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# REGIOSELECTIVE SYNTHESIS OF TRISACCHARIDES BY USE OF A REVERSED HYDROLYSIS ACTIVITY OF α- AND β-D-GALACTOSIDASE

KATSUMI AJISAKA AND HIROSHI FUJIMOTO
*Bioorganic Chemistry Division, Meiji Institute of Health Science, 540 Naruda, Odawara 250 (Japan)*
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ABSTRACT

Incubation of a solution containing D-galactose and a high concentration of sucrose in the presence of α-D-galactosidase from *M. vinacea* afforded raffinose and planteose in a ratio of ~3:2. On the other hand, circulation of a solution of D-galactose and sucrose through a reaction system consisting of columns, in series, of immobilized α-D-galactosidase and activated carbon gave only raffinose, eluted from the activated carbon column in a 17.6% yield. Similarly, only isoraffinose was obtained in 10.6% yield by the continuous method using an immobilized column of β-D-galactosidase from *E. coli*.

## INTRODUCTION

Transglycosylation activity of glycosidases has frequently been used for the synthesis of di- or tri-saccharides[1,2]. However, the isolation of products from the transglycosylation-reaction mixture is very complicated generally, because the incubation solution contains starting materials, reaction products, and hydrolyzed materials.

On the other hand, the production of di- or tri-saccharides by condensation of mono- or di-saccharides by reversed hydrolysis activity of glycosidases has not been fully studied, because the yield of the condensation reaction is very low. Recently, we have introduced a new method for the preparation of disaccharides in high yield by condensation of monosaccharides using various glycosidases[3–5]. In this "continuous method reaction", the solution of substrates is circulated through columns of immobilized enzyme and activated carbon connected in series. The procedure is based on the preferred adsorption, by activated carbon, of di- over mono-saccharides. By eliminating the products from the system, the equilibrium is continually shifted toward disaccharide formation. After an appropriate time of circulation, the disaccharides accumulated in the activated carbon column could be eluted with an aqueous ethanol solution. In the present study, we have applied this procedure to the synthesis of tri- from mono- and di-saccharides by use of α-D-galactosidase from *M. vinacea* or β-D-galactosidase from *E. coli*.

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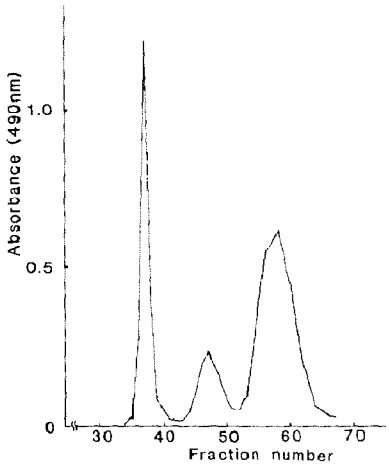



Fig. 1. Activated carbon column chromatography of the 50% ethanol eluate in the continuous method reaction by use of an α-galactosidase–Eupergit column. Vertical scale shows absorbance after reaction with phenol–sulfuric acid according to the method of Dubois *et al.*[9].

## RESULTS AND DISCUSSION

For the synthesis of D-galactosylsucroses, the concentration of D-galactose and sucrose was chosen as 10% (w/v) and 50% (w/v), respectively, in order to avoid the formation of D-galactosyl-D-galactoses. After circulating the solution for an appropriate time through columns of immobilized α-galactosidase from

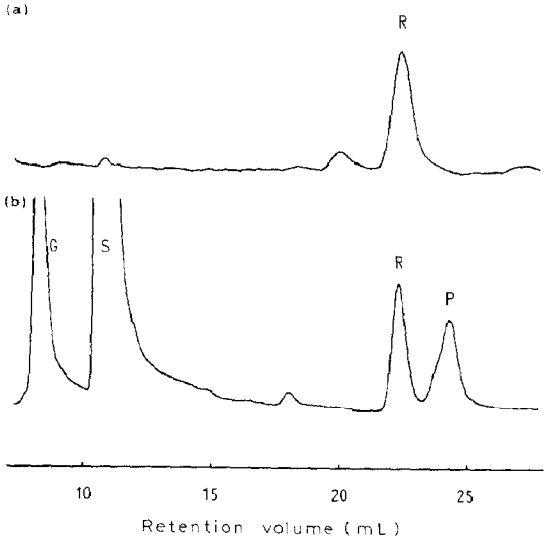
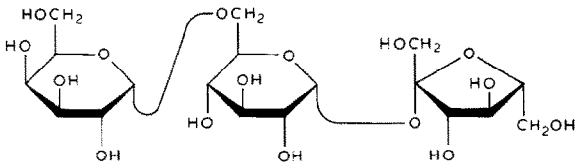



