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Cloning, expression and characterization of a UDP-galactose 4-epimerase from *Escherichia coli*

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Abstract

The gene *galE* encoding UDP-galactose 4-epimerase was cloned into *E. coli* BL21(DE3) from the chromosomal DNA of *E. coli* strain K-12. High expression of the soluble recombinant epimerase was achieved in the cell lysate. In order to evaluate the use of this epimerase in enzymatic synthesis of important α-Gal epitopes (oligosaccharides with a terminal Galα1,3Gal sequence), a new radioactivity assay (α1,3-galactosyltransferase coupled assay) was established to characterize its activity in producing UDP-galactose from UDP-glucose. Approximately 2700 units (100 mg) enzyme with a specific activity of 27 U mg^{-1} protein could be obtained from one liter of bacterial culture. The epimerase was active in a wide pH range with an optimum at pH 7.0. This expression system established a viable route to the enzymatic production of α-Gal oligosaccharides to support xenotransplantation research.

Introduction

UDP-galactose 4-epimerase (UDP-glucose 4-epimerase, EC 5.1.3.2) catalyzes the interconversion of UDP-galactose and UDP-glucose. The epimerase from *E. coli* is a homodimer with an overall molecular weight of 79 kDa (Burke & Frey 1993). Each subunit contains 338 amino acid residues and one irreversibly, noncovalently bound NAD^+ (Thoden *et al.* 1996). Neither NAD^+ nor metal ions such as Mg^{2+} or Mn^{2+} need to be added for catalysis (Wilson & Hogness 1964). The epimerase has been widely employed in oligosaccharide synthesis for *in situ* cofactor regeneration to avoid both the stoichiometric use of expensive UDP-galactose and product inhibition of the transferases (Wong *et al.* 1982, Auge *et al.* 1986, Thiem & Wiemann 1992, Zervosen & Elling 1996). However, large-scale production of the recombinant enzyme has not been reported.

α-Gal epitopes (oligosaccharides with a terminal Galα1,3Gal sequence) are being used in the study of pig-to-primate xenotransplantation, specifically, in the prevention of hyperacute rejection caused by the interaction of natural anti-Gal antibodies in human and α-Gal epitopes expressed on the pig cell surface (Cooper 1998, Bühler *et al.* 1999). α1,3-galactosyltransferase (α1,3GalT, EC 2.4.1.151) is the key enzyme in the synthesis of the terminal structure Galα1,3Gal (Galili *et al.* 1988). In our efforts to develop large-scale enzymatic synthesis of α-Gal epitope for experimental and clinical use in xenotransplantation (Fang *et al.* 1998, Janczuk *et al.* 1999, Zhang *et al.* 1999), the production of large amounts of recombinant epimerase is necessary.

UDP-galactose 4-epimerase activity was usually measured in the reaction producing UDP-glucose from UDP-galactose using the coupled assay developed by Kalckar and modified by Wilson & Hogness (1964). The formation of UDP-glucose was coupled to NADH formation and measured by an UV spectrophotometer at 340 nm. To our knowledge, no method exists for the analysis of epimerase function in the reverse direction. In this paper we report the overexpression of epimerase in *E. coli* and the characterization of its activity using a convenient two-step radioactivity assay (Figure 1). In the first part epimerase is used to generate UDP-galactose. The reaction is stopped while in the initial rate range. The product is then used as

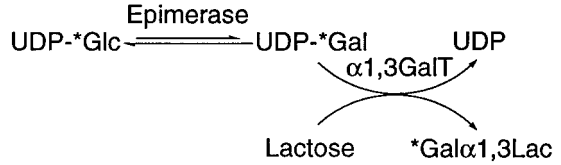

Fig. 1. The principle of a two-step assay for UDP-galactose 4-epimerase. In the first step, the epimerase produces UDP-galactose from UDP-glucose. The reaction is stopped by heating in boiling water. α1,3GalT then catalyzes the second reaction transforming the UDP-galactose to the α-Gal trisaccharide product (* indicates ^{3}H labeled substances).

the donor in the α1,3GalT catalyzed reaction that uses lactose as the acceptor and results in the production of radio labeled Galα1,3Lactose. The amount of the final trisaccharide produced is monitored by a scintillation counter.

