Enzymatic Synthesis of Galactooligosaccharides by the Condensation Action of Thermostable α-Galactosidase from *Pycnoporus cinnabarinus*

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Three kinds of trisaccharides were synthesized using condensation action of α-galactosidase (EC 3. 2. 1. 22) from *pycnoporus cinnabarinus* on the mixture of galactose and sucrose. The condensation products were isolated and their structures were investigated by acid and enzymatic hydrolysis, and methylation analysis. The three trisaccharides isolated were identified as raffinose, planteose, and 3^G-α-galactosylsucrose. Conditions for synthesis of the trisaccharides by condensation action of the α-galactosidase were studied. The yield of the trisaccharides was approximately 15% based on the amount of galactose used, when α-galactosidase (40 units/m*l*) was incubated with 10% (w/v) galactose and 60% (w/v) sucrose for 48 h at pH 5.0 and 60°C.

In our previous paper[1], we reported the isolation and identification of oligosaccharides produced from raffinose by transgalactosylation action of thermostable α-galactosidase (EC 3. 2. 1. 22) from *pycnoporus cinnabarinus*. The enzyme catalyzed the transfer action of galactosyl residues to the C-6 or C-3 hydroxyl group of the terminal galactose moiety of raffinose family oligosaccharides and the C-3 hydroxyl group of the glucose moiety of sucrose.

Galactooligosaccharides containing α-galactosidic linkage, such as raffinose and stachyose, have been reported to promote the growth of bifidobacteria[2]. Galactooligosaccharides synthesized by transgalactosylation action or by condensation action of α-galactosidase are expected to be utilized as a growth factor for bifidobacteria. However, there is no method available to the industrial production of raffinose by transgalactosylation action of α-galactosidase. Therefore, we attempted to synthesize raffinose by condensation action of *P. cinnabarinus* α-galactosidase on the mixture of galactose and sucrose.

Many papers on transgalactosylation action of α-galactosidase have been published[3], and there have been a few reports on condensation action of α-galactosidase. CLANCY and WHELAN[4] synthesized galactobioses from galactose by condensation reaction with yeast α-galactosidase. Recently, AJISAKA and FUJIMOTO[5] reported the synthesis of galactosylsucroses from galactose and sucrose using condensation reaction with α-galactosidase from *Mortierella vinacea*.

This paper deals with the isolation of three kinds of oligosaccharides produced by condensation action of *P. cinnabarinus* α-galactosidase on the mixture of galactose and sucrose and the identification of their structures. Further, conditions for synthesis of oligosaccharides by the condensation reaction of the enzyme are described.

Materials and Methods

Materials

Galactose, sucrose, and raffinose were purchased from Wako Pure Chemical Industries, Ltd. Planteose was a gift from Professor Y. UENO of Gifu University. 3^G-α-Galactosylsucrose was prepared by the procedure described

in our previous paper[1].

α-Galactosidase from *Pycnoporus cinnabarinus* IFO 6139 was purified according to the procedure described in the previous paper[6]. β-Fructofuranosidase from *Candida utilis* was purchased from Seikagaku Kogyo Co., Ltd.

Activated carbon for chromatography and Bio-Gel P-2 (200-400 mesh) were obtained from Wako Pure Chemical Industries, Ltd. and Bio-Rad Laboratories, respectively.

Enzyme assay

α-Galactosidase activity was assayed by the method described previously[7]. One unit of enzyme activity was defined as the amount of enzyme which released one μmol of *p*-nitrophenol per min.

High performance liquid chromatography (HPLC)

The HPLC equipment consisted of an 880-PU pump, an 830-RI RI detector (Japan Spectroscopic Co., Ltd.) a sample injector (Model 7125, Rheodyne Inc.), and a D-2500 Chromato-Integrator (Hitachi Ltd.). Sugars were separated on Radial-PAK μBondapak NH_2 (8.0 × 100 mm, Waters Associates) using acetonitrile-water mixtures in various ratios as the mobile phase, at a flow rate of 2.0 m*l* per min.

