Production of α-Galactosyl Epitopes via Combined Use of Two Recombinant Whole Cells Harboring UDP-Galactose 4-Epimerase and α-1,3-Galactosyltransferase

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α-Galactosyl epitopes (or α-Gal, oligosaccharides with a terminal Galα1,3Gal sequence) are a class of biologically important oligosaccharides in great demand in bulk quantities for basic and clinical studies on preventing hyperacute rejection in pig-to-primate organ xenotransplantaion. A truncated bovine α-1,3-galactosyltransferase, the key enzyme responsible for the biosynthesis of the terminal structure of α-Gal, was cloned and overexpressed previously. The acceptor specificity was further studied in the present paper, and lactose and galactose derivatives were found to be good acceptors. To develop a more proficient reaction process, we report herein an example of an efficient enzymatic synthesis of α-Gal oligosaccharides catalyzed by the combination of two recombinant *Escherichia coli* whole cells harboring the genes of a UDP-galactose 4-epimerase and the α-1,3-galactosyltransferase, respectively. Using lactosyl azide ($LacN_3$) as the acceptor for the glycosyltransferase, the combined use of the two recombinant cells efficiently produced α-Gal epitope Galα1,3$LacN_3$ in 60−68% yield.

Introduction

α-Galactosyl epitopes (α-Gal) are oligosaccharides with a terminal Galα1,3Gal sequence. They are abundantly expressed on the cell surface of mammals other than humans, apes, and Old World monkeys. In contrast, the human body naturally produces a large amount of antibodies, termed anti-Gal antibodies, specifically against α-Gal epitopes. The interaction of α-galactosyl epitopes and natural anti-Gal antibodies (anti-Gal) in the serum of recipients is the main cause of hyperacute rejection (HAR) in pig-to-human xenotransplantation (*1*). To abrogate HAR, removal of antibodies from human serum by antigen-specific immunoadsorption (*2*) and intravenous infusion of α-Gal oligosaccharides (*3*) have been demonstrated in vitro and in vivo to be applicable strategies. However, large quantities of synthetic α-Gal oligosaccharides are required for the application of these procedures. Enzymatic synthesis of the Galα1,3Gal sequence using isolated glycosidases (*4*) or glycosyltransferase (*5*) has been demonstrated. However, the problems associated with glycosidase-catalyzed reactions are low yields and unpredictable regioselectivity. The high specificity in the formation of glycosides by glycosyltransferases makes them a viable strategic choice. The unique enzyme responsible for the formation of α-Gal epitopes in biological system is α-1,3-galactosyltransferase (α1,-3GalT, EC 2.4.1.151) (*6*). However, the high cost of UDP-galactose donor has significantly limited the application of this transferase in large scale synthesis of α-Gal oligosaccharides. A cheaper alternative to UDP-galactose is UDP-glucose, which can be converted to UDP-galactose using UDP-Gal 4-epimerase (GalE, EC 5.1.3.2). Recently we successfully cloned and overexpressed a GalE (*7*), as well as a truncated bovine α-1,3-galactosyltransferase (*5*), in *E. coli* BL21(DE3) strain. To avoid the tedious enzyme purification procedures and reduce the cost of α-Gal oligosaccharide synthesis, in the present report we exploit the feasibility of using the whole α1,3GalT recombinant cells as well as the whole GalE recombinant cells as catalysts. The GalE whole cell converts UDP-Glc to UDP-Gal, which in turn acts as the donor of α1,3GalT for the production of the final α-Gal epitope oligosaccharides (Figure 1). Single types of recombinant cells used for disaccharide synthesis have been studied (*8*). Examples of an alternate metabolic engineering approach for nucleotide-sugar production and oligosaccharide synthesis have been reported by the Kyowa Hakko group using engineered *C. ammoniagenes and E. coli* (*9*−*12*). However, to the best of our knowledge, no example has been reported using a combination of two *E. coli* cells containing different recombinant plasmids in α-Gal trisaccharide synthesis.