Fig. 2. (a) L.c. of the pooled fractions of trisaccharide region in Fig. 1. (b) L.c. of the incubation of D-galactose and sucrose in the presence of α-galactosidase: (G) D-galactose, (S) sucrose, (R) raffinose, and (P) planteose.

*M. vinacea* and activated carbon connected in series, the crude trisaccharides were obtained by elution with 50% (v/v) ethanol from the activated carbon column. The fractions containing the trisaccharides were separated from D-galactose and sucrose by chromatography on activated carbon (Fig. 1). They were pooled and concentrated to give a syrup, which showed a single peak in the region of trisaccharides on l.c., as shown in Fig. 2a. The trisaccharide was identified as raffinose [**1**; α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside] by comparing the $^{13}$C-n.m.r. spectrum with published data[7].



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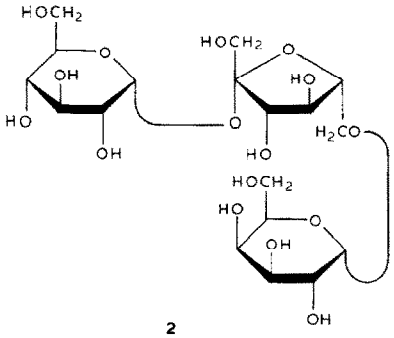

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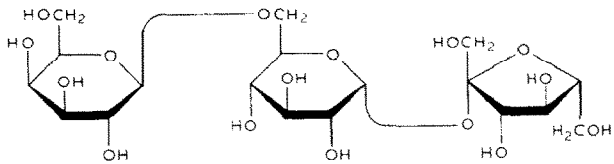
On the other hand, when a solution containing D-galactose (10% w/v) and sucrose (70% w/v) was incubated in the presence of α-galactosidase from *M. vinacea* for 48 h at 37° (a batch-method reaction), two peaks for trisaccharides were observed in l.c. (see Fig. 2b). After separation by preparative l.c. with LiChrosorb-$NH_2$ column, the peaks R and P in Fig. 2b were identified by $^1$H- and $^{13}$C-n.m.r. spectroscopy (Table I), as **1** and planteose [**2**; α-D-glucopyranosyl-(1→2)-[α-D-galactopyranosyl-(1→6)]-β-D-fructofuranoside], respectively. Planteose is a trisaccharide found in sesame seeds[7] and this is the first report of its enzymic synthesis. As raffinose was expected to be a growth factor for bifidobacteria[8], planteose may also be a candidate. It should be noted that although **1** and **2** were synthesized in a ratio of ~3:2 in the batch-method reaction, only **1** was obtained exclusively in the continuous method reaction. Thus, D-galactose reacts regioselectively with O-6 of the D-glucose residue in sucrose in the condensation reaction catalyzed by the immobilized α-galactosidase from *M. vinacea*.