Determination of sugar components

A sample (2 mg) was hydrolyzed in 1.0 m*l* of 0.25N H_2SO_4 at 100°C for 4 h in a sealed tube. The hydrolyzate was neutralized with barium carbonate and the precipitate was removed by filtration. The supernatant was trimethylsilylated[8] and analyzed in a Yanagimoto G180 Gas Chromatograph, connected with a flame ionization detector and a column (0.3 × 200 cm) of 3% SE-52 on Chromosorb W (AW-DMCS, 80 ~ 100 mesh). The column temperature was programmed from 130 to 160°C at a rate of 0.5°C per min. For the determination of fructose, after a sample was hydrolyzed for 2 h, the hydrolyzate was neutralized,trimethylsilylated and then analyzed by gas liquid chromatography in the same manner described above. The trimethylsilyl derivative of methyl-α-mannoside was used as an internal standard.

Methylation analysis

Each oligosaccharide was methylated by the method of HAKOMORI[9], and the methylated product was hydrolyzed with 1 N H_2SO_4 at 100°C for 3 h in a sealed tube. The methylated sugar was reduced with sodium borohydride and then acetylated, and the mixture of alditol acetates was analyzed by gas liquid chromatography, using a column (0.3 × 200 cm) of 3% ECNSS-M on Gas Chrom Q (100 ~ 200 mesh) at 180°C[10]. The partially methylated alditol acetates were identified by comparison of their retention times on gas liquid chromatography with those of the partially methylated alditol acetates derived from authentic raffinose, planteose, and 3^G-α-galactosylsucrose.

Measurement of sugar

The total sugar content in each fraction separated by column chromatography was measured by the phenol-sulfuric acid method[11] using galactose as a standard.

Enzymatic hydrolysis of oligosaccharide.

The reaction mixture consisting of 0.1 m*l* of 2.5% oligosaccharide, 0.1 m*l* of 12.5 mM phosphate buffer (pH 5.8), and 0.05 m*l* of *P. cinnabarinus* α-galactosidase (1.25 units) or *C. utilis* β-fructofuranosidase (1.25 units) was incubated for 24 h at 37°C. The reaction was stopped by boiling for 5 min, the hydrolysis products were analyzed by HPLC.

Optical rotation

Optical rotation was determined with a digital polarimeter DIP-370 (Japan Spectroscopic Co., Ltd.).

Results and Discussion

Isolation of condensation products

A reaction mixture (18.3 m*l*) containing 10% (w/v) galactose, 60% (w/v) sucrose in 0.02 M acetate buffer (pH 5.0), and *P. cinnabarinus* α-galactosidase (732 units) was incubated for 48 h at 60°C. The reaction was stopped by boiling for 5 min. The condensation products were detected by HPLC. As shown in Fig. 1, three kinds of the condensation products (oligosaccharides A, P, and R) were produced. Oligosaccharides A, P, and R showed the same retention time as those of authentic 3^G-α-galactosylsucrose, planteose, and raffinose, respectively.

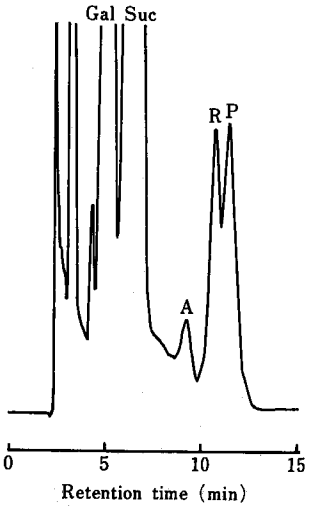

Fig. 1 HPLC of the products formed by condensation action of α-galactosidase on galactose and sucrose

The reaction mixture was filtered and 10 μl of the sample was injected. Sugars were eluted with acetonitrile-water (74 : 26). Gal, galactose; Suc, sucrose; A, oligosaccharide A; P, oligosaccharide P; R, oligosaccharide R

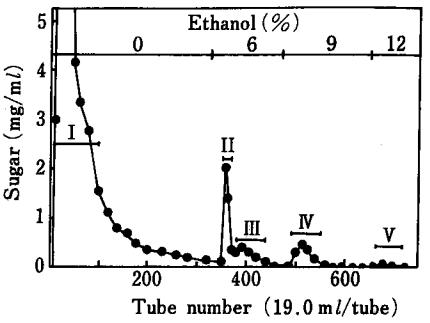

Fig. 2 Carbon column chromatogram of the products formed by condensation action of α-galactosidase on galactose and sucrose

The experimental details are described in the text.

