Enzymatic Syntheses of Glycolipids Catalyzed by a Lipid-coated Glycoside Hydrolase in the Organic-Aqueous Two Phase System

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ABSTRACT: A lipid-coated glycoside hydrolase was prepared in which the enzyme surface is covered with a lipid monolayer and two long alkyl tails serve to solubilize the enzyme in organic solvents. In the two-phase aqueous-organic system, the lipid-coated enzyme exists in the organic phase and acts as an efficient catalyst for transglycosylation of various lipophilic dialkyl alcohols with oligosaccharides in the aqueous buffer solution. When a native glycoside hydrolase was employed in the two-phase system, neither the transgalactosylation nor the hydrolysis reaction proceeded due to the denaturation of the enzyme at the interface. Effects of chemical structures of acceptor alcohols on the transglycosylation catalyzed by the lipid-coated enzyme were also studied.
KEY WORDS Lipid-coated Enzymes / Glycolipid Synthesis / Transglycosylation / Glycoside Hydrolase/ Aqueous-Organic Two-Phase System /

Carbohydrates such as glycolipids and glycoproteins play an important role in living systems, for instance, cell recognition, cell adhesion, immune response, proliferation, and malignant alteration.[1-3] It is important to develop the preparation method of glycolipids in order to study their properties in biological membranes. Over the past decades, a considerable number of researches have been made on the enzymatic glycosylation catalyzed by glycosyl transferases or glycoside hydrolases.[4,5] These enzymatic syntheses include an advantage that reactions occur regioselectively and stereoselectively without using protection and deprotection processes of reactive hydroxyl group in comparison with synthetic chemical methods. In the transglycosylation through the catalytic activity of reverse hydrolysis by glycosidases, water-miscible organic solvents have been added to increase yields of transglycosylations and decrease hydrolysis reactions.[6-11] However, the yield of transglycosylation in these examples has usually been low because the hydrolysis reactions proceeded fast relative to the transglycosylations in homogeneous aqueous-organic media.[6-11] If the reaction could be carried out in nonaqueous organic solvents without the denaturation of enzymes, it is likely that the transglycosylated products could be obtained in high yields.

We have reported a lipid-coated enzyme system in which the enzyme surface is covered with dialkyl amphiphiles, and these lipophilic alkyl tails serve to solubilize the enzyme in hydrophobic organic solvents.[12-18] A lipid-coated lipase was found to act as an efficient catalyst for enantioselective esterifications (the reverse hydrolysis) in dry isooctane.[12,13] The catalytic efficiency was large compared with that of other lipase systems in organic media such as poly(ethyleneglycol)-grafted lipase.[19,20] and a lipase dispersion system.[21,24] In previous papers,[14,17] we reported that a lipid-coated glycosidase could catalyze transglycosylation to hydrophobic alcohols from corresponding p-nitrophenyl glycosides in dry isopropyl ether. The yields of the transgalactosylated compounds were much higher than those catalyzed by a native glycosidase in water-miscible organic and aqueous buffer

media,[25] presumably because the lipid-coating system can be used in dry organic media without hydrolysis side
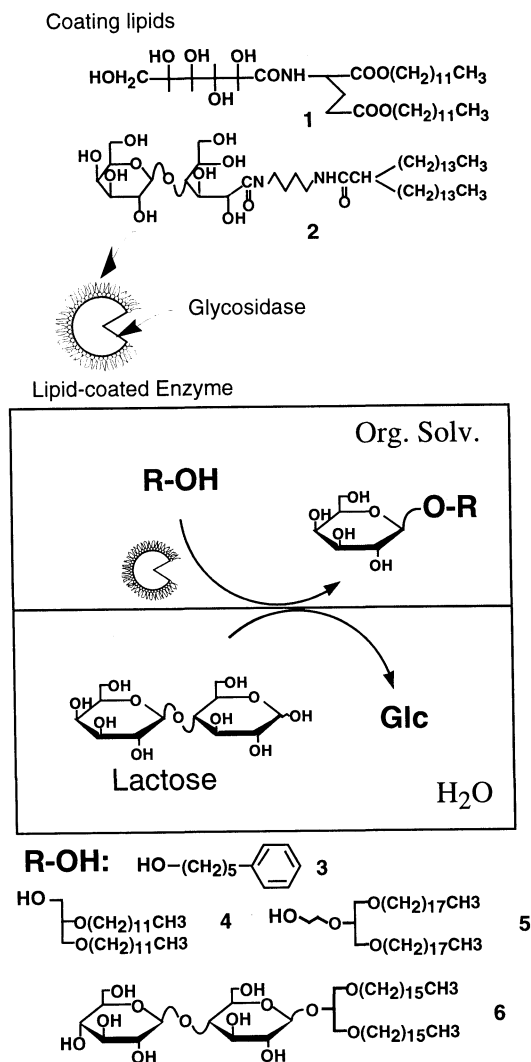

