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Glycosynthase mediated synthesis of psychosine

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ABSTRACT

Globoid cell leukodystrophy (GCL), or Krabbe disease, is a lysosomal storage disorder characterized by a deficiency in galactosylceramidase (GALC), which hydrolyses galactosylceramide and galactosylsphingosine (psychosine). Early detection of GCL in newborns is essential for timely therapeutic intervention and could be achieved by testing infant blood samples with isotopically labeled lysosmal enzyme substrates and mass spectrometry. While isotopically labeled psychosine would be a useful tool for the early diagnosis of GCL, its synthesis is lengthy and expensive. To obviate this problem we developed a one-step chemoenzymatic synthesis of psychosine using a glycosynthase mutant of the *Rhodococcus equi* endogalactosylceramidase (EGALC), α-D-galactopyranosyl fluoride and sphingosine.

1. Text

Psychosine (1-β-D-galactopyranosylsphingosine) is a lysosphingolipid found in trace amounts within the CNS of healthy individuals [1,2]. Patients with globoid cell leukodystrophy (GCL), also known as Krabbe disease, carry mutations in the *GALC* gene that results in a deficiency of galactosylceramide-β-galactosidase, the enzyme that hydrolyses psychosine to D-galactose and sphingosine [3–8]. The resulting accumulation of psychosine in neural tissues is toxic, resulting in the death of oligodendroglial cells, neural signaling dysfunction, neurological impairment and ultimately death [9–13]. Early diagnosis of this disease is important for improving patient outcomes and can be accomplished using isotopically labeled psychosine in conjunction with mass spectrometry to quantify GALC activity in newborns using vanishingly small quantities of blood [14,15]. Obtaining isotopically labeled psychosine for this process is difficult, owing to the length and efficiency of current psychosine syntheses [16]. A direct chemoenzymatic galactosylation of isotopically labeled sphingosine, which is a commercial product, is sorely needed to enhance the accessibility of this promising diagnostic tool.

Glycosynthases, hydrolytically incompetent mutant glycoside hydrolases (GHs) that are capable, instead, of forming glycosidic linkages, have proven to be of great utility in the one-step chemoenzymatic assembly of complex glycoconjugates. When the catalytic nucleophile of a retaining glycosidase is mutated to a small residue (Gly, Ala or Ser), the resulting variant can bind and subsequently transfer sugars from activated α-glycosyl fluorides to an appropriate acceptor group with the aid of the vestigial catalytic acid/base residue. Previous work towards the manufacture of complex glycolipids demonstrated that a glycosynthase mutant of the GH family 5 (www.cazy.org) endoglycosylceramidase II could transfer lactosyl and larger oligoglycosyl units to sphingolipid to form a series of glycosphingolipids [17]. Conceptually, a related glycoside hydrolase capable of processing galactosylceramides may yield a glycosynthase mutant capable of synthesising psychosine.

The endogalactosylceramidase (EGALC) from *Rhodococcus equi* is another GH family 5 enzyme that catalyzes the hydrolysis of 1,6-linked oligogalactopyranosyl sphingolipids and acylglycerides [18]. Despite its promiscuity with respect to the lipid portion of its substrates, it shows a strong preference for oligogalactosides with minor activity on galactosylceramide and psychosine itself [18,19]. Nonetheless, we suspected that catalytic nucleophile mutants of EGALC might have potential as glycosynthases for the assembly of psychosine (Fig. 1).

We first attempted cytosolic expression of a synthetic, codon-optimized EGALC gene in different *Escherichia coli* expression strains but only ever obtained recombinant protein in inclusion

