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Galactosylation of Thiol Group by β-Galactosidase

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β-Galactosidase catalyzed β-galactosylation not only of a hydroxyl group but also of a thiol group in the condensation reaction of D-galactose and 2-mercaptoethanol. The thio-galactosylation product was confirmed as 2-hydroxyethyl *S*-β-D-galactoside on the bases of fast atom bombardment mass spectrometry, infrared spectroscopy, and nuclear magnetic resonance spectorometry. *Aspergillus oryzae* β-galactosidase hydrolyzed *p*-nitrophenyl *S*-β-D-galactoside most rapidly among several β-galactosidases and produced the thio-galactosylation product most efficiently. The *Penicillim multicolor* enzyme was as effective as the *A. oryzae* enzyme. However the enzymes from *Escherichia coli*, *Saccharomyces fragilis*, *Kluyveromyces lactis*, and *Bacillus circulans* galactosylated hydroxyl groups predominantly to produce *O*-galactoside. The thio-galactoside was synthesized most effectively at a 2-mercaptoethanol concentration of about 1.25 M. Galactose concentration at 0.8–2.8 M did not affect the synthetic yield of the thio-galactoside so greatly.

Key words: β-galactosidase; *Aspergillus oryzae*; condensation reaction; thiol compoud; galactosylation

There are many investigations on the synthesis of galactooligosaccharides and galactosides by the transgalactosylation activity of β-galactosidases (EC 3.2.1.23).[1-3] The oligosaccharides, especially those derived from lactose, are of industrially importance because they are used as sweeteners having selective growth activity for Bifidobacteria. In addition to the transfer reaction, condensation (reverse hydrolysis) can be an useful tool for enzymatic synthesis of the saccharides.[4-10] Ajisaka *et al.*, for instance, showed effective synthesis of di- and trisaccharides by reverse hydrolysis activity of immobilized β-galactosidases.[5-7] In spite of such extensive studies, none have been reported for enzymatic galactosylation of thiol (SH) groups.

Thio-glycosides, in which aglycon molecules are bound with the C-1 carbons of saccharide molecules through thio-ether linkage, are often found in nature. For example, sinigrin and sinalbin, both β-thio-glucosides, exist in some mustard plants. They are known to be hydrolyzed by thio-glucosidase (sinigrinase, myrosinase, EC 3.2.3.1). In our preliminarily experiments, a white mustard thio-glucosidase commercially available synthesized no transfer and condensation products. On the other hand, we detected β-thio-galactosidase activities, even though they were not high, in several β-galactosidases, and found that β-galactosylation occurred not only at hydroxyl (OH) groups but also at SH groups of 2-mercaptoethanol (ME) through condensation. This report deals with this versatile activity of β-galactosidase as one possilble and effective method to synthesize β-thio-galactosides.

Materials and Methods

Substrates and chemicals. *p*-Nitrophenyl *O*-β-D-galactopyranoside (Gal-*O*-*p*NP), D-galactose (Gal), 2-mercaptoethanol (ME), dithiothreitol (DTT), L-cysteine hydrochloride (Cys), and *N*-acetyl L-cysteine (Cys·*N*Ac) were purchased from Nakalai Tesque Co. *p*-Nitrophenyl *S*-β-D-galactopyranoside (Gal-*S*-*p*NP), *p*-nitrophenol (*p*NP), and *p*-nitrobenzenethiol (*p*NBT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Aldrich Chemical Co. (Milwaukee, USA), respectively.

Enzymes. β-Galactosidases used were: *Aspergillus oryzae* (Yakult Honsha Co.), *Penicillium multicolor* (Lactase P, KI Kasei Co.), *Bacillus circulans* (Biolacta, Daiwa Kasei Kogyo Co.), *Escherichia coli* (Sigma Chemical Co.), *Saccharomyces fragilis* (Sigma Chemical Co.), and *Klyuveromyces lactis* (GODO-YNL, Godo Shusei Co.).