A quite similar regioselectivity was observed when the reaction conditions

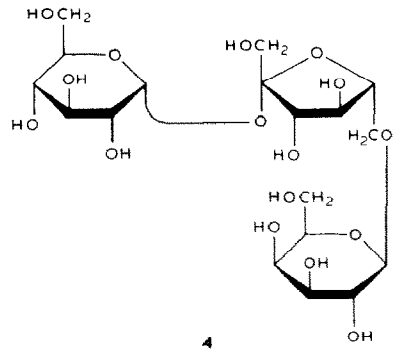
TABLE I

$^{13}$C-N.M.R. CHEMICAL SHIFTS OF TRISACCHARIDES **1–4**

| *Trisaccharide* | *Residue* | *Chemical shifts (δ)* | | | | | |
|---|---|---|---|---|---|---|---|
| | | *C-1* | *C-2* | *C-3* | *C-4* | *C-5* | *C-6* |
| | Glc | 92.6 | 71.5 | 73.2 | 70.1 | 72.0 | 66.6 |
| **1** | Gal | 99.1 | 69.2 | 70.1 | 69.8 | 71.6 | 61.7 |
| | Fru | 62.0 | 104.3 | 77.1 | 74.6 | 81.9 | 63.1 |
| | Glc | 92.0 | 72.2 | 73.8 | 70.6 | 73.4 | 61.6 |
| **2** | Gal | 99.5 | 69.3 | 70.5 | 70.3 | 72.1 | 62.7 |
| | Fru | 62.0 | 105.0 | 77.1 | 75.4 | 80.6 | 69.9 |
| | Glc | 92.7 | 71.6 | 73.2 | 69.8 | 72.1 | 69.0 |
| **3** | Gal | 104.0 | 71.3 | 73.0 | 69.2 | 75.7 | 61.7 |
| | Fru | 61.8 | 104.2 | 76.9 | 74.5 | 81.8 | 63.0 |
| | Glc | 93.4 | 72.2 | 73.7 | 71.5 | 73.6 | 61.5 |
| **4** | Gal | 104.4 | 72.0 | 73.8 | 70.8 | 76.3 | 62.2 |
| | Fru | 62.3 | 105.0 | 77.2 | 75.7 | 81.2 | 72.3 |



**3**



**4**

included a column of immobilized β-galactosidase instead of immobilized α-galactosidase. Although two trisaccharides were synthesized by incubation of D-galactose (10% w/v) and sucrose (70% w/v) at 55° in the presence of β-galactosidase from *E. coli* (Fig. 3b), only isoraffinose [**3**; β-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside] was obtained from the continuous method

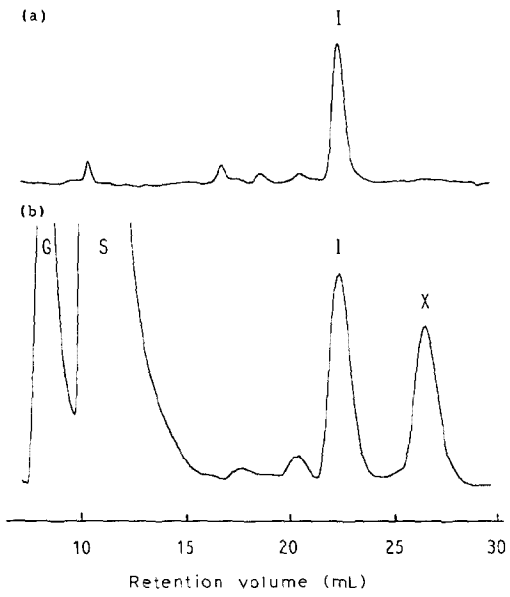



Fig. 3. (a) L.c. of the products by the continuous method reaction by use of a β-galactosidase–Eupergit column. (b) L.c. of the incubation of D-galactose and sucrose in the presence of β-galactosidase: (G) D-Galactose, (S) sucrose, (I) isoraffinose, and (X) new trisaccharide.

reaction using an immobilized β-galactosidase column (Fig. 3a). Peak X in Fig. 3b was identified as α-D-glucopyranosyl-(1→2)-[β-D-galactopyranosyl-(1→6)]-β-D-fructofuranoside (**4**) by $^{1}H$- and $^{13}C$-n.m.r. spectroscopy (Table I). To our knowledge, this trisaccharide is a new compound synthesized for the first time.

The difference in regioselectivity between the two methods may be explained as follows. In the batch method reaction, D-galactose, sucrose, and the trisaccharides are in equilibrium after a prolonged incubation. In the continuous method reaction, on the other hand, the trisaccharides are not in equilibrium because they are immediately adsorbed onto the activated carbon column as soon as they are produced. Consequently, the composition of trisaccharides by the continuous method reaction may be a reflect of an activation free energy of formation or, in other words, an easiness of formation.

This assumption could be verified by the time course of the reaction, as determined by l.c. of the incubated solution in the batch-method reaction with α-galactosidase (Fig. 4). The rate of production of raffinose and planteose was clearly different. Raffinose was produced rapidly by fast equilibrium in solution, whereas planteose might have been produced slowly by transglycosylation from raffinose. Therefore, only raffinose accumulated on the activated carbon column in the continuous method reaction.

