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β-D-Galactopyranosyl Azide

*Its One-Step Quantitative Synthesis Using E461G-β-Galactosidase (*Escherichia coli*) and a Demonstration of Its Potential as a Reagent for Molecular Biology*

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Abstract

A simple one-step synthesis of β-D-galactopyranosyl azide from *o*-nitrophenyl-β-D-galactopyranoside and azide catalyzed by E461G-β-galactosidase is described. The synthesis is quantitative in the presence of excess azide and only the β anomer is produced. The product was purified (71% yield) from the other reaction components by extraction with ethyl acetate, silica gel chromatography, and crystallization. The purity was verified by GLC, TLC, and NMR. Thus, E461G-β-galactosidase is able to specifically and quantitatively form β-D-galactopyranosyl-azide. The purified β-D-galactopyranosyl azide inhibited the growth of *Escherichia coli* that express β-galactosidase but not of *E. coli* that do not. Growth is stopped because β-galactosidase catalyzes the hydrolysis of the β-galactopyranosyl-azide, and the azide that is produced inhibits cell growth. This selective inhibition of growth has potential application in molecular biology screening.

Index Entries: β-Galactosidase; screening; molecular biology; synthesis.

Introduction

β-Galactosidase (EC 3.2.1.23) from *Escherichia coli* catalyzes both hydrolytic and transgalactosidic reactions with β-D-galactopyranosides *(1)*. β-Galactosidases that have substitutions for Glu-461 are much less active (between 1000- and 3000-fold) than wild-type β-galactosidase *(2)*, but if the substitution is with a Gly (or other neutral side chain), there is significant regain of activity in the presence of nucleophiles. The rate increase depends

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on the concentration of the nucleophiles added and follows Michaelis-Menten saturation kinetics, suggesting that the nucleophile binds at the active site. Studies of the nucleophile reaction with E461G-β-galactosidase have shown that the enzyme forms β-D-galactopyranosyl adducts with the nucleophiles and that the increases in rates on addition of the nucleophiles occur because the reaction rates for the formation of the adducts are faster than is the hydrolytic reaction rate *(3)*.

In the present study we made use of the ability of E461G-β-galactosidase to form adducts and developed a simple and rapid synthesis and purification scheme for β-D-galactopyranosyl azide. In addition, we demonstrated that β-D-galactosyl azide is useful to selectively stop the growth of *E. coli* strains that express β-galactosidase. Growth is stopped because wild-type β-galactosidase catalyzes the hydrolysis *(4)* of β-D-galactopyranosyl azide to D-galactopyranose and azide and the azide inhibits cell growth *(5,6)*. The selective inhibition of growth has potential application in molecular biology screening.

Materials and Methods

Bacterial Strains

The following strains were used: ML 308: F^- *lac*I^-, a constitutive producer of β-galactosidase; JM 108: F^- *end*A1 *rec*A1 *gyr*A96(Nal^r) *thi hsd*R17($r_k - m_k^+$) *rel*A1 *sup*E44 Δ*(lac-pro*AB*)*, a strain with a deletion of the *lacZ* gene that produces no β-galactosidase.

Growth Media

Cells were grown in either Luria-Bertani (LB) medium or minimal glucose medium: 96 m*M* KH_2PO_4, 15 m*M* $(NH_4)_2SO_4$, 1 m*M* $MgSO_4$, 1.75 μ*M* $FeSO_4$, 0.005% vitamin B_1, 0.002% L-proline, 100 m*M* glucose. The pH was adjusted to 7.0 for both LB and minimal media.

Purification of Enzyme

E461G-β-Galactosidase was expressed and purified as described previously *(2)*.

Chromatography

The reactions were monitored by thin-layer chromatography (TLC) on K6F Silica Gel 60 Å plates (Whatman). The plates were developed with ethyl acetate, methanol, and water (7:2:1). After development, the plates were dried and *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) was identified under UV light. Compounds that were sugars or that contained sugars were visualized by dipping the plates into 2% H_2SO_4 in methanol and heating until the products became charred. A column (1.5 × 20 cm) packed with Silica Gel 32-63, 60 Å (purchased from ICN) was used in one of the purification steps. Gas liquid chromatography (GLC) was carried out by methods described previously *(7)*.