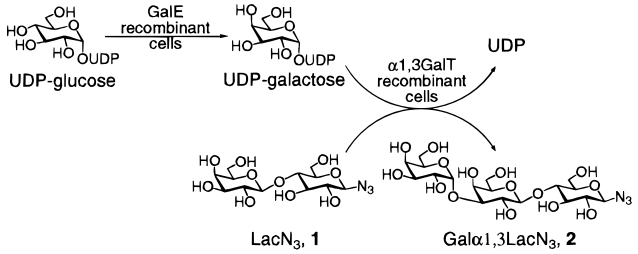

Figure 1. Schematic diagram of the reactions catalyzed by the combination of UDP-Gal 4-epimerase and α-1,3-galactosyltransferase whole cells.

Materials and Methods

Chemicals. Lactosyl azide ($LacN_3$) was synthesized previously (*13*). All other chemicals, including UDP-glucose, UDP-D-[6-^{3}H]glucose, ampicillin, and DOWEX 1 × 8 anion-exchange resin were purchased from Sigma

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Chemical Co. (St. Louis, MO). Deionized water was obtained with Water Pro PS system (Labconco, Kansas, MO).

Plasmids and Strains. The construction of expression plasmid pET15b-α1,3GalT (*5*) and pET15b-GalE (*7*), and the preparation of *E. coli* strain BL21(DE3) harboring the corresponding plasmids have been reported previously. Briefly, the gene of interest was amplified by PCR and inserted into the NdeI and BamHI restriction sites of a pET15b plasmid vector. The resulting plasmids were then transformed into *E. coli* DH5α (cloning host) and BL21(DE3) (expression host) competent cells consequently.

Media and Culture Conditions. Recombinant *E. coli* strains harboring either plasmid pET15b-GalE or pET15b-α1,3GalT were cultivated in Luria-Bertani (LB) media containing 150 μg/mL ampicillin at 37 °C in an incubator shaker (250 rpm) and induced with 0.4 mM IPTG for 3 h. The cells were isolated by centrifugation and stored at −20 °C.

pH Effect for α1,3GalT. This assay was carried out at 37 °C for 15 min in a final volume of 100 μL containing 10 mM $MnCl_2$, 0.1% BSA, 0.3 mM UDP-D-[6-^{3}H]galactose (final specific activity of 1000 cpm/nmol), 50 mM β-D-lactose, 20 μL of purified α1,3GalT (1 μg, 10 mU) in the following buffers at a final concentration of 50 mM: HOAc/NaOAc buffer, pH 4.0 and 5.5; MES (2[*N*-morpholino]ethanesulfonic acid) buffer, pH 6.0; Tris/HCl buffer, pH 7.0 and 8.5; and carbonate buffer, pH 9.0. One unit (U) of α1,3GalT activity is defined as the amount of enzyme that catalyzes the transfer of 1 μmol of galactose from UDP-Gal to lactose per min at 37 °C (*5*).

Acceptor Specificity Assay for α1,3GalT. This assay was performed as described above for the pH effect assay for α1,3GalT except that the acceptor for α1,3GalT was varied and the reaction was carried out in 10 mM Tris/HCl buffer at pH 7.0.

Cell Activity Assay. The efficacy of the recombinant *E. coli* cell catalyzed reaction was first evaluated by radioactive counting assay. Briefly, the reaction was performed at 37 °C for 1 h in a final volume of 100 μL containing 10 mM Tris/HCl pH 7.0, 10 mM $MnCl_2$, 0.1% BSA, 0.3 mM UDP-D-[6-^{3}H]glucose (final specific activity of 1000 cpm/nmol), 50 mM β-D-lactose, 0.5% Triton X-100, phosphatase inhibitors (5 mM ATP, 3 mM theophylline, and 3 mM cimetidine), and 10 μL of each of α1,3GalT and GalE recombinant cells. Lactose was omitted for the blank. The reaction was stopped by adding 100 μL of ice-cold 0.1 M EDTA, and Dowex 1 × 8-200 chloride anion-exchange resin (0.5 mL) was then added to the mixture. After vortexing and centrifugation, the supernatant (350 μL) was collected in a 20 mL plastic vial, and 5 mL of ScintiVerse BD was added. The vial was vortexed completely, and the radioactivity of the mixture was counted in a liquid scintillation counter (Beckmann LS-3801 counter).