Activity assay. The usual β-galactosidase activity was assayed as follows: Reaction mixtures (1.0 ml)

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Abbreviations: Gal, D-galactose; Gal-*O*-*p*NP, *p*-nitrophenyl *O*-β-D-galactopyranoside; *p*NP, *p*-nitrophenol; Gal-*S*-*p*NP, *p*-nitrophenyl *S*-β-D-galactopyranoside; *p*NBT, *p*-nitrobenzenethiol; ME, 2-mercaptoethanol; Gal-*O*-ME, 2-mercaptoethyl *O*-β-D-galactopyranoside; Gal-*S*-ME, 2-hydroxyethyl *S*-β-D-galactopyranoside; DTT, dithiothreitol; Cys, L-cystein hydrochloride; Cys·*N*Ac, *N*-acetyl L-cystein; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Fab-MS, fast atom bombardment mass spectrometry; NMR, nuclear magnetic resonance; IR, infrared spectrometry; HPLC, high pressure liquid chromatography; TLC, thin layer chromatography

containing 5 mM Gal-*O*-*p*NP, appropriate buffers, and enzymes were incubated at 40°C for 10 min. After we stopped the reaction by the addition of 0.2 M Na_2CO_3 (1.0 ml), *p*NP was measured at 410 nm. Buffers were 50 mM acetate (pH 4.5) for the enzymes from *A. oryzae* and *P. multicolor*; 50 mM phosphate (pH 7.0) for the *B. circulans* enzyme, and 50 mM phosphate (pH 7.0) containing 1.0 mM $MgCl_2$ for the enzymes from *E. coli*, *S. fragilis*, and *K. lactis*. One unit was defined as the amount of enzyme that produced 1 μmol of *p*NP per min under these conditions.

β-Thio-galactosidase activity was also assayed as described above except Gal-*S*-*p*NP (5.0 mM) was used as a substrate. The *p*NBS liberated was measured by the absorbance at 410 nm immediately after the addition of Na_2CO_3 solution.

TLC. TLC was done at room temperature by the ascending method using a silica gel 60 (0.5 mm thick) plate (Merck Co.) and a solvent system of ethyl acetate-acetic acid-water (3:1:1, v/v). Spots were detected by spraying sulfuric acid in methanol (1:1, v/v) on them and then heating (150°C). For the detection of thiol compounds, 5% 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Wako Pure Chemical Industries Co.) in acetone was used. After development, the plate was dried well to remove the solvents and the DTNB reagent was sprayed. Compounds containing SH groups were detected as yellow spots.

HPLC. HPLC was done using a Shimadzu LC-10AD and a Shimadzu RID-6A detector under the following conditions: column, Asahipack NH2-P50 (4.6×250 mm, Showa Denko Co.); solvent, 67% (v/v) CH_3CN in water; flow rate, 1.0 ml/min); temperature, 40°C. Samples were filtered through a Chromatodisk 13P (0.45 μm, Kurabo Co.).

Spectrometry for structure analysis. Fast atom bombardment mass spectrometry (Fab-MS) was done in the positive mode using a Jeol JMS 600 mass spectrometer. 3-Nitrobenzyl alcohol (NBA) was used as a matrix. Infrared spectrometry (IR) was measured with a Shimadzu Fourier Transform Infrared Spectrometer FT-IR 8100. Nuclear magnetic resonance (NMR) spectra were recorded with a Jeol AL-300 FT-NMR spectrometer in D_2O (99.9 atom%, Aldrich Chemical Co.). 3-(Trimethylsilyl)-1-propanesulfonic acid, sodium salt (DSS) was used as an internal standard.