The reaction mixture was diluted to 37 ml with distilled water, the mixture was put on a carbon column (5 × 28 cm) and sugars were eluted with successively with distilled water (6.17 l), 6% ethanol (2.74 l), 9% ethanol (0.45 l), and 12% ethanol (1.75 l) at a flow rate of 91 ml per hour. The elution profile is shown in Fig. 2. Fractions I–V were collected, and sugar composition in each fraction was detected by HPLC. Fraction I (No. 10 to 100) was composed of galactose and sucrose, and fraction II (No. 353 to 370) was composed of sucrose and small amounts of oligosaccharides A and P. Oligosaccharides A, P, and R were found mainly in fractions V, III, and IV, respectively.

Fraction III (No. 371 to 440) contained oligosaccharide P and small amounts of sucrose and oligosaccharide A. Fraction III was concentrated to *ca.* 5 ml using a rotary evaporator and was applied to a column (2.6 × 180 cm) of Bio-Gel P-2 and eluted with water at a flow rate of 21 ml per hour. The eluate was fractionated in 5-ml tubes. Collected portion was still contaminated with a small amount of oligosaccharide A, and further purification was done twice by use of carbon column (1.8 × 23 cm) chromatography. After analyzing the sugar composition of each tube by HPLC, pure fractions containing oligosaccharide P were collected and lyophilized. The yield of oligosaccharide P was 173 mg.

Fraction IV (No. 490 to 550) consisting of oligosaccharides R and A was fractionated by gel filtration on Bio-Gel P-2 and carbon column chromatography in the same manner as in the case of purification of oligosaccharide P, and pure oligosaccharide R (173 mg) was obtained.

Oligosaccharide A was also purified by gel filtration on Bio-Gel P-2 from fraction V (No. 670 to 720), and 25 mg of pure oligosaccharide A was obtained.

Structure of condensation products

Complete acid hydrolysis of oligosaccharides A, P, and R gave equimolar parts of galactose, glucose, and fructose. The degree of polymerization of these products was estimated as 3, on Bio-Gel P-2 gel filtration.

The results of hydrolysis of these oligosaccharides with α-galactosidase or β-fructofuranosidase are shown in Fig. 3. Both of

oligosaccharides A and R were completely hydrolyzed by α-galactosidase to galactose and sucrose. β-Fructofuranosidase released fructose from these two oligosaccharides. On methylation analysis, alditol acetates of 1, 3, 4, 6-tetra-*O*-methyl-D-fructose, 2, 3, 4, 6-tetra-*O*-methyl-D-galactose, and 2, 4, 6-tri-*O*-methyl-D-glucose were detected for oligosaccharide A, and 1, 3, 4, 6-tetra-*O*-methyl-D-fructose, 2, 3, 4, 6-tetra-*O*-methyl-D-galactose, and 2, 3, 4-tri-*O*-methyl-D-glucose for oligosaccharide R (Table 1). From above results, oligosaccharides A and R were identified as *O*-α-D-galactopyranosyl-(1-3)-*O*-α-D-glucopyranosyl-(1-2)-β-D-fructofuranoside (3^G-α-galactosylsucrose) and *O*-α-D-galactopyranosyl-(1-6)-*O*-α-D-glucopyranosyl-(1-2)-β-D-fructofuranoside (raffinose), respectively. The specific rotation of oligosaccharide R was $[\alpha]_D^{20}$ + 124° (c = 1.0, in water), which agreed with that of raffinose in the literature[12]. Oligosaccharide A showed $[\alpha]_D^{20}$ + 141° (c = 0.2, in water). SØMME and WICKSTRØM[13] reported that 3^G-α-galactosylsucrose showed $[\alpha]_D^{22}$ + 122° (c = 2.0, in water).

