of: O-α-D- (a) and O-β-D-glucopyranosyl-α,α-trehalose (b) by the batch system with α-D-glucosidase from *Saccharomyces* sp. and β-D-glucosidase from almond, respectively.

The low yields of D-glucopyranosyl-α,α-trehaloses in the column system may result from: (a) A decrease of reactant concentration due to adsorption on activated carbon, as it can adsorb saccharides for ~10% of its weight; in fact, after 3 h of circulation the concentration of monosaccharide decreased to 4–6% and that of trehalose to 15–25%, from initial concentrations of ~10 and 50%, respectively. As the condensation is strongly dependent on the concentration of substrates[1], the decrease of reactant concentration is a serious disadvantage. (b) The adsorption of trehalose on the activated carbon may be stronger than that of α- or β-D-glucopyranosyl-α,α-trehalosesi; but this was disproved experimentally. (c) The velocity of the formation of trisaccharides, as shown by the amounts of **5** or **7** formed in the early stage of the reaction which were extremely small, in contrast to the amounts of **6** or **8** formed later (Fig. 2). In contrast, **4** was formed even in the early stage of the batch system (data not shown), and consequently, the sum of accumulated **2**, **3**, and **4** was relatively large in the column system using β-D-galactosidase from *E. coli*.

Sequential use of β-D-galactosidase for the selective synthesis of **2**. — Recently, Hedbys *et al.*[9] reported the synthesis of O-β-D-galactosyl-(1→3)-2-acetamido-2-deoxy-D-galactose by the sequential use of β-D-galactosidases from bovine testis and from *E. coli*. We applied this technique to the synthesis of **2** by incubating a solution containing 10% of D-galactose and 50% of trehalose with β-D-galactosidase from *A. oryzae*, denaturating the enzyme by boiling, and incubating with β-D-galactosidase from *E. coli*. In the first step, the condensation of β-D-galactose and trehalose gave trisaccharides **1**, **2**, **3**, and **4**, and in the second step, **3** and **4** were rapidly and selectively hydrolyzed; after 24 h, the ratio of the sum of **1**, **3**, and **4** to **2** became < 20% (see Fig. 3). Since it is generally difficult to separate isomeric di- or tri-saccharides on activated carbon and the separation by l.c. is expensive, the best way to obtain a specific oligosaccharide is selective synthesis. Thus, by selection of the appropriate method, various trehalose-containing trisaccharides were synthesized stereo- and regio-selectively, although in low yields.

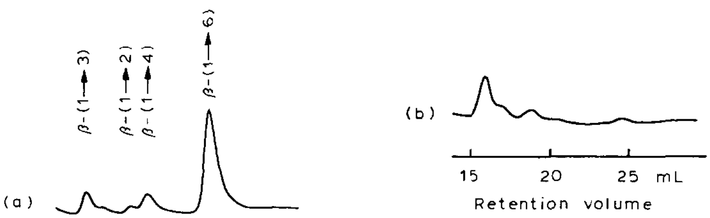


Fig. 3. Trisaccharide region of l.c. tracing of the reaction mixture of 10% D-galactose and 50% trehalose with β-D-galactosidase from *A. oryzae*, before (a) and after (b) hydrolysis with β-D-galactosidase from *E. coli*.

EXPERIMENTAL

Methods. — ^{13}C-N.m.r. spectra were recorded for solutions in D_2O (internal acetonitrile, δ 1.3) with a Varian XL-400 n.m.r. spectrometer. Liquid chromatography was carried out on a LiChrospher-NH_2 column (Merck), which was eluted with 3:1 acetonitrile–water at a flow rate of 1.0 mL min^{-1}. The saccharides eluted were detected with a Shodex SE-51 refractive index monitor (Showa Denko Co., Tokyo).

Materials. — *Saccharomyces* sp. α-D-glucosidase (EC 3.2.1.20; 116 units·mg^{-1}), β-D-glucosidase from almond (EC 3.2.1.21; 14.0 units·mg^{-1}), glucoamylase from *R. niveus* (EC 3.2.1.3; 38.3 units·mg^{-1}), and β-D-galactosidases (EC 3.2.1.23) from *A. oryzae* (108 units·mg^{-1}) and from *E. coli* (680 units·mg^{-1}) were purchased from Toyobo Co., Ltd. (Osaka, Japan). These enzymes were used without further purification. Eupergit C, glucoamylase-Eupergit (Plexazym AG, 25 units·g^{-1}), and β-D-galactosidase (*A. oryzae*)- Eupergit (Plexazym LA-1, 305 units·g^{-1}) were from Röhm Pharma (Weiterstadt, FRG). The other enzymes were immobilized on Eupergit and the enzyme activity was determined as described previously[4,5]. Activated carbon was a product of Merck (Darmstadt, FRG).