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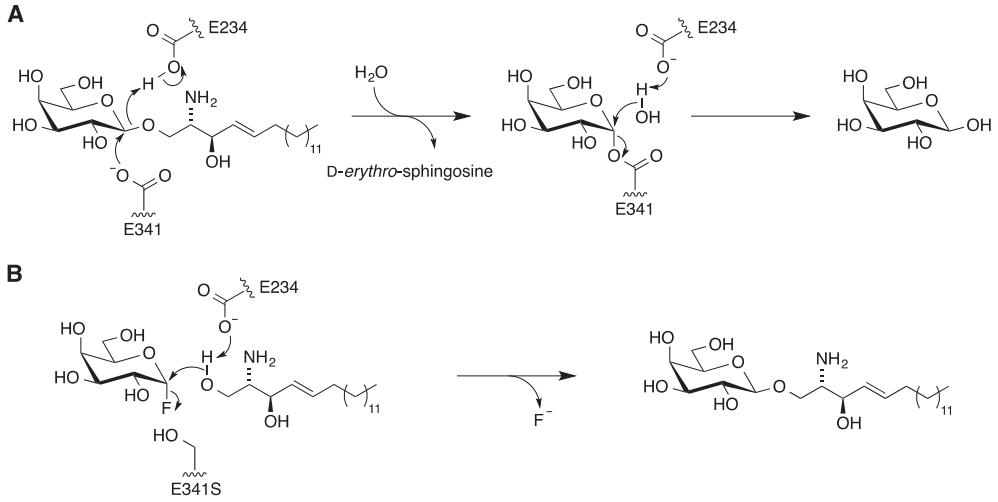

Fig. 1. Mechanism of EGALC galactosylceramidase and its glycosynthase mutant. (A) The proposed mechanism for the hydrolysis of psychosine by the retaining glycosidase EGALC. (B) Upon mutating the catalytic nucleophile, the enzyme cannot carry out hydrolysis but can instead mediate glycoside bond formation when supplied with an activated glycosyl fluoride donor.

bodies. We transitioned to using the secretory pathway in *E. coli* by the addition of the PelB signal peptide to target EGALC to the periplasmic space (Figure S1). After much optimization we could repeatedly obtain high yields (20 mg l^{-1}) of recombinant EGALC from the culture supernatant, though prolonged incubation (30 h) in autoinduction media was absolutely essential for reproducible yields. Our recombinant EGALC was capable of hydrolyzing psychosine, in line with previous reports on the specificity of this enzyme [18].

Based upon previous experience in generating other glycosynthases, site-directed mutagenesis was performed to produce three catalytic nucleophile mutants: E341G, E341A, and E341S. The EGALC E341A/S mutants expressed very well, comparable to the wild-type enzyme, while the glycine mutant failed to provide any protein. Neither the E341A nor the E341S mutant had any hydrolytic activity on psychosine, as anticipated. Both mutants were evaluated as glycosynthases using sphingosine and α-D-galactopyranosyl fluoride. Only low activity was observed during the initial trial reactions, though a small amount of psychosine was observed by mass spectrometry and thin layer chromatography. Qualitatively, E341S appeared to be the more active mutant and so we set about optimizing reaction conditions with this variant.

Two large issues that we had to contend with were the poor solubility of psychosine and the propensity for EGALC to precipitate from solution under the reaction conditions. We exhaustively explored the use of different buffering agents, pH, co-solvents, detergents, molecular crowding agents and amphiphilic host molecules to increase substrate and enzyme solubility and to maximize conversion of substrate to product. Optimized conditions were established as: 200 mM α-D-galactopyranosyl fluoride, 10 mM sphingosine, 500 mM MOPS (pH 7.5), 20 mM α-cyclodextrin and 2.5% methanol. The order of operations was critical for the reaction. The sphingosine was first dispersed in methanol at 40 °C with the aid of sonication. The glycosyl fluoride and cyclodextrin were concurrently dissolved in the MOPS buffer and the resulting solution was added to the sphingosine in methanol. This mixture was sonicated at 40 °C for 15 min then cooled to 37 °C and the enzyme added. The resulting turbid mixture was gently agitated at 37 °C for 3 days or until all the galactosyl fluoride had been depleted. The psychosine product was extracted using chloroform and purified by chromatography on silica and C-8 silica to provide pyschosine in 21% yield, along with recovered sphingosine. Although the yield of psychosine was relatively low, the ability to produce the glycolipid in a single step and to readily recover the unused sphingosine makes the EGALC glycosynthase reaction a viable method for the synthesis of isotopically labeled psychosine for diagnostic purposes.

2. Experimental

2.1. Materials

All chemical reagents were purchased from Sigma-Aldrich at >95% purity unless otherwise stated. ^{1}H and ^{13}C NMR spectra were recorded using a 400 MHz instrument. All signals were referenced to solvent peaks ($CDCl_3$: δ 7.26 ppm for ^{1}H NMR). TLC analysis used aluminum backed Merck Silica Gel 60 F_{254} sheets, detection was achieved using UV light, 5% H_2SO_4 in MeOH, or ceric ammonium molybdate solution with heating as necessary.