Isolation of the products. A mixture (25 ml) of 1.0 M Gal, 1.0 M ME, and the *A. oryzae* β-galactosidase (5,860 units) in 50 mM acetate buffer (pH 4.5) was incubated at 37°C for 4 days. The mixture was boiled for 10 min to stop the reaction, diluted to about 150 ml, and precipitates were removed by centrifugation. The supernatant was put on a column (3.2×29 cm) of activated carbon (Wako Pure Chemical Industries Co.). The column was washed with water (about 500 ml) to remove any Gal that remained, and eluted with a linear gradient of ethanol concentration (0–40%, v/v, 500 ml each) into 5-ml fractions at a flow rate of 21 ml/h. By testing the fractions with TLC, those containing the product P2 (Gal-*S*-ME) were collected. The product P1 (Gal-*O*-ME) was eluted at higher ethanol concentrations. After evaporation, a small amount of disaccharides still contaminating the preparation of Gal-*S*-ME was digested with the *A. oryzae* β-galactosidase at 40°C for 6 h. The digested sample was chromatographed on activated carbon again under the same conditions. The purified preparation was lyophilized and kept at −20°C (isolated yield, 506 mg). Gal-*O*-ME was further purified by gel filtration on a Bio-Gel P-2 (Bio-Rad Laboratories Co., California, USA) column (2.3×92 cm) equilibrated with 5% (v/v) ethanol. The saccharide was eluted at a flow rate of 7.5 ml/h and 2-ml fractions were collected. Fractions containing Gal-*O*-ME were collected, evaporated to a small volume (about 5 ml) and kept at −20°C (isolated yield, 114 mg).

Other analytical methods. Enzymatic measurement of Gal was done by F-kit for lactose/D-galactose (Boehringer Mannheim GmbH, Mannheim, Germany).

Results and Discussion

Hydrolysis of pNP-S-Gal by several β-galactosidases

p-Nitrophenyl *S*-β-D-galactoside (Gal-*S*-*p*NP), a substrate containing a thio-galactosidic linkage, was hydrolyzed by β-galactosidases from several origins. Figure 1 shows the ratios of β-thio-galactosidase activity measured by the liberation of *p*NBT to the usual β-galactosidase activity measured by the liberation of *p*NP from Gal-*O*-*p*NP. The *A. oryzae* enzyme had the highest activity, though it was only 0.8

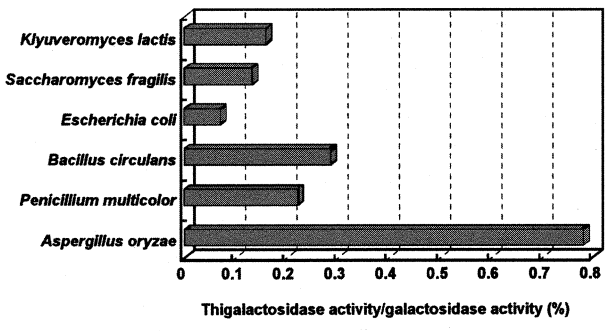

Fig. 1. Hydrolysis of Gal-*S*-*p*NP by Several β-Galactosidases.
The thio-galactosidase and the galactosidase activities were measured using 5.0 mM Gal-*S*-*p*NP and Gal-*O*-*p*NP as substrates, respectively, as described in "Materials and Methods".

% of the activity on the *O*-galactosidic linkage. The thio-galactosidase activities of the *K. lactis*, *S. fragilis* and *E. coli* enzymes were considerably lower compared to their usual galactosidase activities.

In glycosidase-catalyzed condensation reactions, oligosaccharides that are hydrolyzed rapidly do not necessarily accumulate after a long reaction. For example, glucoamylase hydrolyzes maltose more rapidly than isomaltose, and therefore, the enzyme affords the former more rapidly than the latter in an early stage of condensation of glucose. As the reaction proceeds, however, isomaltose accumulates gradually due to its slower hydrolysis and synthesis rates, and the lower potential energy.[4,8,10] Likewise we considered that some of these β-galactosidases may accumulate thio-galactoside by a condensation reaction, just because of its low, if not naught, hydrolysis activities on thio-galactosidic linkages.