O-*β*-D-*Galactopyranosyl-α,α-trehaloses by column system.* — A solution of D-galactose (0.5g, 2.8 mmol) and trehalose (2.5 g, 7.3 mmol) in 0.1M sodium acetate-acetic acid buffer solution (pH 5.0, 5 mL), was circulated through a column of immobilized *β*-D-galactosidase (*A. oryzae*)-Eupergit (2g) and a column of activated carbon (5g) connected in series for 24 h at room temperature. The carbon was washed in sequence with water (50 mL), with 1:19 ethanol–water (50 mL), and with 2:3 ethanol–water (50 mL) to elute the trisaccharides. Concentration of the eluate afforded a syrup, which was chromatographed on the activated carbon column with a gradient of 0–40% ethanol in water. Each fraction was analyzed[10] with the phenol-H_2SO_4 reagent for saccaride content at 490 nm. The fractions containing trisaccharides were collected and analyzed by l.c. to estimate the ratio of isomeric trisaccharides; the yields calculated from the peak areas were 0.7, 0.5 and 5.9% for **2**, **3**, and **4**, respectively.

O-*β*-D-*Galactopyranosyl-α,α-trehaloses by batch system.* — A 0.1M acetate buffer solution (pH 5.0, 10 mL) containing D-galactose (1 g, 5.6 mmol) and trehalose (5 g, 14.6 mmol) was incubated at 55° with *β*-D-galactosidase from *A. oryzae* (10 mg). After 24 h, the solution was heated for 5 min in a boiling-waterbath, and the denatured enzyme was filtered off. After concentration, the residual syrup was chromatographed on activated carbon eluted with a gradient of 0–40% ethanol in water. Concentration of the eluate afforded a colorless syrup (504 mg) containing **1**, **2**, **3**, and **4** in 1.6, 1.8, 1.2, and 13.3% yield, respectively (by l.c.). Each trisaccharide was isolated by preparative l.c. and its structure was identified by ^{13}C-n.m.r. spectroscopy.

Anal. Calc. for $C_{18}H_{32}O_{16}$: C, 42.86; H, 6.39. Found (for **4**): C, 42.96; H, 5.95.

O-*β*-D-*Galactopyranosyl-(1→3)-α*-D-*glucopyranosyl-α*-D-*glucopyranoside* (**2**). — A solution of 0.1M acetate buffer (pH 5.0, 50 mL) containing D-galactose (7.5 g, 41.7 mmol) and trehalose (25 g, 73 mmol) was incubated at 45° with *β*-D-galactosidase from *A. oryzae* (50 mg). After 48 h, the solution was heated in a boiling-waterbath for 5 min, and the same buffer (450 mL) was added. The solution was incubated for 24 h at 37° with *β*-D-galactosidase from *E. coli* (5 mg), and then heated in a boiling-waterbath for 5 min. The denatured enzyme was filtered off and concentration of the solution gave a syrup which was chromatographed in an activated carbon column (5 × 40 cm). Elution with a gradient of 0–30% ethanol in water gave colorless syrup (136 mg) containing mainly **2** (~ 80% purity based on l.c.). Purification by preparative l.c. gave an amorphous solid (97 mg, ~ 96% purity, 0.46% yield).

Anal. Calc. for $C_{18}H_{32}O_{16}$: C, 42.86; H, 6.39. Found: C, 42.78; H, 6.21.

O-*α*-D-*Glucopyranosyl-(1→4)-α*-D-*glucopyranosyl α*-D-*glucopyranoside* (**5**). — (*a*). *By use of α*-D-*glucosidase from* Saccharomyces *sp.* — A 0.1M phosphate buffer solution (6 mL, pH 6.0) containing D-glucose (600 mg, 3.3 mmol) and trehalose (3 g, 8.8 mmol) was circulated through the columns of *α*-D-glucosidase (*Saccharomyces* sp.)-Eupergit (3.6 g, 56 units.g^{-1}) and activated carbon (5 g) connected in series. After 3 days, the carbon column was washed in sequence with water (100 mL), and 2:3 ethanol–water (100 mL). Concentration of the last eluate gave a syrup (~ 500 mg) which was rechromatographed on an activated carbon column (2 × 50 cm). Elution with a gradient of 0–40% ethanol in water, gave **5** (~ 95% purity) as a colorless syrup (36 mg, 2.1% yield).

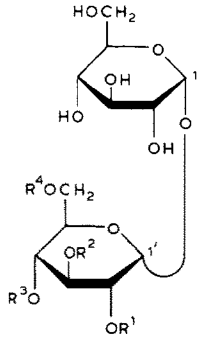
(*b*) *By use of glucoamylase from* R. niveus. — A 0.1M acetate buffer solution (2 mL, pH 5.0) containing D-glucose (400 mg, 2.2 mmol) and trehalose (800 mg, 2.3 mmol) was circulated through columns of glucoamylase-Eupergit (1 g) and activated carbon (3 g) for 24 h. After a treatment as just mentioned, **5** (~ 95% purity) was obtained as a syrup (23 mg, 2.0% yield).