Synthesis and Purification of β-D-Galactopyranosyl Azide

E461G-β-Galactosidase (200 μL containing 6 mg) was added to a mixture (pH 7.0) of 15 mmol of *o*NPG and 30 mmol of NaN_3 in 350 mL of water also containing $MgSO_4$ (20 m*M*) and NaCl (145 m*M*) at room temperature. An additional aliquot of enzyme (4.5 mg) was added after 24 h. When TLC indicated that the reaction was complete (3 to 4 d), the reaction mixture was brought to pH 5.0 and the solution was extracted with ethyl acetate (3 × 100 mL). The aqueous phase was evaporated to dryness, and the residue was chromatographed on the silica gel column (ethyl acetate: methanol:water [7:2:1]). The fractions containing β-D-galactopyranosyl azide were identified by TLC and pooled and evaporated to dryness. The residue was dissolved in methanol (room temperature), and ethyl acetate was added dropwise until white crystals were obtained.

Identification of β-D-Galactopyranosyl Azide by Nuclear Magnetic Resonance

The purified product was dissolved in D_2O and lyophilized (twice). Spectra were recorded on a Bruker AMX-500 spectrometer operating at 500.13 MHz for ^1H and 125.77 MHz for ^{13}C. The spectra were acquired at 27°C using a 5-mm probe. A one-dimensional ^1H spectrum was acquired in D_2O with presaturation of the residual hydrogen deuterium oxide (HOD) resonance at 4.8 ppm and was referenced to internal sodium 4,4-dimethyl-4-silapentanesulfonate (0 ppm). A one-dimensional ^{13}C spectrum was acquired with composite pulse proton decoupling. Two-dimensional heteronuclear multiple-quantum correlation (HMQC) with decoupling of ^{13}C and double-quantum-filtered correlated spectroscopy (COSY) nuclear magnetic resonance (NMR) spectra were acquired using standard Bruker pulse programs. The data obtained were used to assign the signals.

Cell Growth

Cells grown overnight in LB media in a rotary shaker at 37°C (225 rpm) were harvested, washed three times with minimal medium, and resuspended in glucose minimal medium. Equal aliquots were inoculated into glucose minimal medium (100 m*M*) cultures containing 0, 10, and 50 m*M* β-D-galactopyranosyl azide. These cultures were grown in a rotary shaker at 37°C (225 rpm), and cell growth was monitored by withdrawing samples and measuring the light scattering at 600 nm.

Cell Growth on Agar Plates

Filter-sterilized β-D-galactopyranosyl azide (100 m*M*) was spread on the surface of agar plates containing glucose minimal medium. These plates were inoculated with a loop of overnight culture and incubated for 18 h (37°C).

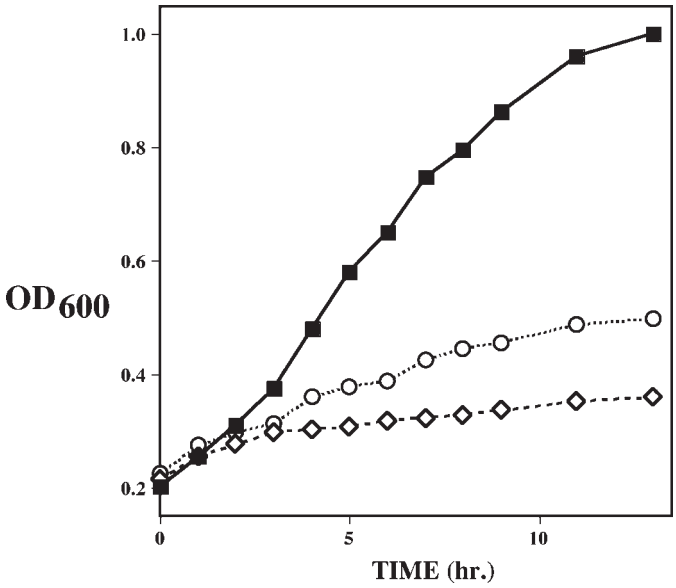

Fig. 1. Growth of *E. coli* ML 308: (■), no β-D-galactosyl azide; (○), 10 m*M* β-D-galactosyl azide; (◇), 50 m*M* β-D-galactosyl azide. Growth was estimated by the OD_{600}. Values relative to the maximum OD_{600} achieved are reported.