Materials and methods

Materials

Ni^{2+}-NTA (nitrilotriacetic acid) agarose, PCR purification kit, QIA II gel extraction kit, QIAamp tissue kit and DNA miniprep spin kit were from Qiagen (Santa Clarita, CA). pET15b plasmid vector was purchased from Novagen. All restriction enzymes, 1 kb DNA ladder, Taq DNA polymerase and T4 DNA ligase were obtained from Promega (Madison, WI). UDP-D-[6-^{3}H]galactose was purchased from Amersham, β-lactose, UDP-glucose, UDP-galactose, UDP-D-[6-^{3}H]glucose, ampicillin and Dowex 1$\times$ 8 anion exchange resin were obtained from Sigma. ScintiVerse BD was from Fisher. All other chemicals were obtained in reagent grade from commercial sources.

Bacterial strains and plasmids

E. coli strain K-12 (substrain MG1655) was from ATCC (#47076). Plasmid α1,3GalT-pET15b was constructed as described previously (Fang *et al.* 1998). *E. coli* plasmid host strain DH5α was from Gibco-BRL and *E. coli* expression host strain BL21(DE3) was from Novagen.

Construction of pET15b-galE

The chromosomal DNA of *E. coli* was purified using QIAamp tissue kit (Qiagen, Santa Clarita, CA). PCR reaction was performed in a 50 μl reaction mixture containing K-12 chromosomal DNA (2 μg), *N*-terminal primer GalE-M1N: 5′-GGAATTCCATATGAGAGTTCTGGTTACC-3′ (containing *Nde*I restriction site, 1 μM), *C*-terminal primer GalE-D338C: 5′-CGCGGATCCTTAATCTGGGATATCCCTG-3′ (containing *Bam*HI restriction site, 1 μM), $MgCl_2$ (2.5 mM), buffer B [10 mM Tris/HCl, pH 8.3 (at 25 °C); 50 mM KCl], dNTPs (1 mM), and 2.5 units of Taq polymerase. The reaction mixture was overlayed with 50 μl of mineral oil and subjected to 30 cycles of amplifications with annealing temperature of 55 °C in a Thermolyne Amplitron I thermal cycler (Barnstead Thermolyne corporation, Dubque, IA). Restriction enzyme digestions and DNA ligation were performed as directed by the enzyme manufacturers. The resulting plasmid was transformed into *E. coli* DH5α and subsequently into BL21(DE3) competent cells. Selected clones were grown for minipreps and characterization by restriction mapping and DNA sequencing.

Expression of recombinant epimerase

BL21(DE3) cells harboring the pET15b-galE plasmid were cultured in 50 ml LB medium containing 150 μg ampicillin ml^{-1} overnight (16–18 h) with rapid shaking (250 rpm) at 37 °C in a C25 incubator shaker (New Brunswick Scientific Co., Inc., Edison, NJ). Overnight cell culture (20–30 ml) was transferred into 1 l LB media. IPTG (isopropyl-1-thio-β-D-galactopyranoside) was added to a concentration of 400 μM to induce the expression of recombinant proteins when the $A_{600\ nm}$ of the culture reached 0.8 to 1.0 (3–5 h). After shaking at 37 °C (250 rpm) for 3 h, the cells were harvested by centrifugation at 5000 rpm for 20 min and washed once with washing buffer (pH 8.5, 20 mM Tris/HCl, 20% sucrose). Lysis buffer (pH 8.5, 20 mM Tris/HCl, 1 mM EDTA, 1% Triton X-100, 200 μg lysozyme ml^{-1}) was added and the mixture was stirred vigorously for 10 min at room temperature. DNaseI (2 μg enzmye ml^{-1}) was then added and the mixture was shaken at 37 °C in a water bath for an additional 40 min. Cell lysate was obtained by centrifugation at 12 000 rpm for 20 min.

