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ENZYMIC SYNTHESIS OF DISACCHARIDES BY USE OF THE REVERSED HYDROLYSIS ACTIVITY OF β-D-GALACTOSIDASES*

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ABSTRACT

D-Galactosyl disaccharides have been synthesized by utilizing the reversed hydrolysis activity of β-D-galactosidases from *E. coli* and from *A. oryzae*, respectively. In order to shift the equilibrium towards the formation of disaccharide, solutions of monosaccharides were circulated through columns of immobilized β-D-galactosidase and activated carbon in series. After 24 h, the disaccharides were eluted with aqueous 50% ethanol from the column of activated carbon and analyzed by h.p.l.c. and ^{13}C-n.m.r. spectroscopy. In this way, β-D-galactosyl-D-glucose, β-D-galactosyl-2-acetamido-2-deoxy-D-glucose, and β-D-galactosyl-D-fructose were produced in yields of 6.0, 16.0, and 11.3%, respectively, when the immobilized β-D-galactosidase from *E. coli* was used. The possible mechanism of the synthesis of the disaccharides is discussed.

INTRODUCTION

D-Galacto-oligosaccharides have been synthesized mainly by utilizing the transglycosylation activity of β-D-galactosidase of various origins[1–4]. However, it is difficult to separate the products from the starting disaccharides and also to determine the optimum time to terminate the reaction in order to obtain high yields of the desired compounds[5].

We have reported the preparation of disaccharides by utilizing the reversed hydrolysis activity of glycosidases[6,7]. The equilibrium between mono- and disaccharides in solution can be shifted markedly towards the disaccharides by using high concentrations of monosaccharides in a batch method[6], or by adsorption of the disaccharides produced on a column of activated carbon in a continuous process[7]. Disaccharides are adsorbed on activated carbon in preference to monosaccharides[8].

We now report on the synthesis of disaccharides by two procedures using the reversed hydrolysis activity of two β-D-galactosidases and on the structure of the products in relation to the origin of the enzyme.

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RESULTS AND DISCUSSION

Synthesis of D-*galactosyl*-D-*glucose.* — High concentrations of monosaccharides favour the formation of disaccharide and, for D-galactosyl-D-glucose derivatives, the ratio of D-glucose/D-galactose must be as high as possible in order to minimize the formation of D-galactosyl-D-galactoses. Therefore, a solution containing 10% of D-galactose and 50% of D-glucose was used together with β-D-galactosidase from *E. coli* and from *A. oryzae*. The optimum pH for the reversed hydrolysis reaction with almond β-D-glucosidase is different from that of the hydrolysis reaction[6]. Since the optimum pH of β-D-galactosidase and immobilized β-D-galactosidase used in the present study was a plateau between pH 7 and 9, and the immobilized β-D-galactosidase exhibited maximum stability at pH 6.0, the reaction was conducted at pH 7.3.

The solution of monosaccharides was circulated (peristaltic pump) for 24 h at room temperature through columns in series of immobilized enzyme and activated carbon (see Experimental). H.p.l.c. of the eluate of the column of activated carbon with aqueous 50% ethanol solution then afforded the pattern shown in Fig. 1. In the batch process, the solution of monosaccharides was incubated for 24 h at 55° with the β-D-galactosidase. After removal of the heat-denatured enzyme, h.p.l.c. of the filtrate gave a pattern similar to that in Fig. 1.

In Fig. 1, peak A contained D-galactose and D-glucose, and the peaks B–E contained β-D-galactosyl-D-glucose with (1→3), (1→4), (1→2), and (1→6) linkages, respectively, which were identified on the basis of the retention volume in h.p.l.c.