Results

Synthesis of β-D-*Galactopyranosyl Azide*

TLC analysis of the synthetic reaction incubation mixture with E461G-β-galactosidase showed that *o*NPG was totally depleted after 3 to 4 d of incubation. The only product that charred (*o*NP does not contain a sugar and thus does not char) moved to the position (R_f = 0.55) that one would expect β-galactopyranosyl azide to migrate. No free D-galactose was detected. The large amount of *o*NP present was removed by extraction with ethyl acetate. Purification of the product on silica gel and crystallization with methanol/ethyl acetate yielded 2.2 g of white crystals of β-D-galactopyranosyl azide (71% yield). The product was shown to be pure by both TLC (only one band was seen) and GLC (only one symmetrical peak was present). In addition, the following NMR results also showed that the product was pure: ^{1}H-NMR: δ 4.66 (d, 1 H, $J_{1,2}$ 8.6 Hz, H-1), 3.51 (dd, 1 H, $J_{2,3}$ 9.7 Hz, H-2), 3.68 (dd, 1 H, $J_{3,4}$ 3.0 Hz, H-3), 3.95 (d, 1 H, $J_{4,5}$ ~0, H-4), 3.74–3.78 (m, 3 H, H-5,6); ^{13}C NMR: δ 91.4 (1 C, C-1), 71.2 (1 C, C-2), 73.5 (1 C, C-3), 69.5 (1 C, C-4), 78.5 (1 C, C-5), 61.8 (1 C, C-6). The chemical shifts and the coupling constants identified the adduct as β-D-galactopyranosyl azide. The $J_{1,2}$ value of 8.6 Hz clearly shows that the product has a β configuration. There was no evidence for the presence of any α product.

Effects of β-D-*Galactopyranosyl Azide on Growth of* E. coli

Figure 1 shows that the addition of both 10 and 50 m*M* β-D-galactopyranosyl azide inhibited the growth of *E. coli* ML 308. As expected, the

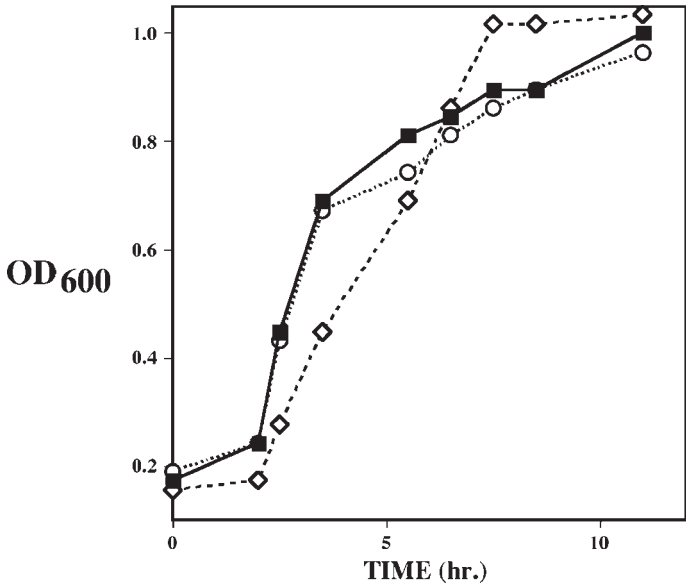

Fig. 2. Growth of *E. coli* JM 108: (■), no β-D-galactosyl azide; (○), 10 m*M* β-D-galactosyl azide; (◇), 50 m*M* β-D-galactosyl azide. Growth was estimated by the OD_{600}. Values relative to the maximum OD_{600} achieved are reported.

inhibition was greater with 50 than with 10 m*M* β-D-galactopyranosyl azide. Figure 2 shows that *E. coli* JM 108 grew well in the presence of both 10 and 50 m*M* β-D-galactopyranosyl azide. There was a slight delay of growth at the higher concentration, but the overall growth was comparable with that without β-D-galactopyranosyl azide. Figure 3 shows that *E. coli* ML 308 cells did not grow on agar plates (minimal glucose) with 100 m*M* β-D-galactopyranosyl azide. On the other hand, these ML 308 cells grew well on agar plates with no added β-D-galactopyranosyl azide. Growth with *E. coli* JM 108 cells (not shown) on agar plates (minimal glucose) was the same in the presence of 100 m*M* β-D-galactopyranosyl azide as in its absence.

Discussion

Glu-461 is an active site residue of β-galactosidase (*E. coli*). It is probably the acid catalyst of the reaction *(8)*. β-Galactosidase with a substitution of Gly (or of another neutral residue) for Glu-461 catalyzes the transfer of the galactopyranosyl part of substrates to nucleophiles and thereby forms β-D-galactopyranosyl adducts *(2,3)*. Glu-461 is also an inner-sphere ligand to the Mg^{2+} that is essential for full β-galactosidase activity *(9–12)*. It was thus necessary to carry out the reaction with a relatively high concentration of Mg^{2+} (20 m*M*).