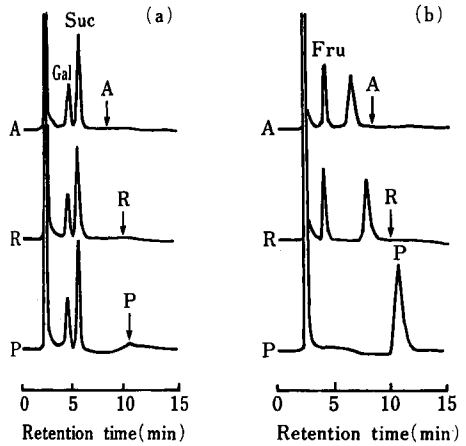

Fig. 3 HPLC showing hydrolyzated products from the oligosaccharides by the action of *P. cinnabarinus* α-galactosidase (a) and *C. utilis* β-fructofuranosidase (b)

The experimental details are described in Materials and Methods. Sugars were eluted with acetonitrile-water (76 : 24). Fru, fructose; Gal, galactose; Suc, sucrose; A, oligosaccharide A; P, oligosaccharide P; R, oligosaccharide R

Oligosaccharide P was hydrolyzed to galactose and sucrose by α-galactosidase, but it was not attacked by β-fructofuranosidase (Fig. 3). Methylation analysis of oligosaccharide P gave 2, 3, 4, 6-tetra-*O*-methyl-D-glucose, 2, 3, 4, 6-tetra-*O*-methyl-D-galactose, and 1, 3, 4-tri-*O*-methyl-D-fructose (Table 1). The specific rotation of oligosaccharide P was $[\alpha]_D^{20}$ + 130° (c = 1.0, in water), which agreed with that of planteose in the literature[12]. From these results, a possible structure of oligosaccharide P is *O*-α-D-galactopyranosyl-(1-6)-*O*-β-D-fructofuranosyl-(2-1)-α-D-glucopyranoside (planteose).

The oligosaccharides synthesized by condensation action of α-galactosidase from *P. cinnabarinus* on the mixture of galactose and sucrose were trisaccharides in which galactose was attached to the C-6 or C-3 hydroxyl group

Table 1 Molar ratios of alditol acetates derived from permethylated oligosaccharides

Oligosaccharides	1, 3, 4, 6-Tetra-*O*-Me-D-Fru	2, 3, 4, 6-Tetra-*O*-Me-D-Glc	2, 3, 4, 6-Tetra-*O*-Me-D-gal	1, 3, 4-Tri-*O*-Me-D-Fru	2, 4, 6-Tri-*O*-Me-D-Glc	2, 3, 4-Tri-*O*-Me-D-Glc
Oligosaccharide A	0.8		1.0		1.2	
Oligosaccharide P		1.2	1.0	0.7		
Oligosaccharide R	0.8		1.0			1.1
3^G-α-galactosylsucrose	0.8		1.0		1.1	
Planteose		1.2	1.0	0.8		
Raffinose	0.8		1.0			1.0

of the glucose moeity of sucrose, and the C-6 hydroxyl group of the fructose moeity of sucrose. Thus, the oligosaccharide with α (1-6) galactosidic linkage was preferentially synthesized by this enzyme. The yield of the oligosaccharide having α (1-3)-galactosidic linkage was very low. We have reported[1] that the *P. cinnabarinus* α-galactosidase transfer galactosyl residue from raffinose to the C-6 or C-3 hydroxyl group of the galactose moiety of raffinose and the glucose moiety of sucrose. However, in the presence of this enzyme and high concentration of raffinose, planteose has not been formed as the transfer product[1]. AJISAKA and FUJIMOTO[5] have reported that raffinose and planteose are formed, when a solution containing galactose and sucrose is incubated in the presence of α-galactosidase from *M. vinacea,* and only raffinose is obtained by circulation of the reaction mixture through an activated carbon column. However, formation of 3^G-α-galactosylsucrose has not been reported.

Three kinds of trisaccharides containing α-galactosidic linkage isolated by the condensation reaction in this study have been widely distributed in the plant kingdom[14]. YAZAWA *et al.* have reported that raffinose and stachyose promote the growth of bifidobacteria[2]. Therefore, these condensation products are expected to be utilized as a growth factor for bifidobacteria.