Figure 1. Schematic illustrations of transglycosylation in the two-phase organic-aqueous system catalyzed by a lipid-coated glycosidase, and chemical structures of the employed acceptor alcohols.

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reactions. However, this system could not be applied to a large-scale synthesis because of the low solubility of the glycosyl donor in hydrophobic organic media. In the preceding papers,[15] we reported a new transgalactosylation system by using a lipid-coated glycosidase in the two-phase aqueous-organic system in which both the lipid-coated enzyme and hydrophobic acceptor alcohols exist in the organic phase and an excess amount of inexpensive lactose as galactose donor in the aqueous buffer solution. A schematic illustration of the transgalactosylation is shown in Figure 1. What has to be noted is that the enzyme exists in the organic solvent phase. Although native β-D-galactosidase may be used to the transglycosylation at a first glance, as a result, it was found not to catalyze the transglycosylation in this system.

In this study, syntheses of glycolipids from the hydrophobic alcohols having two long alkyl chains through the glycosylation using the two-phase system were examined. These reactions are also compared with the two-phase aqueous-organic system using a conventional native enzyme.

EXPERIMENTAL

Materials

β-D-galactosidase [EC 3.2.1.23] from *Bacillus circulans* (Daiwa Kasei Co., Osaka) were used without further purification. β-D-glucosidase [EC 3. 2. 1. 21] from *Almond* and α-D-mannosidase [EC 3. 2. 1. 24] from *Jack beans* were purchased from SIGMA and used without further purification. Preparations of dialkyl amphiphiles as coating lipids having gluconoamide (1) and lactonoamide (2) head groups have been reported elsewhere.[26] The chemical structures of these amphiphiles are shown in Figure 1. Acceptor alcohols, 1,2-didodecyl-glycerol (4), 1,3-dioctadecyl-2-hydroxyethyl glyceryl triether (5), and 1,3-dihexadecyl glyceryl β-cellobioside (6) were synthesized according to conventional methods.[26,27] α-Mannan was purchased from SIGMA. Other chemicals and organic solvents were purchased from Tokyo Kasei Co., Tokyo, Nacalai Tesque Co., Kyoto, and Kanto Chemicals Co., Tokyo.

Preparation of a lipid-coated glycosidase

A lipid-coated D-glycosidase was prepared in a manner similar to that reported in our previous papers.[12-18] A typical procedure is as follows. An aqueous buffer solution (50 mL, 0.01 M phosphate, pH 5.1) of the β-D-galactosidase (50 mg) was mixed with an aqueous dispersion (50 mL) of dialkyl amphiphiles (1) (50 mg) at 4 °C and stirred for 1 day at 4 °C. Precipitates were gathered by centrifugation at 4 °C (5000 rpm, 15 min) and washed with buffer solution and distilled water, repeatedly, and then lyophilized. The resulting white powder was soluble in most organic solvents such as acetonitrile, benzene and isopropyl ether, but insoluble in aqueous buffer solution. The protein content of the lipid-enzyme complex was determined from both the elemental analysis (C, H, and N) and the UV absorption by aromatic amino acid residues of proteins at 280 nm in chloroform solution.[12-18] Results are summarized in Table I.

Table I. Preparation of a lipid-coated glycosidase and its enzymatic activity

| Glycosidase + Lipids | Enzymatic activity | | |
	yields[a] mg	protein content[b] wt%	conversion after 6 days / %[c]
β-galactosidase + 1	32.6	8.8	66
α-mannosidase + 1	16.4	7.0	60
β-glucosidase + 2	14.4	2.4	23

[a] Both aqueous solutions of each glycosidase (50 mg) and lipids (50 mg) were mixed and precipitates were lyophilized. [b] Obtained from UV absorption of aromatic amino acid residues in the protein, which was consistent with the protein content obtained from C/N ratio of elemental analyses. [c] Transglycosylation to 5-phenyl-1-pentanol was carried out under the same conditions as Figure 2.