Cell Reaction for Galα1,3LacN_3 Production. The two-cell catalyzed reaction was carried out in a total of 5 mL at room temperature with stirring. To UDP-glucose (186 mg, 0.3 mmol) and $LacN_3$ (92 mg, 0.25 mmol) in a 50 mL centrifuge tube were added 0.5 mL of 0.2 M Tris/HCl (pH 7.0), 0.5 mL of 0.1 M $MnCl_2$, 0.5 mL of 1% BSA, 0.1 mL of 5% Triton X-100, 0.5 mL of phosphatase inhibitor (50 mM ATP, 30 mM theophylline ,and 30 mM cimetidine), α1,3GalT cells (1 mL in 50 wt % 20 mM Tris/HCl buffer, pH 7.0, 0.8 unit), and GalE cells (1 mL in 50 wt % 20 mM Tris/HCl buffer, pH 7.0, 30 unit). Deionized water (0.9 mL) was added to bring the total reaction mixture volume to 5 mL. The reaction was monitored by TLC [*i*-PrOH/H_2O/NH_4OH = 7:3:2 (v/v/v)]. After 25 h, the *E. coli* cells were removed by centrifugation at 10,000*g* for 20 min. The pellet was washed twice with 2.5 mL of deionized water, and the combined supernatants were passed through a Dowex 1 × 8-200 chloride anion exchange column. The elute was mixed with 20 mL of MeOH and stirred for 20 min. After centrifugation for 20 min, the supernatant was concentrated and loaded to a G-15 gel filtration column (120 cm × 4 cm) with water as the eluent. The desired fraction was collected and lyophilized to yield the product. One unit (U) of GalE activity is defined as the amount of enzyme that catalyzes the transfer of 1 μmol of UDP-Glc to UDP-Gal per min at 24 °C (*7*).

Galα1,3LacN_3 Analysis. ^{1}H and ^{13}C NMR (500 MHz) spectra were obtained using a 500-MHz Varian Unity spectrometer with the chemical shift expressed as parts per million downfield using deuterated water as solvent. Mass spectra (FAB or ESI) were run at the mass spectrometry facility at the University of California, Riverside. Thin-layer chromatography was conducted on Baker Si250F silica gel TLC plates with a fluorescent indicator. The following data were obtained. ^{1}H NMR (D_2O standard): δ 3.25 (t, J = 9.0 Hz, 1H), 3.46−3.84 (m, 15H), 4.00 (d, J = 2.5 Hz, 1H), 4.01 (t, J = 6.5 Hz, 1H), 4.34 (d, J = 8.0 Hz, 1H), 4.59 (d, J = 8.5 Hz, 1H), 4.96 (d, J = 3.5 Hz, 1H). ^{13}C NMR (D_2O): δ 59.78, 60.80, 60.90, 64.69, 68.08, 69.00, 69.16, 69.45, 70.71, 72, 38, 74.30, 74.94, 76.55, 77.03, 77.77, 89.84, 95.30, 102.67 (see Supporting Information). HRFABMS calculated for $C_{18}H_{31}N_3O_{15}$ (M + Na): 552.1652. Found: 552.1639.

Results and Discussion

To determine the optimal reaction conditions and achieve high-yielding production, the effects of pH, phosphatase inhibitors, and Triton were investigated. Although the activity assay for α1,3GalT was reportedly performed at pH 6.0 (*14*), our recombinant α1,3GalT displayed optimal activity at pH 7.0. At pH 6.0 in 50 mM MES (2[*N*-morpholino]ethanesulfonic acid) buffer, the recombinant α1,3GalT only has about 55% activity compared to that at pH 7.0 in 50 mM Tris/HCl buffer. There was only a 20% change in activity level within the range from pH 7.0 to 8.5, and an abrupt decrease in activity was observed when the pH value exceeded 8.5. We previously demonstrated that the optimal pH for GalE activity was also 7.0 in Tris/HCl buffer (*7*). Therefore, the reactions catalyzed by two recombinant strains were carried out at pH 7.0 in 10 mM Tris/HCl buffer.