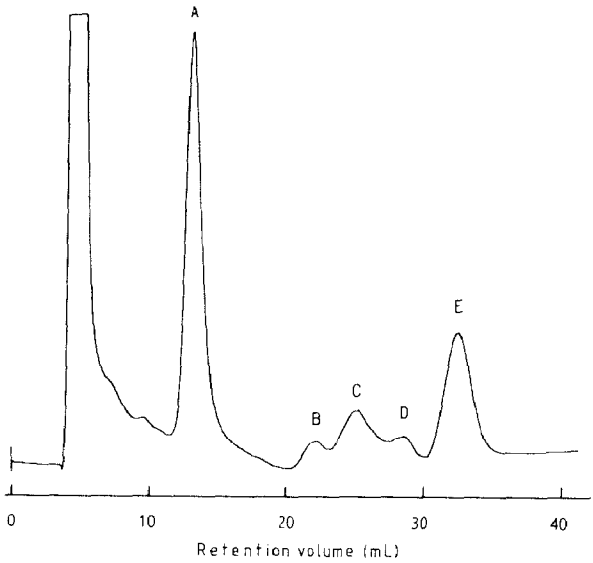

Fig. 1. H.p.l.c. of the aqueous 50% ethanol eluate of the column of activated carbon after the circulation of aqueous 10% D-galactose and 50% D-glucose through a column of β-D-galactosidase (*E. coli*)–Eupergit.

TABLE I

β-D-GALACTOPYRANOSYL-D-GLUCOSES PRODUCED BY THE BATCH AND CONTINUOUS METHODS USING β-D-GALACTOSIDASE FROM *E. coli*

Method	*Yield (%)*	*Composition (%)*			
		(1→2)	*(1→3)*	*(1→4)*	*(1→6)*
Batch	2.9	13	6	27	54
Continuous	6.0	9	9	28	54

and comparison of the ^{13}C-n.m.r. data with the literature data[10–12]. The yields of disaccharides are summarized in Table I. The yield of disaccharides by the continuous process was about twice that of the batch method.

Synthesis of D-*galactosyl-2-acetamido-2-deoxy*-D-*glucose.* — An aqueous solution containing 10% of D-galactose and 30% of 2-acetamido-2-deoxy-D-glucose was used. H.p.l.c. of the aqueous 50% ethanol eluate of the column of activated carbon used in the continuous method afforded two peaks in the ratio ~1:4 in the region for disaccharides (Fig. 2). A similar result was obtained with the batch method and with the β-D-galactosidase from *E. coli* or *A. oryzae.*

The ^{13}C-n.m.r. spectrum (Fig. 3A) of the product in peak C corresponded to that of authentic 2-acetamido-2-deoxy-4-*O*-β-D-galactosyl-D-glucose[10] (Fig. 3C). The signals at 61.1 and 62.0 p.p.m. in Fig. 3C were assigned to C-6 of 2-acetamido-2-deoxy-D-glucose and D-galactose residues, respectively. In the spectrum of the product in peak D (Fig. 3B), the signal for C-6 of the 2-acetamido-2-deoxy-D-glucose residue is shifted to lower field; therefore, the galactosyl residue must be attached to position 6. Thus, peak D contains 2-acetamido-2-deoxy-6-*O*-β-D-galactosyl-D-glucose. The yields of these disaccharides are summarized in Table II.

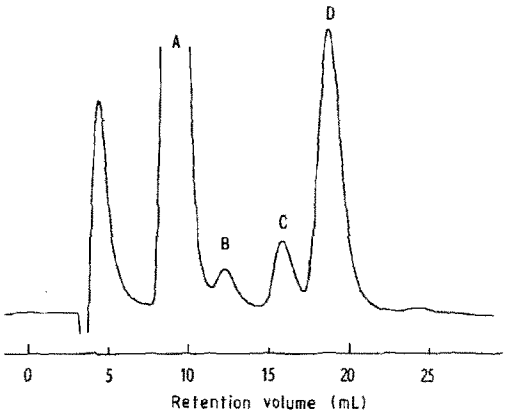

Fig. 2. H.p.l.c. of the aqueous 50% ethanol eluate of the column of activated carbon after the circulation of aqueous 10% D-galactose and 30% 2-acetamido-2-deoxy-D-glucose through a column of β-D-galactosidase *(E. coli)*–Eupergit.