Catalytic activity of a lipid-coated β-D-galactosidase in the two-phase system

The two-phase system of aqueous buffer solution (10 mL, 0.01 M phosphate, pH 5.1) of lactose monohydrate (36 mg, 10 mM) and isopropyl ether solution (10 mL) of a lipid-coated enzyme (1-2 mg, 0.1 mg of protein) and an alcohol substrate (6.5-8.5 mg, 1.0 mM) was vigorously stirred at 30 °C. Within the prescribed time interval, the reduction of alcohol acceptors was followed by liquid chromatography: TSK-gel ODS-80Ts (4.6 mm i. d. x 25 cm) in a TOSOH CCPS-system liquid chromatography equipped with an UV (at 254 nm) and RI detector (elution, 1:1 acetonitrile/water; flow rate, 1 mL/min). Identification and quantification of the substrates and the products were made by comparison of the HPLC retention time and the HPLC peak area to those of the authentic samples, respectively. Production of D-galactose in the aqueous phase was followed with an enzymatic detection using D-galactose dehydrogenase [D-galactose: NAD$^+$ 1-oxido-reductase, EC 1.1.1.48].[25]

The transgalactosylation products (β-D-Gal-OR) were isolated from the reaction mixture by evaporating the organic phase and purified by a large scale separation HPLC column (TSK-gel ODS-80Ts, 21.5 mm i. d. x 30 cm). Their chemical structures of isolated products were confirmed by elemental analyses (C and H), ^{1}H NMR spectroscopy, and FAB mass spectroscopy.

RESULTS AND DISCUSSION

Transglycosylation to 5-phenyl-1-pentanol

In order to confirm the transglycosylation activity of the lipid-coated enzyme in the organic-aqueous two-phase system, 5-phenyl-1-pentanol (3) was selected as a simple acceptor alcohol. Figure 2 shows typical time courses of transgalactosylation catalyzed by a β-D-galactosidase from a 10-fold excess of lactose as a galactosyl donor in the aqueous phase to 5-phenyl-1-pentanol (PhC$_5$OH, 3) as a galactosyl acceptor in the isopropyl ether phase at 30 °C.

When the lipid-coated enzyme was solubilized in the organic phase, the transglycosylated product (5-phenyl-1-pentyl β-D-galactopyranoside, Gal-O-C$_5$Ph) was obtained in 70% yield after eight days as the only product in the organic phase. Galactose, the hydrolyzed product of lactose, was not obtained even after eight days in the aqueous phase (Figure 2a). It was confirmed from ^{1}H and ^{13}C NMR spectra that the chemical structure of Gal-O-C$_5$Ph was in the

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β–configuration of D-galactose. The amount of the consumed PhC_5OH corresponds to the production of Gal-O-C_5Ph. On the contrary, when a water-soluble native enzyme was employed in the same two-phase system, either the transgalactosylation (the reduction of PhC_5OH) or the hydrolysis of lactose (the production of glucose) was hardly observed (Figure 2b). This shows that the native enzyme seems to be easily denatured at the aqueous-organic interface. These results indicate that the lipid-coated β-D-galactosidase can act as an efficient glycosylation catalyst in the isopropyl ether-water two-phase system.
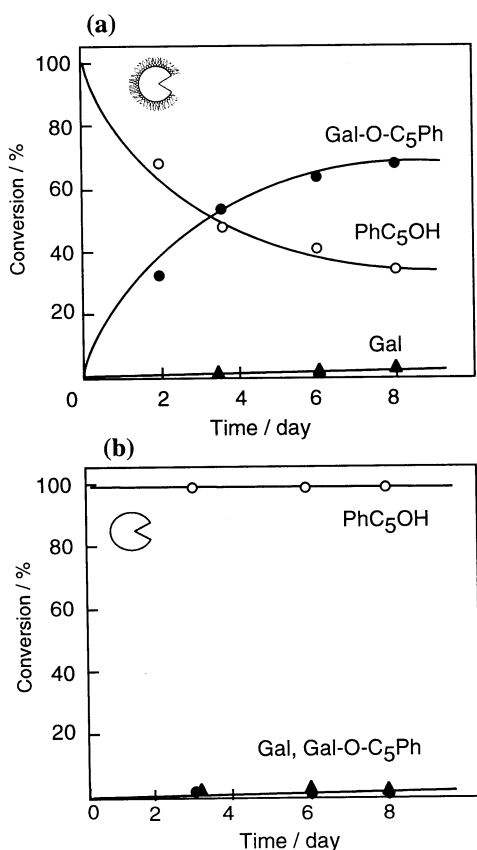