Condition for condensation reaction

Conditions for synthesis of the trisaccharides (3^G-α-galactosylsucrose, planteose, and raffinose) from galactose and sucrose by condensation reaction were investigated. The trisaccharides formed by the condensation reaction were analyzed by HPLC. The effect of pH on the formation of the trisaccharides is shown in Fig. 4. The rate of the total trisaccharides formation increased as pH decreased. The yield of the trisaccharides gave the highest value at pH 4.0 near the optimal pH 5.0 for the hydrolysis activity of this enzyme[15]. Fructose would be released by acid hydrolysis of β-fructofuranoside linkage to decrease the yield of the trisaccharides below pH 4.0 at high temperature.

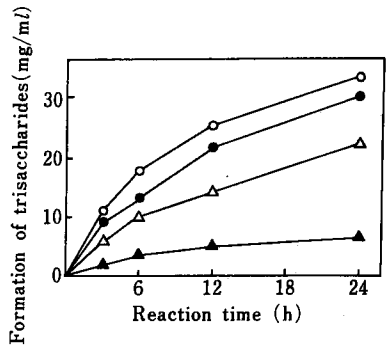

Fig. 4 Effect of pH on the formation of the trisaccharides by condensation action of α-galactosidase.

The mixture of α-galactosidase (20 units/ml), 10% (w/v) galactose, and 60% (w/v) sucrose was incubated at 60°C in various buffers of pH 4.0 to 7.0.

Buffers: pH 4.0 (○) and 5.0 (●), 20 mM acetate buffer; pH 6.0 (△) and 7.0 (▲), 20 mM sodium phosphate buffer

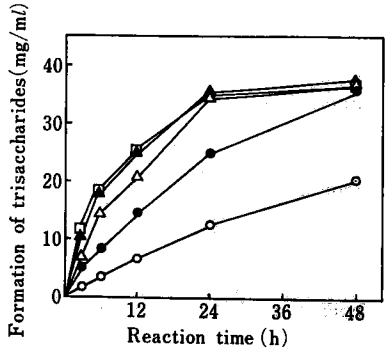

Fig. 5 Effect of temperature on the formation of the trisaccharides by condensation action of α-galactosidase

The mixture of α-galactosidase (20 units/ml), 10% (w/v) galactose, and 60% (w/v) sucrose was incubated in 20 mM acetate buffer (pH 5.0) at various temperatures from 40 to 80°C.

○, 40°C; ●, 50°C; △, 60°C; ▲, 70°C; □, 80°C.

Figure 5 shows the effect of temperature on the condensation reaction. The reaction mixture was incubated at various temperatures

and pH 5.0. The amount of the total trisaccharides increased with the rise of temperature. However, when the reaction mixture was incubated for 48 h at 60°C, 70°C, and 80°C, the amount of the trisaccharides formed was almost the same (about 37 mg/m*l*). The ratio of 3^G-α-galactosylsucrose, raffinose, and planteose was approximately 1 : 4 : 5, and was little affected by the change of temperature.

The effect of sucrose concentration on the formation of the total trisaccharides by condensation reaction was examined. The mixture of 10% (w/v) galactose, various concentrations of sucrose, and the enzyme was incubated. As shown in Fig. 6. The yield of the trisaccharides increased as the initial concentration of sucrose increased. However, the amount of 3^G-α-galactosylsucrose was extremely low,compared with those of planteose and raffinose.

The synthesis of the trisaccharides with various enzyme concentrations was examined. The amount of the total trisaccharides formed by condensation reaction was shown in Fig. 7. The rate of the trisaccharides formation increased as the enzyme concentration in creased.