HPLC and TLC analysis of the products

In our study, 2-mercaptoethanol (ME) was adopted as a thiol compound to be galactosylated because of its simple structure and high solubility. The reactions were done in capped vials to minimize the oxidation of ME and the products with SH groups. A high concentration of Gal (*e.g.* 2.0 M) and ME (*e.g.* 0.5 M) were used as substrates to shift equilibrium to synthesis. Figure 2 shows HPLC analysis of the products formed by *A. oryzae* β-galactosidase. In addition to oligosaccharides derived from the condensation only of Gal (retention time, 8–12 min), two unknown products (P1 and P2) were detected. The preparation of P1 isolated as described in "Materials and Methods" gave two peaks in HPLC analysis (data not shown): one had the same retention time as P1 (about 3.5 min) and the other was eluted later (about 4.8 min). After the reduction of this preparation with 2.5% ME, the latter peak disappeared and was integrated into the former peak (Fig. 2B). By TLC analysis, the product P1 gave a yellow spot with DTNB reagent. These results suggested the product P1 was formed by *O*-galactosylation of ME and its free SH group, which was oxidized partly during the isolation procedures to form a dimer through a disulfid bond. The dimer could be reduced again to the monomer (P1) by ME as indicated by Fig. 2B.

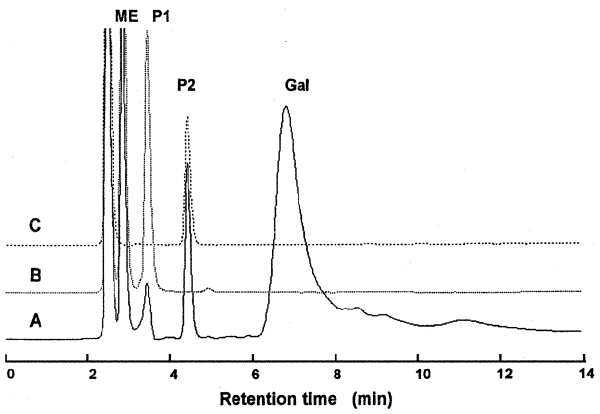

Fig. 2. HPLC Analysis of the Condensation Products from Gal and ME.

A, reaction mixture; B, the product P1 (Gal-*O*-ME) treated with 2.5% ME; C, the product P2 (Gal-*S*-ME). A reaction mixture containing 2.0 M Gal, 1.0 M ME, 25 mM acetate buffer (pH 5.5), and *A. oryzae* enzyme (202 units/ml) was incubated at 37° C for 72 h in a capped vial. After boiling for 5 min, the sample was diluted 10 times, centrifuged, passed through a membrane filter, and analyzed by HPLC. The products P1 and P2 were isolated as described in "Materials and Methods". The preparation of the product P1 was treated with 2.5% ME at room temperature for 10 min before HPLC analysis.

On the other hand, the product P2, which had a slightly faster mobility on TLC than Gal and a slower mobility than the product P1, was not stained by DTNB. The retention time on HPLC (about 4.5 min, Fig. 2C) and the mobility on TLC were not affected by the reduction by ME, indicating the product had no free SH group. The structure of P2 was verified by spectrometric analysis as described later.

The enzyme from *A. oryzae* was reacted with Gal (1.0 M) and several thiol compounds (0.5 M) such as DTT, Cys, and Cys·*N*Ac at 37°C for 72 h, and the product was analyzed by TLC (data not shown). However we could not detect any products that were considered to contain Cys and Cys·*N*Ac as components. DTT gave at least two products with different mobilities on TLC. However both were stained with DTNB reagent and we could not ascertain which spot was the thio-galalctosylation product.

Structure of thio-galactosylation product

The structure of the product P2 was analyzed further by spectrometric analysis. By Fab-MS, an adduct ion with sodium ion was observed at m/z 263 ($[M+Na]^+$) corresponding the sum of sodium ion (23) and the estimated molecular weight of the product (240). By FT-IR spectrum of P2, adsorption peaks were observed at 1,231 cm^{-1}, and 706 cm^{-1}, indicating the presence of S–CH_2 and C–S bonds respectively, but it lacked a peak at 2,550 cm^{-1} which suggested the absence of S–H.