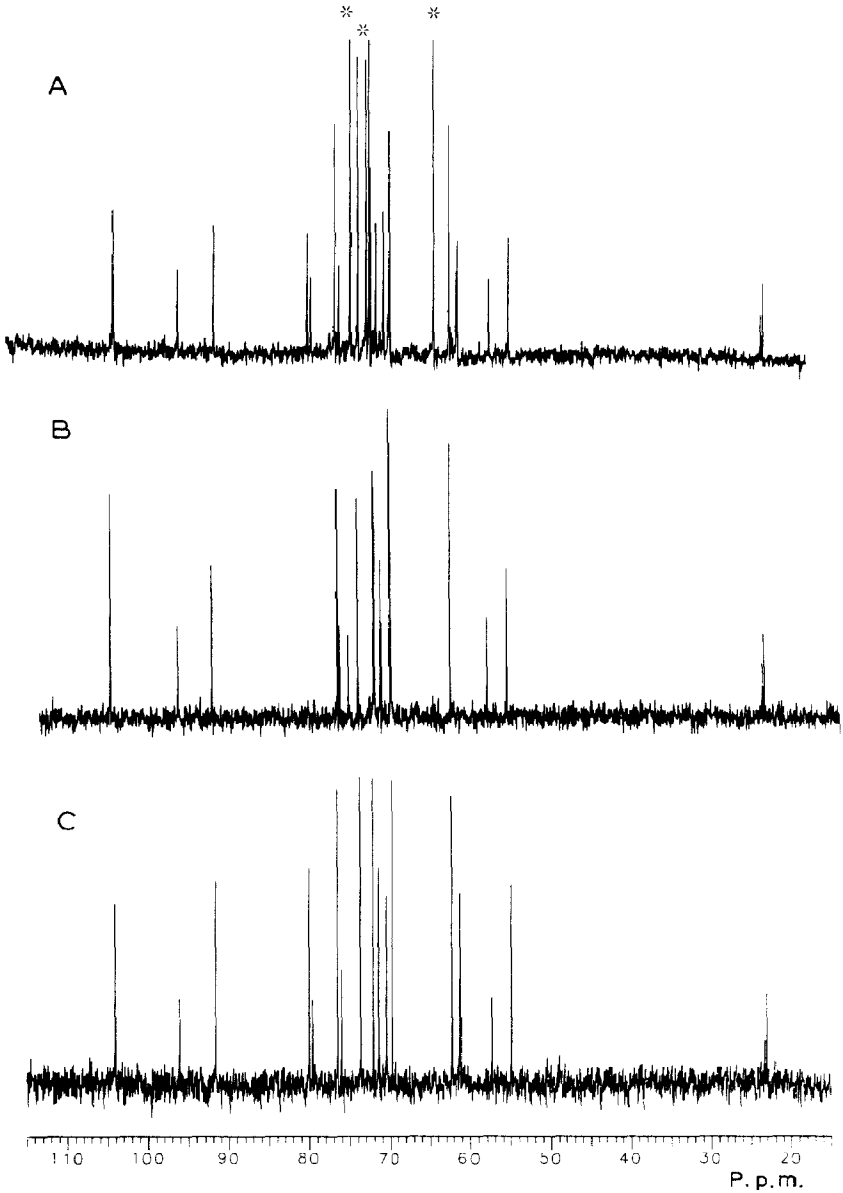

Fig. 3. ^{13}C-N.m.r. spectra of *A*, peak C; *B*, peak D (see Fig. 2); and *C*, authentic 2-acetamido-2-deoxy-4-*O*-β-D-galactopyranosyl-D-glucose; * denotes an unidentified impurity.

The total yield of the disaccharides (14.0–16.0% on the basis of D-galactose) by the column method was about twice that (7.7–8.9%) of the batch method.

Synthesis of D-*galactosyl*-D-*fructose*. — The viscous solution containing 100% of D-fructose and 10% of D-galactose in 0.1M phosphate buffer (pH 7.3) was incubated with the β-D-galactosidase from *E. coli* and from *A. oryzae* for 24 h at 55°.