O-*α*-D-*Glucopyranosyl-(1→6)-α*-D-*glucopyranosyl α*-D-*glucopyranoside* (**6**). — (*a*) *By use of α*-D-*glucosidase from* Saccharomyces sp. — A 0.1M phosphate buffer solution (3 mL, pH 6.0) containing D-glucose (300 mg, 1.7 mmol) and trehalose (1.5 g, 4.4 mmol) was incubated with *α*-D-glucosidase from *Saccharomyces* sp. (10 mg) for 72 h, at 37°. The solution was heated in a boiling-waterbath for 5 min, and the denatured enzyme was filtered off. The filtrate was concentrated and chromatographed in an activated carbon column (2 × 50 cm). The trisaccharides were eluted by a gradient of 0–40% ethanol in water to give, after concentration, a syrup (60 mg, 7.1% yield) containing a ratio of **5** to **6** of ~ 1:10. Compound **6** was isolated as an amorphous solid (~ 94% purity) by preparative l.c.

Anal. Calc. for $C_{18}H_{32}O_{16}$: C, 42.86; H, 6.39. Found: C, 43.10; H, 6.36.

(*b*) *By use of glucoamylase from* R. niveus. — A 0.1M acetate buffer solution (1 mL, pH 5.0) containing D-glucose (100 mg, 0.56 mmol) and trehalose (500 mg, 1.5 mmol) was incubated at 35° for 14 h with glucoamylase from *R. niveus* (5 mg). Chromatography on activated carbon as just described gave a syrup (39 mg) containing **5** and **6** in a ~ 1:19 ratio (~ 1 and 13% yield, respectively).

O-*β*-D-*Glucopyranosyl-(1→4)-α*-D-*glucopyranosyl α*-D-*glucopyranoside* (**7**). — A 0.1M phosphate buffer solution (pH 6.0, 6 mL) containing D-glucose (600 mg, 3.3 mmol)



	R^1	R^2	R^3	R^4
1	β-D-Gal*p*	H	H	H
2	H	β-D-Gal*p*	H	H
3	H	H	β-D-Gal*p*	H
4	H	H	H	β-D-Gal*p*
5	H	H	α-D-Glc*p*″	H
6	H	H	H	α-D-Glc*p*″
7	H	H	β-D-Glc*p*″	H
8	H	H	H	β-D-Glc*p*″

and trehalose (3 g, 8.8 mmol) was circulated through columns of *β*-D-glucosidase (almond)-Eupergit (3 g, 29.0 units.g^{-1}) and activated carbon (5 g) connected in series. After 3 days, the carbon column was washed with water (100 mL) and 1:19 ethanol–water (100 mL). Concentration of the eluate with 2:3 ethanol–water gave a syrup (~ 320 mg) which was rechromatographed in an activated-carbon column (2 × 50 cm) with elution by a gradient of 0–40% ethanol in water. Compound **7** was obtained as a colorless syrup (~ 96% purity; 16 mg, 0.9% yield).

O-*β*-D-*Glucopyranosyl-(1→6)-α*-D-*glucopyranosyl α*-D-*glucopyranoside* (**8**). — A 0.1M phosphate buffer solution (pH 6.0; 3 mL) containing D-glucose (300 mg, 1.7 mmol) and trehalose (1.5 g, 4.4 mmol) was incubated with *β*-D-glucosidase from almond (40 mg). After 72 h, the solution was treated by the same procedure as that for the preparation of *O*-*α*-D-glucopyranosyl *α*,*α*-trehaloses. A colorless syrup was obtained, and **8** was further purified to 95% by preparative l.c. (42 mg, 5.0% yield).

Adsorption of trehalose and trehalose-containing trisaccharides on carbon. — A mixture (1 mL) of 10% of trehalose (100 mg) and 10% of trehalose-containing trisaccharide (*α*- or *β*-D-glucopyranosyl*α*,*α*-trehalose, or *β*-D-galactopyranosyl-*α*,*α*-trehalose; 100 mg) was circulated through a carbon column (500 mg) for 16 h, after which, the carbon column was washed with water repeatedly, the adsorbed trehalose and trisaccharide were eluted with 40% (v/v) ethanol, and the amounts, calculated from the peak areas of the l.c. chart, were 4–6 mg for trehalose and 26–37 mg for the trisaccharide.

Anal. Calc. for $C_{18}H_{32}O_{16}$: C, 42.86; H, 6.39. Found: C, 42.98; H. 6.56.

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