Enzyme purification from cell lysate

The enzyme was purified directly from the cell lysate using a Ni^{2+}-NTA affinity column which binds to the epimerase's *N*-terminal hexahistidine sequence. All procedures were performed in the cold room (4–7 °C). After loading the cell lysate, the Ni^{2+} column was washed with six column volumes of binding buffer

(5 mM imidazole, 20 mM Tris/HCl pH 7.9, 0.5 M NaCl), followed by six volumes of washing buffer (20 mM imidazole, 20 mM Tris/HCl pH 7.9, 0.5 M NaCl). The overexpressed protein was eluted with eight volumes of elution buffer (200 mM imidazole, 20 mM Tris/HCl pH 7.9, 0.5 M NaCl). Fractions containing the purified enzyme were detected by a UV/Vis spectrometer at 280 nm, collected and then dialyzed three times against dialysis buffer (10% glycerol, 20 mM Tris/HCl, pH 7.9). Glycerol was added to a final concentration of 50% to stabilize the enzyme. The enzyme solution was then stored in -20 °C freezer.

SDS-PAGE

SDS-PAGE was performed in a 15% gel using Bio-Rad mini-protean 3 cell gel electrophoresis unit (Bio-Rad, Hercules, CA) at DC = 150 V. Low range SDS-PAGE Standards (Bio-Rad) were used as molecular weight standards. The gel was stained with Coomassie Blue.

Enzyme activity assay

The enzyme activity assay was carried out in duplicate at 24 °C in a two-step assay in which the formation of UDP-galactose was coupled to α-Gal epitope production. The reaction mixture of the first step consisted of UDP-D-[6-^{3}H]glucose (0.6 mM, final specific activity was 2000 cpm nmol^{-1}), Tris/HCl buffer (20 mM), pH 7.0, BSA (0.2%), purified epimerase (2×10^{-4} mg). The reaction was allowed to proceed for 5 min, then 50 μl of the mixture was placed in a boiling water bath for 3 min. After cooling to room temperature, $MnCl_2$, lactose and α1,3GalT was added to final concentrations of 10 mM, 50 mM and 0.01 mg enzyme ml^{-1} respectively, and to a total volume of 100 μl. In the second step, α1,3-galactosyltransferase activity assay was carried out in a 37 °C water bath for 1 h. Lactose (the acceptor of α1,3GalT) was omitted in the blank. The reaction was stopped by adding 100 μl ice cold EDTA (0.1 M) and the mixture was passed through a Dowex 1× 8–200 chloride anion exchange column, washed with 0.5 ml and 1 ml H_2O consecutively. The flow-through was collected in a 20 ml plastic vial and ScintiVerse BD (5 ml) was added. The radioactivity of the mixture was determined in a liquid scintillation counter (Beckmann LS-3801 counter). One unit (U) of the epimerase activity is defined as the amount of the epimerase required to produce 1 μmol UDP-galactose from UDP-glucose per minute at room temperature.

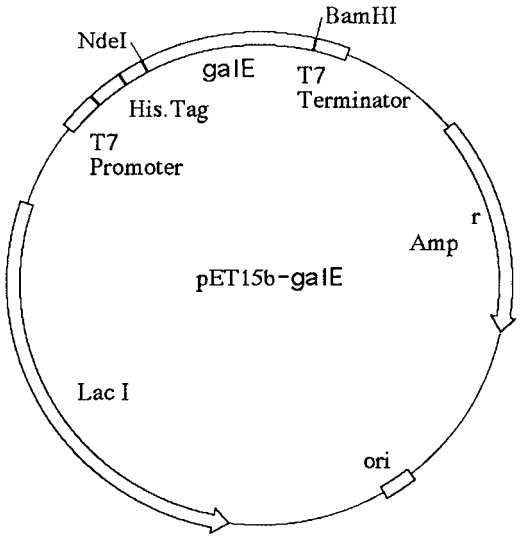

Fig. 2. Plasmid map of pET15b-galE bearing the *E. coli galE* gene encoding UDP-galactose 4-epimerase.