^{13}C-NMR and ^{1}H-NMR data of the product P2 are summarized in Table 1. Signals of ^{13}C-NMR were assigned exactly as follows: Signals of the product P2 in ^{1}H-NMR spectrum were assigned by the homogate decoupling irradiation technique and H–H shift correlation spectroscopy. The primary carbon signals for C6, Cα, and Cβ were assigned by the distortionless increase by polarization transfer (DEPT) technique. All signals were further assigned by H/C shift correlated spectroscopy. The large coupling constant (doublet, $^{3}J_{1,2}=9.5$ Hz) and high field shift (4.37 ppm) of the anomeric proton suggested β-configuration in $^{4}C_1$ form. From these results, we concluded the product P2 was 2-hydroxyethyl *S*-β-D-galac-

Table 1. NMR Data on the Product P2 (Gal-*S*-ME)

	Position	^{1}H-NMR	^{13}C-NMR
Gal	1	4.37 (*doublet*, $^3J_{1,2}=9.5$ Hz)	86.6
	2	3.43 (*doublet doublet*, $^3J_{1,2}=9.5$ Hz, $^3J_{2,3}=9.4$ Hz)	70.3
	3	3.52 (*doublet doublet*, $^3J_{2,3}=9.4$ Hz, $^3J_{3,4}=3.1$ Hz)	74.5
	4	3.84 (*doublet*, $^3J_{3,4}=2.9$ Hz)	69.4
	5	3.60–3.54 (*multiplet*)	79.6
	6	3.60–3.54 (*multiplet*) × 2	61.7
ME	α	2.82 (*multiplet*) and 2.50 (*multiplet*)	33.0
	β	3.67 (*triplet*, $^3J=6.4$ Hz)	61.7

topyranoside (Gal-*S*-ME, Fig . 3), in which the Gal residue was linked to the SH group in the ME molecule.

Thio-galactosylation of ME by several β-galactosidases

The condensation of Gal and ME was done by several β-galactosidases having nearly the same activities on Gal-*O*-*p*NP. The yields of Gal-*O*-ME and Gal-*S*-ME are shown in Fig. 4. The enzymes from *A. oryzae* and *P. multicolor* showed almost the same yields of the Gal-*S*-ME (about 20 mM) and Gal-*O*-ME (about 10 mM). The galactosylation of OH group of ME was predominant in the reactions of the enzymes from *K. lactis*, *S. fragilis*, *B. circulans*, and *E. coli*, and their productivity in Gal-*S*-ME was roughly 10 to 20 times lower than the fungal enzymes.

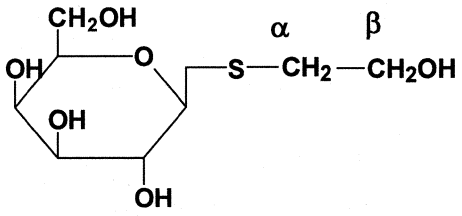

Fig. 3. Structure of Gal-*S*-ME.

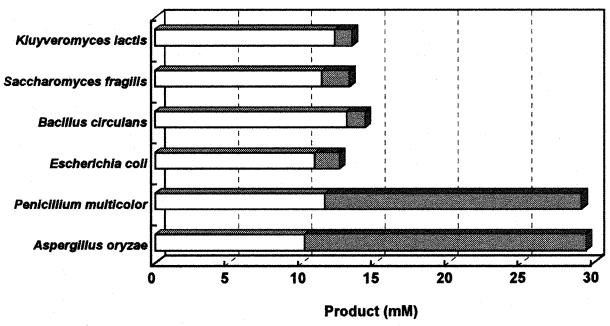

Fig. 4. Galactosylation of ME by Several β-galactosidases.
Reaction mixtures containing 2.0 M Gal, 0.25 M ME, and the enzymes in appropriate buffers were incubated at 37°C for 24 h. Following activities (units of Gal-*O*-*p*NP hydrolysis) were added per ml of the reaction mixtures; *A. oryzae* enzyme, 101 units; *P. multicolor* enzyme, 127 units; *B. circulans*, 108 units; *E. coli*, 122 units; *K. lactis*, 119 units □, Gal-*O*-ME; ■, Gal-*S*-ME.