In our study, we took advantage of the ability of E461G-β-galactosidase to form β-D-galactopyranosyl adducts to synthesize large amounts of β-D-galactopyranosyl azide in one step. The synthesis was quantitative,

Fig. 3. Growth of *E. coli* ML 308 on agar plates in **(top)** presence and **(bottom)** absence of 100 m*M* β-D-galactosyl azide.

and β-D-galactopyranosyl azide was isolated from the other reaction components (*o*NP, NaCl, and Mg^{2+}) as pure crystals in 71% yield. NMR showed that only the β-anomer of D-galactopyranosyl azide was formed. In addition, both TLC and GLC showed that the product was pure and that no galactose was present. The quantitative synthesis with no evidence of the formation of any free galactose indicates that the reaction is extremely efficient. It also indicates that β-D-galactopyranosyl azide is a very poor substrate of E461G-β-galactosidase or there would have been hydrolysis. Synthesis of β-D-galactopyranosyl azide by this method rather than by clas-

sic chemical methods *(4,13)* eliminates the need to chemically protect/deprotect the hydroxyl groups of galactose. Furthermore, it is not necessary to specifically activate the anomeric carbon of galactose in such a way that only the β form of galactopyranosyl azide is produced.

β-D-Galactopyranosyl azide inhibited the growth of *E. coli* cells (ML 308) that expressed β-galactosidase. This inhibition was dependent on the concentration of β-D-galactopyranosyl azide. TLC studies showed that β-galactopyranosyl azide is broken down during growth of these cells (β-galactopyranosyl azide is known to be a reasonably good substrate of wild-type β-galactosidase *[4]*) and, thus, that it was the azide product that inhibited the growth, not the β-D-galactopyranosyl azide itself. The normal growth of JM 108 cells on β-D-galactopyranosyl azide (discussed subsequently) is also evidence that β-D-galactopyranosyl azide itself is not the molecule that causes inhibition of growth.

E. coli JM 108 cells that do not express β-galactosidase because of a deletion of the *lac Z* gene grew well in the presence of β-D-galactopyranosyl azide (Fig. 2). TLC studies showed that there was no detectable breakdown of β-D-galactopyranosyl azide during growth of this strain. There was a short delay of growth at early times at 50 m*M* β-D-galactopyranosyl azide, but growth thereafter proceeded rapidly. It is possible that trace amounts of β-D-galactopyranosyl azide had broken down nonenzymatically (in amounts too small to be detected by TLC or GLC) and that the very small amount of azide thus present initially caused the delay. Growth of *E. coli* JM 108 on agar plates that had β-D-galactopyranosyl azide (100 m*M*) spread on the surface was also normal (not shown).

These results show that β-galactopyranosyl azide can be used for the selection of *E. coli* cells that lack functional β-galactosidase. This has potential application in molecular biology as a replacement for blue/white screening. In blue/white screening, an inactive form of β-galactosidase (M15 β-galactosidase) with a deletion of residues 11–41 is important. M15 β-galactosidase is inactive because it is a dimer rather than a tetramer. Addition of a peptide (called α-peptide) that includes residues 3–41 (minimum, longer peptides also work as long as residues 3–41 are included) causes the enzyme to take up a tetrameric structure *(14)* and thereby restores activity (called α-complementation). For blue/white screening, mutant chromosomal DNA codes for the expression of M15 β-galactosidase. If a gene fragment that codes for the α-peptide is present on the plasmid, the α-peptide that is expressed complements the M15 β-galactosidase. 5-Bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) that is spread on the surface of the plate is hydrolyzed and the colonies turn blue. The plasmid also includes a "multicloning region" at the beginning of the α-peptide gene fragment. This region includes numerous unique restriction sites. DNA from external sources can be inserted into the multicloning region if the external DNA was cleaved using the same restriction enzyme(s) used to cleave within the multicloning region. The insertion disrupts the expression of the α-peptide. M15 β-galactosidase is not complemented and

is therefore inactive. X-Gal that is on the plate is not hydrolyzed, and as a result, the colonies are white, rather than blue. There are usually many blue colonies on plates with a few white colonies, and the white colonies can be hard to find. In addition, one has to be careful when removing the colonies to prevent contamination from blue colonies. Growth on plates with β-D-galactopyranosyl azide would allow only the cells with DNA inserts to grow because those cells would not have active β-galactosidase. This has potential as a reagent in molecular biology for blue/white screening.

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