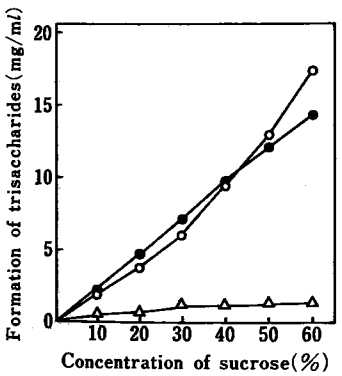

Fig. 6 Effect of sucrose concentration on the formation of the trisaccharides by condensation action of α-galactosidase

The mixture of α-galactosidase (100 units/m*l*), 10% (w/v) galactose, and 10–60% (w/v) sucrose was incubated in 20 mM acetate buffer (pH 5.0) for 24 h at 60°C.
△, 3^G-α-galactosylsucrose ; ○, planteose ; ●, raffinose

The maximum yield of the trisaccharides was obtained, when 40 units/m*l* of the α-galactosidase was used at the concentration of 10% (w/v) galactose and 60% (w/v) sucrose, at pH 5.0 and 60°C (Fig. 7), and the yield was approximately 15% on the basis of the amount of galactose added. This yield was relatively low, compared with that of the transfer products[15] formed by transgalactosylation action of this enzyme.

Generally, the condensation products can be obtained in good yield by increasing the concentration of substrate, by decreasing the concentration of water, or by eliminating the products from the reaction mixture. Mannooligosaccharides[16] and glucooligosaccharides[17] have been obtained in good yield by the incubation of extremely high concentration of substrate with jack bean α-mannosidase and almond β-glucosidase, respectively. On the other hand, the amount of the disaccharides formed by condensation of galactose and *N*-acetylglucosamine with β-galactosidase has also increased by use of columns of immobilized enzyme and activated carbon connected in series[18]. The products formed in the immobilized enzyme column were adsorbed on the

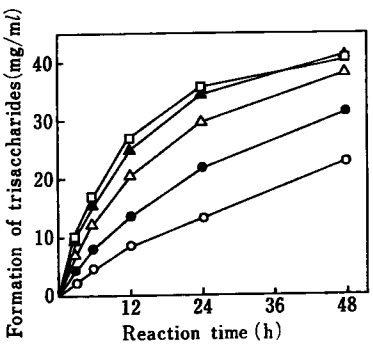

Fig. 7 Effect of enzyme concentration on the formation of the trisaccharides by condensation action of α-galactosidase

The mixture of α-galactosidase (5 ~ 80 units/m*l*), 10% (w/v) galactose, and 60% (w/v) sucrose was incubated in 20 mM acetate buffer (pH 5.0) at 60°C.
○, 5 units/m*l* ; ●, 10 units/m*l* ; △, 20 units/m*l* ; ▲, 40 units/m*l* ; □, 80 units/m*l*

carbon column, and the nonreacted substrate was recycled to accumulate the products on the carbon column.

In our study, the amounts of the trisaccharides enzymatically synthesized from galactose and sucrose gave the highest values around the optimal pH 5.0 and high temperatures from 60 to 80°C in the presence of 10% (w/v) galactose and 60% (w/v) sucrose. The concentration of galactose was fixed to 10% (w/v) in order to avoid the formation of galactobioses. α-Galactosidase from *P. cinnabarinus* used in this study is highly thermostable[7]. Therefore, this α-galactosidase is advantageous for the production of galactooligosaccharides by condensation reaction at high temperature, allowing the use of extremely high concentration of substrate and low concentration of water.

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Pycnoporus cinnabarinus 耐熱性 α-ガラクトシダーゼの縮合反応によるガラクトオリゴ糖の酵素合成

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Pycnoporus cinnabarinus 耐熱性 α-ガラクトシダーゼの触媒する縮合反応を用いてガラクトオリゴ糖の合成を試みた. α-ガラクトシダーゼを高濃度のガラクトースおよびシュクロースに作用させたところ 3 種類の三糖類が生成した. 生成したオリゴ糖は活性炭カラムクロマトグラフィーおよび Bio-Gel P-2 ゲル濾過により単離した. 得られたオリゴ糖は酸分解, 酵素分解およびメチル化分析により Gal α 1-3 Glc α 1-2 β Fru, Gal α 1-6 Glc α 1-2 β Fru (ラフィノース), および Gal α 1-6 Fru β 2-1 α Glc (プランテオース) と同定された. さらに縮合反応によるこれら三糖類の合成条件について検討した. 10% (w/v) ガラクトース, 60% (w/v) シュクロースおよび α-ガラクトシダーゼ (40 units/ml) からなる反応液を pH 5. 0, 60℃ で 48 時間反応させたとき, 縮合生成物の収率は約 15% (対ガラクトース) であった.