The hydrolysis activities on Gal-*S*-*p*NP (Fig. 1) of the enzymes were not necessarily coincidental with the productivity of Gal-*S*-ME. The following factors seemed to affect the productivity; (1) the sensitivity of the enzymes to the substrates, Gal or ME, or (2) to the products especially thio-galactoside, or (3) the hydrolysis activities toward Gal-*S*-ME, not toward Gal-*S*-*p*NP. In the condensation reaction, high substrate concentrations were used to shift the reaction equilibrium. The enzyme tended to be inhibited by such elevated concentrations of substrates.[9,10] For example, the Gal-*O*-*p*NP hydrolyzing activities of *S. fragilis* and *E. coli* enzymes were inhibited (or inactivated) by 250 mM ME to almost 5% and 61% of the original activities, respectively, while inhibition was not evident in *A. oryzae*, *P. multicolor*, or *B. circulans* enzymes. Such different sensitivity of the enzymes to ME, and probably to Gal, seemed to be one reason for the different productivity. Some thio-galactosides are known to inhibit β-galactosidases and, for instance, *p*-aminophenyl *S*-β-galactoside is widely used as a ligand molecule for affinity chromatography of these enzymes.[11,12] In our experiment, Gal-*S*-ME (10 mM) reduced the activity of the *E. coli* enzyme to about 73%. On the contrary, no inhibition was observed in the *A. oryzae* enzyme at the same concentration. These indicated the different productivity can also arise from different sensitivity of the enzyme toward Gal-*S*-ME. As for the third possibility, it is not curious that substrates with different aglycone moieties (ME and *p*NBT) were hydrolyzed at different rates, and therefore, synthesized at different rates in the reverse reaction. Gal-*S*-ME (10 mM) was hydrolyzed by rather high activities (100 units for Gal-*O*-*p*NP/ml) at 40°C for 6 h and Gal liberated was measured by a enzymatic measurement kit. The activities were considerably lower than those toward Gal-*S*-*p*NP, and 6.85, 6.26, 0.52, 0.31 0.22, and 0.26 % of the substrate were degraded by the enzymes from *A. oryzae*, *P. multicolor*, *B. circulans*, *E. coli*, *S. fragilis*, and *K. lactis*, respectively. The fungal enzymes hydrolyzed Gal-*S*-ME faster than the other enzymes and this was probably one reason for their higher productivity of Gal-*S*-ME. We concluded, therefore, that these enzyme characteristics may decide the productivity of Gal-*S*-ME.

Synthesis under various conditions

The *A. oryzae* enzyme was reacted with different concentrations of Gal (0.8 M–2.8 M) and 0.5 M ME at 37°C for 120 h. The yields of Gal-*S*-ME was increased slightly (from 130 mM to 154 mM) with the Gal concentration. On the other hand, ME concentration considerably affected the synthesis of Gal-*S*-ME. As shown in Fig. 5, Gal-*S*-ME increased with ME concentration and reached an optimum at around 1.25 M ME. At higher concentrations (1.5 M–2.0 M), the decrease may be due to the denaturation (inactivation) of the enzyme by ME, which was noticed by precipitates in the reaction mixtures. The productivity of Gal-*O*-ME and Gal-*S*-ME was reversed at 2.0 M ME. As shown in Fig. 6, Gal-*O*-ME was synthesized rapidly and reached a maximum concentration within 12 h. The yield of Gal-*S*-ME exceeded that of Gal-*O*-ME at 30–40 h and the increase still continued after 120 h-incubation, indicating Gal-*S*-ME had not reached the equilibrium concentration under these reaction conditions. In general, oligosaccharides that are hydrolyzed fast are synthesized rapidly in condensation.[4,8,10] Therefore Gal-*O*-ME, a usual and preferable substrate for the enzyme, was considered to be synthesized speedily. The slower synthetic rate of the thio-galactoside was coincidental with the low hydrolysis activities of the enzyme on Gal-*S*-*p*NP (Fig. 1) and Gal-*S*-ME. Considering such different synthetic rates, the inactivation of the enzyme before sufficient accumulation of Gal-*S*-ME may be responsible for the reversal of the productivity at high ME concentrations (Fig. 5). In condensation reactions, the final composition of oligosaccharides are considered to depend on the potential energy of each product: Saccharides with lower energy levels tend to be predominant after the reaction reaches a equilibrium.[4-8] The larger final yield of Gal-*S*-ME than that of Gal-*O*-ME, therefore, suggested a lower potential energy, and thus the thermodynamically greater stability of the thio-galactoside.