The yields of **1** (17.6%) and **3** (10.6%) are comparable to the yield of iso-

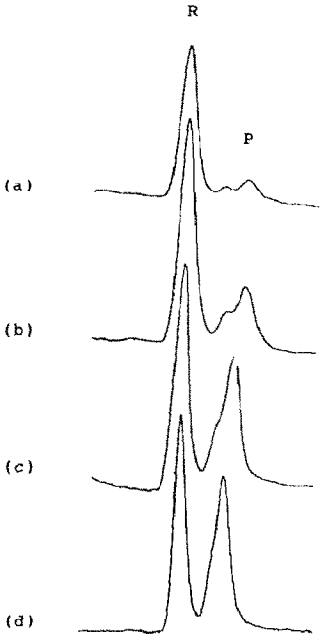



Fig. 4. L.c. analysis of the trisaccharide region of the time-course reaction by the batch-method reaction using α-galactosidase: (a) 12 h, (b) 24 h, (c) 36 h, and (d) 48 h. (R) raffinose and (P) planteose.

raffinose (17.0%) reported by Suyama *et al.*[2], who have synthesized the trisaccharide by use of transglycosylation activity of β-galactosidase from *E. coli.* However, it was very cumbersome to isolate the products from the starting material or by-products in the transglycosylation reaction. In contrast, in the present procedure, the trisaccharides could be obtained directly from the activated carbon column, and the excess mono- and di-saccharides could be used again.

In conclusion, in the synthesis of trisaccharides by use of the reversed hydrolysis activity of glycosidases, the poor yield could be improved by a combined use of columns of immobilized enzyme and activated carbon. Furthermore, an unexpected regioselectivity was observed in the continuous method reaction. Therefore, the continuous method reaction may be of great interest for the regioselective synthesis of biologically important di- or tri-saccharides.

## EXPERIMENTAL

*Materials.* — *M. vinacea* α-galactosidase (EC 3.2.1.22, 200 units/mg) and β-galactosidase from *E. coli* (EC 3.2.1.23, 750 units/mg) were purchased from Seikagaku Kogyo Co. (Tokyo) and Sigma Chemical Co. (St. Louis), respectively. Eupergit C was from Röhm Pharma (Weiterstadt).

*Methods.* — $^{13}$C-N.m.r. spectra (100 MHz) were recorded with a Varian XL-

400 spectrometer for solutions in $D_2O$ (methyl signal of internal acetonitrile, $\delta$ 1.829). Analytical l.c. was performed on a Pharmacia P-3500 pump system equipped with Shodex SE-51 refractive index monitor (Showa Denko Co., Tokyo) and a column (4 × 250 mm) of LiChrospher-$NH_2$ (Merck). The preparative l.c. was performed on a Hitachi 655A-11 pump system with Shodex SE-52 RI monitor and LiChrosorb-$NH_2$ column (10 × 300 mm). The mobile phase was 3:1 (v/v) acetonitrile–water in both cases.

*Immobilization of enzymes.* — Dry Eupergit C (800 mg) was added to a solution of α-galactosidase (1.25 mg) in M potassium phosphate buffer (pH 7.3, 7 mL). The suspension was mixed upside down by a Rotator III (Taiyo Co., Tokyo) for 72 h at room temperature. The gel was then collected on a glass filter and washed with the same buffer to give wet immobilized α-galactosidase on Eupergit (2.9 g). The activity of the immobilized enzyme was 0.02 unit/mg when *o*-nitrophenyl α-D-galactopyranoside was used as a substrate. Immobilization of β-galactosidase was carried out in a similar way by use of dry Eupergit C (1.5 g) and β-galactosidase from *E. coli* (17.5 mg). Filtration on a glass filter gave wet β-galactosidase–Eupergit gel (4.9 g; 0.21 unit/mg of gel).