Kinetics

Kinetic assay was similar to the enzyme assay described above except that the concentration of UDP-glucose was varied (as the final concentration in the first-step reaction mixture) to obtain the apparent kinetic parameters. The concentrations of the UDP-glucose in the first-step reaction mixture were: 0.2 mM, 0.6 mM, 1 mM, 1.4 mM, 2 mM, 3 mM and 4 mM. Epimerase (2×10^{-4} mg) was used for each assay.

pH dependence

This assay was carried out as described in enzyme activity assay but the buffer pH was varied. The following buffers were used: HOAc/NaOAc buffer, pH 4.0 and 5.5; MES buffer, pH 6.0; Tris/HCl buffer, pH 7.0 and 8.5 and carbonate buffer, pH 9.0. The concentration of each buffer solution was 50 mM. After the first step, the pH value of the reaction mixture was adjusted to 7.0 before continuing with the second step.

Results and discussion

Cloning, overexpression and purification of epimerase

The *galE* gene was cloned directly from *E. coli* K-12 into the *Nde*I (*N*-terminal) and *Bam*HI (*C*-terminal) multiple cloning site of pET15b vector. The T7 promoter in advance of the multiple cloning site allows the IPTG controlled expression of the epimerase. The ampicillin resistance gene is for the colony selection. A hexa-histidine tag sequence at the 5′ of the target gene facilitates the purification of expressed protein (Figure 2). DNA sequencing of the gene indicated that the cloned *galE* sequence was identical to that reported in the GenBank database. A large quantity of the epimerase was expressed in the pET15b-galE transformed strain after the IPTG induction (Figure 3, lane 3). However, as a negative control, no band corresponding to the recombinant epimerase was found in the SDS-PAGE for non transformed strain (Figure 3, lane 2). More interestingly, the epimerase dominantly expressed as soluble protein in the cell lysate (about 90%) (Figure 3, lane 5) and only very small part of the epimerase expressed as insoluble cell inclusion bodies (Figure 3, lane 4). This means that our expression system and conditions are suitable for obtaining the soluble active epimerase. The Ni^{2+}-NTA column efficiently bound the histidine tagged enzyme and more than 95% of the total epimerase was obtained after one pass through the column (Figure 3, lane 6). The molecular weight of purified epimerase was determined to be around 40 kDa as expected. Generally, about 100 mg epimerase l^{-1} was obtained after purification. The concentration of protein was estimated from the absorbance at 280 nm. A value of 1.2 for $E_{280}^{0.1\%}$ was calculated based on the amino acid composition of the enzyme.

Time course

We demonstrated previously that 0.3 μmol UDP-galactose can be completely transformed to lactose (transferase acceptor) to produce trisaccharide product after 50 min at 37 °C with 0.075 mg α1,3GalT ml^{-1} (data not shown). Based on this result, 1 h was used as the reaction time for the second step reaction in α1,3GalT coupled epimerase assay. This way, all the radio labeled UDP-galactose obtained from UDP-glucose catalyzed by the epimerase in the first step can be totally transformed to the product in the α1,3GalT catalyzed second reaction. This made the present method a more accurate choice than the one reported (Wilson & Hogness 1964). The reaction reached the equilibrium in the first 15 min (Figure 4).