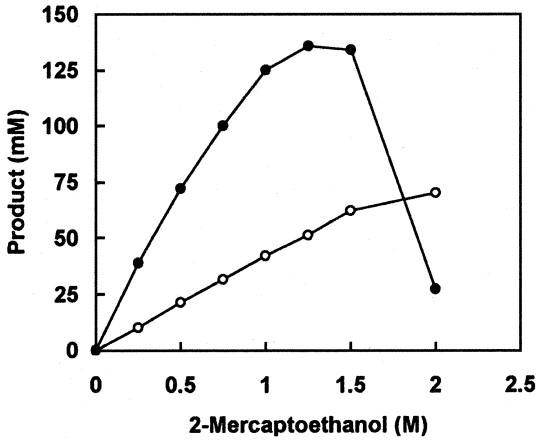

Fig. 5. Galactosylation of Various Concentration of ME by *A. oryzae* β-Galactosidase.

Reaction mixtures containing 2.0 M Gal, various concentrations of ME, and the *A. oryzae* enzyme (202 units/ml) in 50 mM acetate buffer (pH 5.5) were incubated at 37°C for 50 h. ○, Gal-*O*-ME; ●, Gal-*S*-ME.

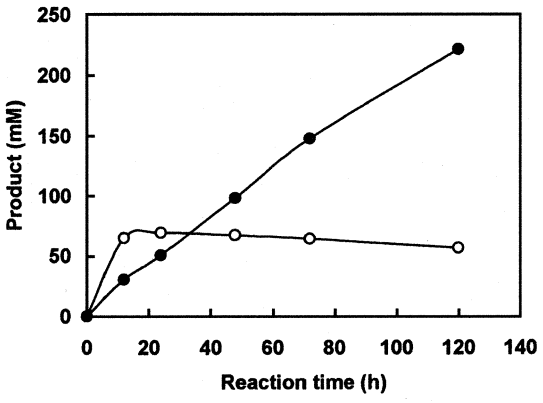

Fig. 6. Course of the Reaction.

Reaction mixtures containing 2.0 M Gal, 1.0 M ME and the *A. oryzae* enzyme (202 units/ml) in 50 mM acetate buffer (pH 5.5) were incubated at 37°C. At intervals, the reaction was stopped by boiling for 5 min. ○, Gal-*O*-ME; ●, Gal-*S*-ME.

The reaction temperatures at around 45°C were suitable for the synthesis (the yield, 129 mM) from 2.0 M Gal and 1.0 M ME by the enzyme after 48 h of incubation. At 50°C, the yield of Gal-*S*-ME decreased because of the heat-inactivation and, at 60°C, the synthesis stopped completely.

The *A. oryzae* enzyme was reacted with lactose (0.1 M) in the presence of ME (0.5 M) at 40°C and the products were measured periodically by HPLC. In such transgalactosylation system, Gal-*O*-ME was formed predominantly and the yields of Gal-*S*-ME were extremely low (about 2.5–3.3 mM). The condensation system proposed in this study, therefore, is much more effective for the galactosylation of thiol groups.

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