The effects of phosphatase inhibitors and Triton X-100 are depicted in Figure 2. In the presence of phosphatase inhibitors, adding or omitting Triton X-100 yielded similar amounts of the product (column 1 and 4). It is interesting to notice that the presence of phosphatase inhibitors appeared to be crucial to this reaction. In the absence of phosphatase inhibitors, the product formation decreased by almost 50% (column 2). Omitting Triton X-100 further decreased the reaction activity (column 3). Cimetidine and theophylline have been demonstrated to be effective uncompetitive inhibitors for alkaline phosphatase (*15*). In our results, phosphatase inhibitors (consisting of 5 mM ATP, 3 mM theophylline, and 3 mM cimetidine) greatly decreased the blank radioactive count. This suggests that these inhibitors might participate in the inhibition of the hydrolysis of UDP-Gal and/or UDP-Glc by UDP-sugar pyrophosphatase in *E. coli* host cells. Although UDP-sugar pyrophosphatase/phosphodiesterase

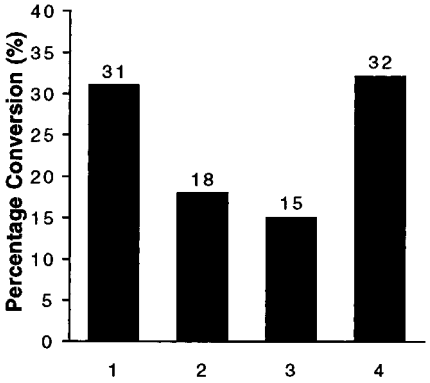


Figure 2. The effects of phosphatase inhibitor and Triton X-100 on the reaction catalyzed by GalE and α1,3GalT recombinant cells. Column 1, with phosphatase inhibitors (5 mM), with Triton X-100 (0.5%); column 2, no phosphatase inhibitors, with Triton X-100 (0.5%); column 3, no phosphatase inhibitors, no Triton X-100; column 4, with phosphatase inhibitors, no Triton X-100. α1,3GalT and GalE recombinant *E. coli* cells were used in the assay.

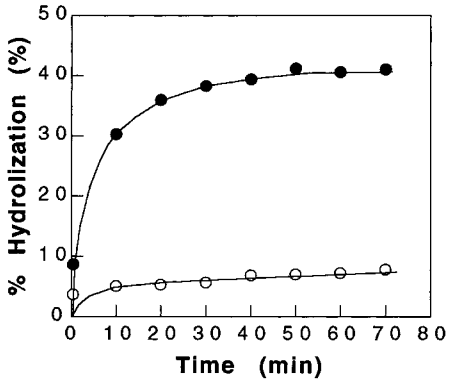


Figure 3. Time course of nucleotide-sugar phosphatase in *E. coli* BL21 (DE3) cells harboring GalE. Radiolabeled UDP-glucose was treated with *E. coli* cells in the absence (closed circles) or presence (open circles) of phosphatase inhibitors. *E. coli* cells were omitted for blank.