TABLE II

β-D-GALACTOPYRANOSYL-2-ACETAMIDO-2-DEOXY-D-GLUCOSES PRODUCED BY THE BATCH AND CONTINUOUS METHODS USING THE β-D-GALACTOSIDASES FROM *E. coli* AND *A. oryzae*

Method	*β-D-Galactosidase*	*Yields(%)*	*Composition (%)*	
			(1→4)	*(1→6)*
Batch	*E. coli*	7.6	20	80
	A. oryzae	8.9	18	82
Continuous	*E. coli*	16.0	20	80
	A. oryzae	14.0	18	82

For the continuous method, a solution of 10% of D-galactose and 50% of D-fructose was used. H.p.l.c. of the products by the continuous method using β-D-galactosidase from *E. coli* and *A. oryzae* gave the results shown in Fig. 4. The products in the various peaks were identified on the basis of the comparison of their ^{13}C-n.m.r. spectra with literature data[10–12]. The peaks C–F contained β-D-galactopyranosyl-D-fructose with (1→5), (1→4), (1→6), and (1→1) linkages, respectively. In the (1→5)-linked β-D-galactosyl-D-fructose, the fructose residue must be pyranoid. The yields of the disaccharides are summarized in Table III.

In the preliminary report[7] for the reaction of D-galactose and D-fructose by the column method, the peaks D and E overlapped in h.p.l.c. and the yield of the (1→6)-linked disaccharide was smaller than in the present experiment and its presence was not detected.

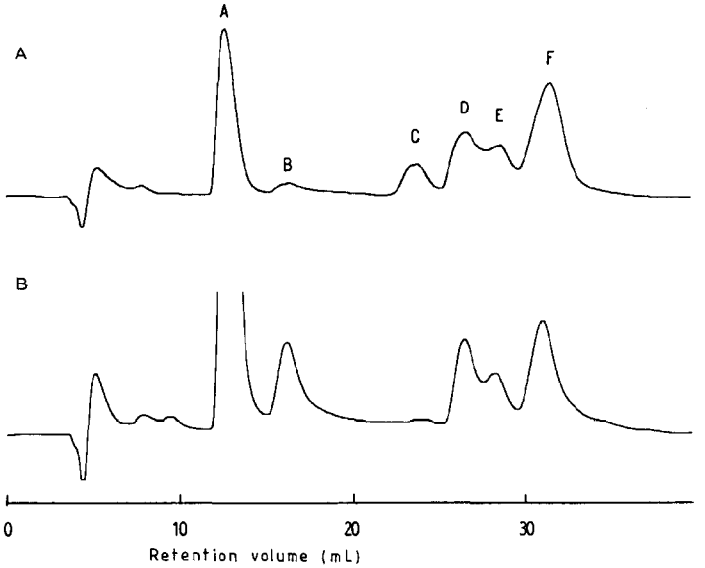

Fig. 4 H.p.l.c. of the aqueous 50% ethanol eluate of the column of activated carbon after the circulation of aqueous 10% D-galactose and 50% D-fructose through columns of *A*, β-D-galactosidase *(E. coli)*–Eupergit; and *B*, β-D-galactosidase *(A. oryzae)*–Eupergit.

TABLE III

β-D-GALACTOPYRANOSYL-D-FRUCTOSES PRODUCED BY THE BATCH AND CONTINUOUS METHODS USING THE β-D-GALACTOSIDASES FROM *E. coli* AND *A. oryzae*

Method	*β-D-Galactosidase*	*Yield(%)*	*Composition (%)*			
			(1→1)	*(1→4)*	*(1→5)*	*(1→6)*
Batch	*E. coli*	55.0	61	19	12	8
	A. oryzae	57.6	57	18	16	9
Continuous	*E. coli*	11.3	51	24	10	15
	A. oryzae	8.0	51	29	1	19

The yield of the (1→5)-linked disaccharide was negligibly small when the β-D-galactosidase from *A. oryzae* was used. The composition of the mixture of disaccharides obtained by the continuous method was quite different from that obtained by the batch method. In particular, the yield of the (1→6)-linked disaccharide obtained by the continuous method was about twice that obtained by the batch method. Furthermore, the yield of disaccharides by the batch method was >50% on the basis of D-galactose. Thus, for the synthesis of disaccharides containing D-fructose as the "aglycon", the batch method with a high concentration of D-fructose is the most favorable.

Thus, in the syntheses of the D-galactosyl-D-glucose and D-galactosyl-2-acetamido-2-deoxy-D-glucoses, the compositions of the mixtures of disaccharides were similar for the batch and the continuous methods, and were not affected by the origin of the enzyme. On the other hand, in the synthesis of the D-galactosyl-D-fructoses by the continuous method, the proportions of the (1→5)-linked disaccharides were markedly different for the two enzymes and, for both β-D-galactosidases, the ratios of (1→5)- and (1→6)-linked disaccharides associated with the batch and continuous methods were different.