Figure 2. Typical time-courses of transgalactosylation from lactose (10 mM) to 5-phenyl-1-pentanol (PhC_5OH, 1.0 mM) catalyzed by (a) a lipid 1-coated β-D-galactosidase and (b) a native β-D-galactosidase from E. coli, in isopropyl ether-aqueous buffer two-phase system: 30 °C, 10 mL of isopropyl ether and 10 mL of buffer solution (0.01 M phosphate, pH 5.1), and [Enzyme] = 0.1 mg of protein.

It should be mentioned that the yield of the Gal-O-C_5Ph product plateaued around 70%, even after a week as shown in Figure 2a. Furthermore, when the lipid-coated enzyme was added to the reaction mixture after the reaction reached the equilibrium, the yield did not increase. When the lipid-coated enzyme had been soaked in isopropyl ether for two days in advance, and both substrates were then added, the transgalactosylation proceeded in 65% yield in eight days and reached the equilibrium as observed for the conventional reaction. These results indicate that the lipid-coated enzyme was not deactivated during the reaction in the organic solvents. When the product, Gal-O-C_5Ph, was added to the organic phase in advance, the yield of the transglycosylation product decreased with increase in the

amount of the added Gal-OC_5Ph. For example, when an equivalent amount of Gal-OC_5Ph was added to the substrate, the reaction was completely stopped. The maximizing yield near 65% is explained by the product inhibition and not by the deactivation of the lipid-coated enzyme.

Transglycosylation to Dialkyl Alcohols

Since the combination of the lipid-coated enzyme and the aqueous-organic two-phase system was effective as the transglycosylation to hydrophobic alcohols, we applied it to the synthesis of glycolipid molecules. Figure 3 shows a representative TLC plate of the reaction mixture of transmannosylation from α-mannan to alcohol molecules having two long alkyl chains after the incubation for 6 days at 37 °C.
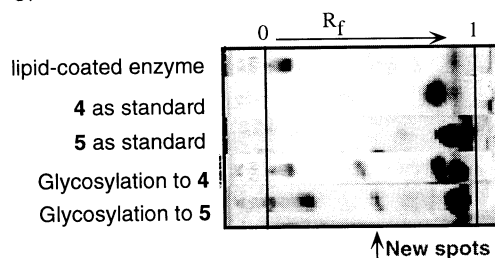

Figure 3. TLC analysis of the reaction mixture of transmannnosylation from a-mannan (10 mM) to dialkyl alcohols (4 and 5) (1.0 mM) after the incubation for 6 days at 37 °C, eluting with $CHCl_3$:CH_3OH = 9:1, and stained with $H_3[P(Mo_3O_{10})_4]$.

The components in each spot were identified by comparison of the R_f values of authentic standards. The new spot was isolated by the reverse phase HPLC (TSK-gel, elution with 1:1 CH_3CN/H_2O). FAB mass spectrum and ^1H NMR spectrum of the isolated product were shown in Figure 4 and Figure 5, respectively.

From the result of the molecular weight and the assignment of the signals, the purified compounds were found to be the object glycolipids in the yield of 39 %. The possibility of employing a broad range of acceptor alcohols in transglycosylation reaction was examined with respect to the various glycosidases. The conversions of transglycosylation were summarized in Table II.
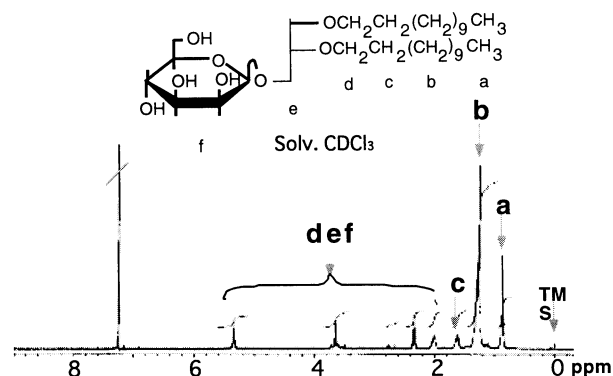