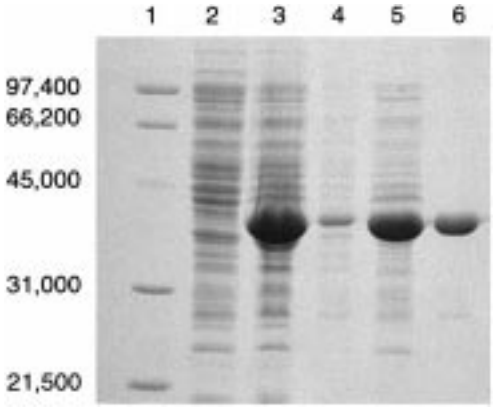

Fig. 3. SDS-PAGE of the epimerase. Lanes: 1, protein standards; 2, BL21(DE3) host cell induced with 0.4 mM of IPTG; 3, BL21(DE3) containing the plasmid pET15b-galE induced with 0.4 mM IPTG; 4, inclusion bodies after induction (washed with lysis buffer twice); 5, cell lysate after induction; 6, purified epimerase. Same volume of lysis buffer was used to prepare each sample.

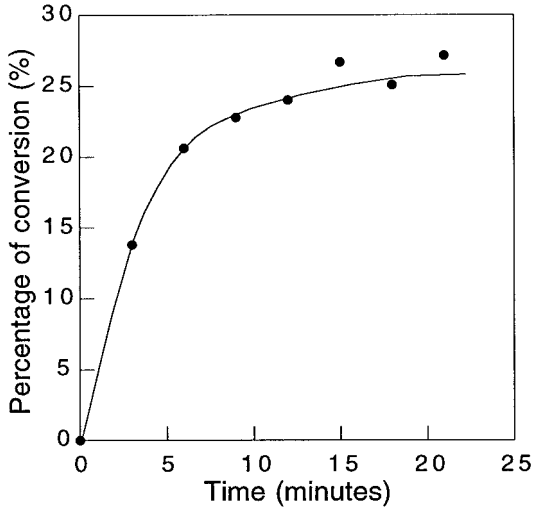

Fig. 4. Time course of the epimerase catalyzed conversion of UDP-glucose to UDP-galactose. The first step assay was carried out in a 1 ml reaction mixture containing 0.004 mg epimerase, 0.2% BSA and 0.6 mM radio-labeled UDP-glucose (the reaction started by adding UDP-glucose) in a 100 mM pH 8.0 Tris/HCl buffer. At 0, 3, 6, 9, 12, 15, 18 and 21 min, 50 μl aliquots of the reaction mixture were placed in a boiling water bath for 3 min. The second step assay was the same as described in the experimental section.

pH dependence of the reaction

The optimal pH value for α1,3GalT was determined to be 7.0 in Tris/HCl buffer (data not shown). Thus, the

pH dependence of epimerase was assayed with various pH values and then adjusted to pH 7.0 before performing the second step of the assay involving α1,3GalT. The epimerase is active in a wide pH range with an optimal pH of 7.0 in Tris/HCl. No epimerase activity was observed at pH 4.0 in 100 mM acetic acid-sodium acetate buffer.

Apparent kinetic parameters

Under the experimental conditions (24 °C and pH 7.0), the kinetics of the reaction are consistent with the classical Michaelis–Menten model. The k_{cat} and K_m for UDP-glucose were determined to be 18 s^{-1} and 1.2 mM, respectively. The k_{cat} and K_m of epimerase for UDP-galactose were reported to be 500 s^{-1} and 0.16 mM, respectively (Wilson & Hogness 1964). These results indicate that the binding of the epimerase to UDP-glucose (K_m = 1.2 mM) is weaker than its binding to UDP-galactose (K_m = 0.16 mM). Furthermore, the rate of conversion from UDP-glucose to UDP-galactose is slower than the conversion from UDP-galactose to UDP-glucose. Thus, if using the conventional definition of producing UDP-glucose from UDP-galactose to describe the activity of the epimerase, the activity should be 28 times (500 s^{-1}/18 s^{-1}) higher than we reported. The expression level of the epimerase in the system described herein would be 75 000 units protein l^{-1}.

Conclusion

High level overexpression of soluble recombinant epimerase was achieved by using an IPTG-inducible expression system. A new α1,3GalT-coupled radioactive assay was established to determine UDP-galactose production. This expression system and the related assay protocol established a viable route to the large-scale enzymatic production of α-Gal oligosaccharides to support a variety of biomedical research activities in the field of transplantation.

Acknowledgement

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