If the transglycosylation reactions involved in the reversed hydrolysis reaction are rapid, then the compositions of the mixtures of disaccharides will be determined kinetically. However, if they are relatively slow, then the disaccharides produced will be adsorbed by the activated carbon before extensive transglycosylation can occur. If the first assumption is correct, the compositions of the mixtures of D-galactosyl-D-fructoses associated with the continuous method may represent the intrinsic ratio of the disaccharide formation by the β-D-galactosidases. Further work is necessary in order to clarify the situation fully.

EXPERIMENTAL

Materials. — β-D-Galactosidases from *E. coli* and from *A. oryzae* were purchased from Sigma and Toyobo (Osaka, Japan), respectively. Eupergit C was purchased from Röhm Pharma (Weiterstadt). The activated carbon was a product of Merck.

Analytical procedures. — H.p.l.c. was performed on a Pharmacia P-3500 pump system equipped with a Shodex SE-51 refractive index monitor (Showa Denko Co. Ltd., Tokyo) and a column (4 × 250 mm) of LiChrosorb-NH_2. The mobile phase was 3:1 acetonitrile and deionized, distilled water. The separation of the peaks was assessed by sampling the fraction repeatedly (5–10 times) with the same system as for the analytical experiment. ^{13}C-N.m.r. spectra (100 MHz) were recorded with a Varian XL-400 spectrometer on solutions in D_2O (internal acetonitrile, 18.29 p.p.m.).

Assay of immobilized enzyme. — To 0.1M sodium phosphate buffer (pH 7.3, 14.5 mL) containing 3.36M 2-mercaptoethanol, 0.03M $MgCl_2$, and 5 mg of wet β-D-galactosidase–Eupergit gel was added 68mM *o*-nitrophenyl β-D-galactopyranoside (0.5 mL). The solution was incubated at 37° and the absorbance was measured at 410 nm every 30 s on ~2-mL samples. Under these conditions, 1 unit of enzyme activity liberates 1 μmol of *o*-nitrophenol/min.

Immobilization of β-D-galactosidase with Eupergit C. — To a suspension of dry Eupergit C (0.8 g) in M potassium phosphate buffer (pH 7.4, 4 mL) was added a solution of β-D-galactosidase from *A. oryzae* (100 mg, 107 units/mg) or *E. coli* (7.5 mg, 580 units/mg) in 1 mL of the buffer. Each suspension was mixed using a rotator III (Taiyo Co., Tokyo) for 24 h at ~23°. Each gel was then collected on a glass filter and washed thoroughly with the buffer to give ~2.3 and 4 g, respectively, of wet gels containing the β-D-galactosidases from *A. oryzae* and *E. coli* with activities of 0.49 and 0.19 unit/mg of solid, respectively.

Synthesis of disaccharides. (*a*) *By the batch method.* To an aliquot (1 mL) of 0.1M sodium phosphate buffer (pH 7.3, 100 mL) containing D-galactose (10 g) and appropriate amounts of "aglycon" monosaccharide was added β-D-galactosidase from *E. coli* (2 mg, 580 units/mg) or *A. oryzae* (10 mg, 107 units/mg), respectively, and each solution was incubated at 55°. After 24 h, the enzyme was heat-denatured, and the filtrate was analyzed by h.p.l.c.

(*b*) *By the continuous method.* Wet β-D-galactosidase–Eupergit gels from *E. coli* (760 units, 4 g) and *A. oryzae* (1130 units, 2.3 g), respectively, were packed into columns (12 × 100 mm) and each column was connected in series to a column (22 × 150 mm) of activated carbon (15 g). The solution (10 mL) containing D-galactose and the "aglycon" monosaccharide was circulated by a peristaltic pump at ~0.5 mL/min. After 24 h, the column of activated carbon was washed with water (500 mL) and then aqueous 5% ethanol (300 mL). The disaccharides were eluted with aqueous 50% ethanol (300 mL). Concentration of the eluate gave 0.2–1.7 g of a syrup containing mono- and di-saccharides.

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