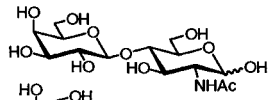
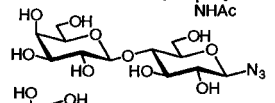
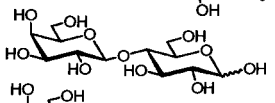
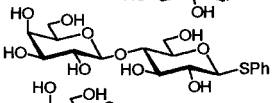
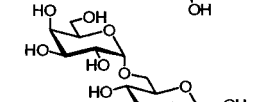
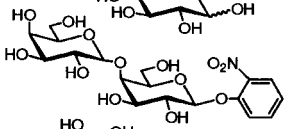
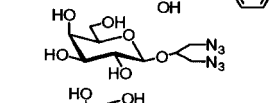
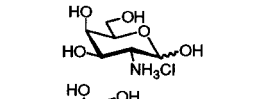
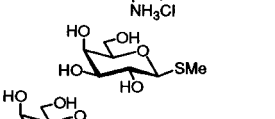
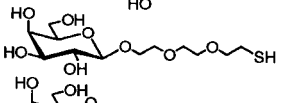
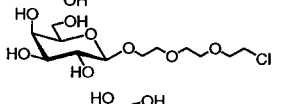
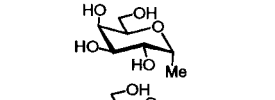
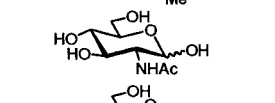
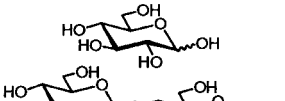
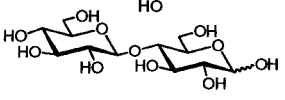
have been found in rat retina (*16*, *17*), no similar findings were reported for prokaryotic systems. To confirm the presence of the phosphatase, a time course of UDP-glucose hydrolization by *E. coli* BL21 (DE3) cells harboring GalE was carried out both in the presence and absence of phosphatase inhibitors. Decomposition of UDP-sugar in the presence of the cells was observed (Figure 3). Compared to the blank (no cells), about 30% of the radiolabeled UDP-glucose was hydrolyzed within 10 min of incubation. The hydrolization was saturated within 20 min, where 40% decomposition of the nucleotide-sugar was observed. However, in the presence of phosphatase inhibitors, the hydrolization was significantly decreased, and only 8% of UDP-glucose was hydrolyzed by the cells. It would be interesting to identify the phosphatase in *E. coli*. The presence of Triton X-100 may increase the permeability of the recombinant cells. No significant activity change between the reactions in the presence and absence of Triton X-100 indicates that during storage under −20 °C and under the reaction conditions, the cell membrane was permeabilized to the substrates as well as the products.

Naturally, the α1,3GalT mediates the transfer of galactose from UDP-galactose to *N*-acetyllactosamine terminated glycoproteins or glycolipids. To facilitate the enzymatic synthesis of α-Gal oligosaccharides and, more importantly, their derivatives and to widen the scope of the application, we believe that it is necessary to study the acceptor specificity of our recombinant α1,3GalT. Therefore, derivatives of lactose, galactose, and glucose were assayed as potential unnatural acceptors for α1,3GalT. Unlike the absolute specific structure requirement of Galβ1,4GlcNAc for Galβ1,4GlcNAc-R α2,6 sialyltransferases from bovine colostrum (*18*) and rat liver (*19*), the recombinant α1,3GalT can accept a wide range of substrates (Table 1). We found that Galβ1,4Glc disaccharide derivatives (entries 2−4) were very good acceptors for α1,3GalT. Compared to the natural acceptor (100%), the enzyme activities for these compounds were ranged from 47% to 96%. The activity was affected by the nature of the aglycons (N_3 > OH > SPh). In addition, the β1,4 linkage between Gal and Glc was important but not critical. Melibiose (Galα1,6Glc, entry 5) and Galβ1,-4Gal-*O*-nitrophenon (entry 6) exhibited reasonable activity. Galactose β-1-modified derivatives (entries 7−11) were found to be good or fair acceptors with activities in the range of 6−45%. Galactose α-1-modified derivatives such as α-1-methyl-galactopyranoside (entry 12) were not acceptors for α1,3GalT. It also appeared that the presence of the axial hydroxyl group at the C4 position of the Gal residue was essential for the enzyme activity. Cellobiose (Glcβ1,4Glc, entry 15) differs from lactose (entry 3) only at the configuration of the hydroxyl group at the C4′ position and is not a substrate for the recombinant enzyme. Generally, glucose derivatives (entries 13 and 14) were not substrates for α1,3GalT either. In a general sense, the disaccharide derivatives of lactose were better acceptors than galactose derivatives, whereas glucose derivatives were not acceptors.