2.2. Cloning and expression

A codon-harmonized gene encoding the PelB signal peptide, residues 23–488 of EGALC (BAF56440.1) and a C-terminal hexahistidine tag was synthesized (BioBasic) and cloned into pET29b(+) (Novagen). The sequence of the gene and the protein it encodes is detailed in Figure S1 (Supplementary Data). Site directed mutagenesis of EGALC was accomplished using the four-primer PCR method [20] and the primers detailed in Table S1 (Supplementary Data). Sequence-validated plasmids were transformed into BL21*(DE3), selecting with kanamycin. Single colonies were used to inoculate 10 ml of LB media (50 μg ml^{-1} Kan), which was incubated overnight at 37 °C. The overnight culture was used to inoculate two 500 ml cultures of LBE-5052 auto-induction media (50 μg ml^{-1} Kan). These were incubated at 30 °C and shaken at 250 rpm for 36 h. The cultures were centrifuged at 17,000 × g for 20 min at 4 °C. The culture supernatants were collected and treated with protease inhibitor cocktail. The supernatant was filtered through a glass fiber filter then concentrated to a volume of 40 ml using stirred cells and a membrane with a 10 kDa NMWL. This concentrate was diluted with 4.5 ml of 8 × binding buffer (160 mM Tris, 4 M NaCl, 40 mM ImH, pH 8). The sample was centrifuged at 17,000 × g for 20 min and filtered before loading on a 1 ml HisTrap column. The protein was eluted from the column using a gradient of

binding (20 mM Tris, 500 mM NaCl, 5 mM ImH, pH 8) and elution (20 mM Tris, 500 mM NaCl, 500 mM ImH, pH 8) buffers. Fractions containing product were dialysed into 50 mM MOPS, 150 mM NaCl at pH 7.5 then concentrated using an Amicon centrifugal filter unit (10 kDa NMWL). The yield of wild type and mutant EGALC was typically 20 mg l^{-1}.

2.3. Glycosynthase reaction

D-*erythro*-Sphingosine (6.0 mg, 20 μmol) was dispersed in methanol (50 μl) within a glass vial using sonication at 40 °C for 15 min. α-D-Galactopyranosyl fluoride (73 mg, 0.4 mmol) and α-cyclodextrin (40 mg, 40 μmol) were dissolved in MOPS buffer (1.870 ml, 150 mM, pH 7.5) and this solution added to the sphingosine in methanol. This mixture was sonicated at 40 °C for a further 15 min. It was cooled to 37 °C and then EGALC-E341S (100 mg ml^{-1}, 80 μl) was added. The resulting turbid mixture was nutated at 37 °C for 3 days or until all the α-D-galactopyranosyl fluoride had been depleted. The mixture was extracted with chloroform:methanol (1:1, 5.0 ml). The aqueous phase was then extracted a further two times with chloroform (2.0 ml). The combined organic extracts were dried under a stream of nitrogen. The residue was purified by flash chromatography ($CHCl_3$/MeOH/H_2O, 1:1:0 → 5:4:1) and C_8 reversed phase chromatography (H_2O/CH_3CN, 1:0 → 0:1), affording 1.9 mg pyschosine (21% yield) and 3.8 mg of sphingosine (63% recovery). R_f: 0.35 ($CHCl_3$/MeOH, 1:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.79 (t, 3H, CH_3), 1.05–1.40 (m, 22H, CH_2), 1.90–2.05 (m, 2H, CH=CHCH_2), 2.90–3.75 (m, 14H, H-2, H-3, H-4, H-5, H-$6_{a,b}$, CH_2O, CH=CHC*H*O, NH_2, OH), 3.97–4.17 (m, 2H, H-1, C*H*NH_2), 4.85–5.15 (m, 2H, NH_2, OH), 5.40 (dd, 1H, C*H*=CHCH_2, J = 8.0, 15.0 Hz), 5.55–5.70 (m, lH, CH=C*H*CH_2); HRMS (ESI)$^+$ *m/z* 462.6483 [$C_{24}H_{48}NO_7$ $(M + H)^+$ requires 462.6480]. These spectroscopic data were in agreement with those previously reported [21].

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.carres.2016.09.013.

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