Figure 4. ^1H NMR spectrum of the product of trans-mannnosylation from α-mannan to the dialkyl alcohol 4. An internal standard was TMS.
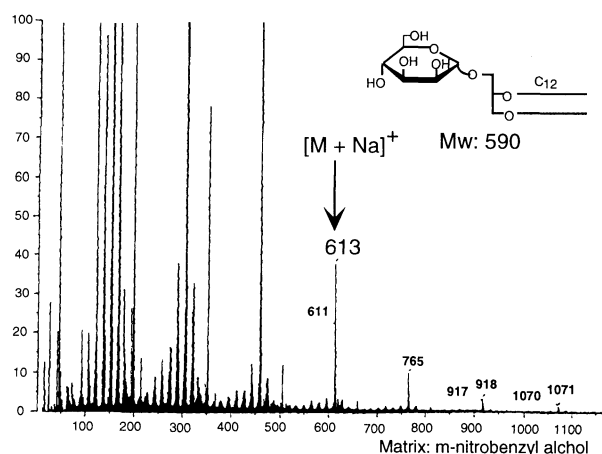
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Figure 5. FAB mass spectrum of the isolated product of transglycosylation from α-mannan to the dialkyl alcohol 4.

Table II. Conversion of transglycosylations in the combination of enzymes and acceptor dialkyl alcohol[a]

Lipid-coated glycosidase	Conversion after 6 days / %		
	4	5	6
α-mannosidase[b]	39	17	0
β-glucosidase[c]	39	19	0
β-galactosidase[d]	0	0	0

[a]Transglycosylation from corresponding oligosaccharides as a donor (10 mM) in phosphate buffer solution (1 mL) and acceptor alcohols (1.0 mM) in isopropyl ether (2 mL) catalyzed by a lipid glycosidase ([enzyme] = 0.1 mg of protein) at 37 °C. [b] α-mannan was used as saccharide donor. [c] cellobiose was used as saccharide donor. [d]lactose was used as saccharide donor.

When the primary alcohol having two longer alkyl chains was used as the acceptor, β-D-glucosyl and α-D-mannosyl transfer occurred, but β-galactosyl transfer did not. Although the transgalactosylation to the simple alcohol (3) could be succeeded in 70% yield, it did not occur to the primary alcohols having two long alkyl tails (4 and 5). This means that galactosidase could not recognize the bulkyl alcohols as a substrate.

A glycosyl transfer to the glycolipid (6) having cellobiose as a head group did not proceed in any kind of case using the water / organic two phase system. Up to the present, there may be two possibilities for the failure. One is to assume that the substrate specificity of these glycosidases is restricted: the substrate having the large cellobiose unit could not be recognized in the enzyme. Another possibility is that the lipophilic and hydrophilic glycolipid (6) acts as a surfactant and forms their aggregates or reversed micelles in the water-containing organic phase. Therefore, nucleophilic attack of glycolipids as an acceptor toward the glycosyl oxocarbenium ion intermediate could not proceed. Recently, we observed that these glycosidases could catalyze the transglycosylation to the glycolipids in supercritical fluoroform, as noted elsewhere. There is no conclusive proof that glycolipids are micro-environmentally homogeneous in supercritical fluoroform. From the fact that these glycosidases can recognize the glycolipids,

however, we may say that the latter possibility is entirely fair.

CONCLUDING REMARKS

By using the lipid-coated enzyme in the organic-aqueous two phase system, we could prepare stereoselectively and without using protecting group several kinds of glycolipids such as 1-α-mannosyl-2,3-didodecyl glycerol, 1-β-glucosyl-2,3-didodecyl glycerol, 2-α-mannosyl-1,3-dioctadecyl glycerol, 2-β-glucosyl-1,3-dioctadecyl glycerol. This technique is obviously advantageous for the preparation of glycolipids through the transglycosylation, as compared with the conventional method using a native enzyme in aqueous-organic solvents.

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