Although both the recombinant enzyme and the natural enzyme purified from calf thymus (*10*) were not active with Galα-*O*-methyl or GlcNAc, the recombinant enzyme showed a much better acceptance for LacOH (84% activity compared to LacNAc) than the natural α1,3GalT, which has only 20% activity for LacOH compared to LacNAc. It is worth noting that the recombinant α1,-3GalT exhibits a high activity toward $LacN_3$ (96% relative activity), which is a versatile oligosaccharide building block. Introduction of the anomeric azido group makes the trisaccharide easier to be transformed to other derivatives and glycoconjugates. Hence we selected $LacN_3$ as the synthetic acceptor for α1,3GalT for two cells catalyzed reactions.

In our repeated synthesis of α-Gal trisaccharide **2** (Galα1,3$LacN_3$, Figure 1) with the two recombinant cells, the product was consistently obtained in good yields (60−68% yield). The ^1H and ^{13}C NMR (500 MHz, D_2O standard) spectra of the product were consistent with our previous results utilizing purified α1,3GalT. Since the culture media and the periplasmic fraction of both recombinant cells did not exhibit enzyme activity, the active enzyme actually was overexpressed inside the cell plasmid. It seemed that the cell membrane was permeablized during the storage at −20 °C or under the reaction condition, especially in the presence of Triton X-100. There also exists another possibility that cells had certain mechanisms to efflux (import/export) UDP-Gal or UDP-Glc between the growth media and the cytoplasmic portion and to export the α-Gal trisaccharide from the cytoplasm to the reaction media (out of the cell membrane). If this was the case, then the reaction catalyzed by two recombinant *E. coli* cells would be a six-step reaction sequence (Figure 4): (i) UDP-glucose was transported into the *E. coli* cell containing UDP-Gal 4-epimerase; (ii) GalE converted UDP-glucose to UDP-galactose; (iii) UDP-glucose was transported out of the

Table 1. Acceptor Specificity of α1,3GalT[a]

Entry	Structure	% Activity
1		100
2		95.6
3		84.1
4		47.2
5		12.7
6		6.8
7		44.8
8		29.3
9		16.1
10		7.7
11		6.3
12		< 0.1
13		< 0.1
14		< 0.1
15		< 0.1

[a] Purified α1,3GalT enzyme (1 μg, 10 mU) was used in the assay. Each compound was used at a final concentration of 50 mM

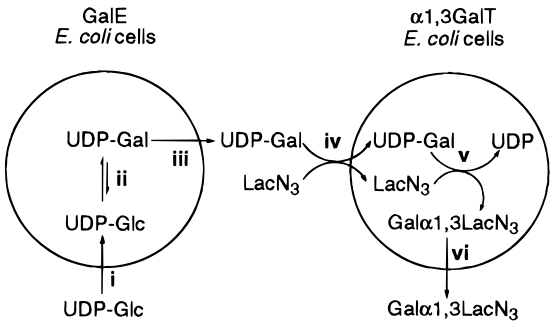


Figure 4. Proposed pathway for the reaction catalyzed by two recombinant *E. coli* cells.

E. coli cells; (iv) UDP-galactose and $LacN_3$ were transported into *E. coli* cells containing α1,3GalT; (v) α1,3GalT transferred galactose from UDP-galactose to $LacN_3$ to produce the α-Gal epitope; (vi) α-Gal epitope product was transported out of the *E. coli* cells.

In summery, this report established the convenient use of two recombinant cells in oligosaccharide synthesis without the tedious procedure of enzyme purification. Since the cost for preparation of α-Gal oligosaccharides was decreased by more than 40-fold with the use of UDP-Gal 4-epimerase recombinant cells, it is now economically feasible to use this technology to synthesize α-Gal trisaccharides on a multigram scale.

Acknowledgment

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Supporting Information Available: Spectral data (^1H, ^{13}C) for compound **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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