*α-D-Galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside* (**1**). — Wet α-galactosidase–Eupergit (2.9 g) and activated carbon (2 g) were packed into columns (10 × 70 mm and 12 × 100 mm, respectively) that were then connected in series. A 0.1M potassium phosphate buffer solution (pH 6.0, 3 mL) containing D-galactose (300 mg) and sucrose (1.5 g) was circulated through the columns by a peristaltic pump at a speed of ~0.5 mL/min. After 48 h, the activated carbon column was disconnected from the system, and washed first with water (200 mL) and next with 5% ethanol (200 mL). The trisaccharides were eluted with aqueous 50% (v/v) ethanol (200 mL). The concentrated eluate was applied to an activated carbon column (20 × 600 nm), and the trisaccharide was eluted with a linear gradient of 0–50% (v/v) ethanol in water (700 mL). The fractions (7 mL) were collected by a fraction collector. The content of carbohydrates in each fraction was measured with the procedure of Dubois *et al.*[9]. Fractions containing trisaccharides were pooled and concentrated with an evaporator to give syrupy **1** (150 mg, 17.6% yield).

*Anal.* Calc. for $C_{18}H_{32}O_{16}$: C, 42.86; H, 6.39. Found: C, 42.63; H, 6.38.

*α-D-Galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside* (**1**) *and α-D-glucopyranosyl-(1→2)-[α-D-galactopyranosyl-(1→6)-]-β-D-fructofuranoside* (**2**). — A solution (1 mL) of 0.1M potassium phosphate buffer (pH 6.0) containing D-galactose (100 mg), sucrose (700 mg), and α-galactosidase (0.25 mg) was incubated at 37°. After 48 h, the enzyme was denatured by heating in boiling water and filtered off. The filtrate was applied to an activated carbon column (20 × 600 mm), and the trisaccharide were separated with a linear gradient of 0–50% (v/v) ethanol in water (700 mL). Fractions containing trisaccharides were pooled and concentrated with an evaporator to give a mixture of **1** and **2** as a syrup (78 mg). From a portion of the syrup, the two trisaccharides were purified by preparative l.c. to give a sample for a $^{13}C$-n.m.r. measurement and an elemental analysis.

*Anal.* Calc. for $C_{18}H_{32}O_{16}$: C, 42.86; H, 6.39. Found (for **2**): C, 42.60; H, 6.45.

*β-D-Galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside* (**3**). — Wet β-galactosidase–Eupergit gel (4.9 g) and activated carbon (5 g) were packed into columns of 12 × 100 mm and 15 × 120 mm, respectively. 0.1M Potassium phosphate buffer (pH 7.3) containing D-galactose (800 mg) and sucrose (2.4 g) (4 mL) was circulated through the columns in series at a speed of ~0.5 mL/min. After 72 h, the activated carbon column was disconnected from the system, and was washed first with water (200 mL) and next with 5% ethanol (200 mL). The products were then eluted from the activated carbon column with 50% ethanol (200 mL). The eluate was concentrated to a syrup and the syrup was applied to an activated carbon column (20 × 600 mm). The products were eluted with a linear gradient of 0–50% ethanol in water (800 mL). The fractions containing trisaccharide were pooled and concentrated to give syrupy **3** (237 mg, 10.6% yield).

*Anal.* Calc. for $C_{18}H_{32}O_{16}$: C, 42.86; H, 6.39. Found: C, 42.87; H, 6.43.

*β-D-Galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside* (**3**) *and α-D-glucopyranosyl-(1→2)-[β-D-galactopyranosyl-(1→6)-]-β-D-fructofuranoside* (**4**). — A solution (2 mL) of 0.1M potassium phosphate buffer (pH 6.0) containing D-galactose (500 mg), sucrose (1.2 g), and β-galactosidase from *E. coli* (3 mg) was incubated at 55°. After 48 h, the enzyme was denatured by heating in boiling water and filtered off. The filtrate was applied to an activated carbon column (20 × 600 mm), and trisaccharides were eluted with a linear gradient of 0–50% ethanol in water (1000 mL). Fractions containing trisaccharides were pooled and concentrated with an evaporator to give a mixture of **3** and **4** (185 mg) as a syrup. From a portion of the syrup, the two trisaccharides were purified by preparative l.c. to give a sample for a $^{13}C$-n.m.r. spectroscopic measurement and an elemental analysis.

*Anal.* Calc. for $C_{18}H_{32}O_{16}$: C, 42.86; H, 6.39. Found (for **4**): C, 42.99; H, 6.49.

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