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Short Communication

Synthesis of novel galactose containing chemicals by β-galactosidase from *Enterobacter cloacae* B5

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ABSTRACT

The β-galactosidase from *Enterobacter cloacae* B5 was employed to synthesize novel galactose containing chemicals (GCCs) using mannitol, sorbose, and salicin as acceptors in the presence of *o*-nitrophenyl-β-D-galactopyranoside (*o*NPGal) as donor. The influences of the process parameters on GCC synthesis using mannitol as an acceptor, including effects of variations in initial substrate concentration, reaction time, and temperature, were studied in detail. The mannitol derivative reached a yield of 14.6% when the enzyme was used in the presence of 30 mM *o*NPGal and 60 mM mannitol at 50 °C for 10 min. The sorbose and salicin derivatives reached yields of 19.4% and 25.2%, respectively, under the same conditions except for acceptor concentrations. Through analysis of ESI-MS and NMR spectroscopy, the three derivatives were identified to be β-D-galactopyranosyl-(1 → 1′)-D-mannitol, β-D-galactopyranosyl-(1 → 1′)-L-sorbose, and 2-(hydroxymethyl) phenyl β-D-galactopyranosyl-(1 → 6′)-β-D-glucopyranoside.

1. Introduction

The synthesis of oligosaccharides is currently receiving worldwide attention because oligosaccharides play important roles in many biological processes, including cell–cell recognition, communication, and viral infections. The classic chemical approach to such synthesis is usually laborious and prohibitively expensive for industrial carbohydrate manufacturers because it requires complicated protection and de-protection steps due to the presence of multiple hydroxyl groups with similar reactivity (Sears and Wong, 2001; Daines et al., 2004). The process also often leaves chemical residues that are subject to health and safety concerns (Jia and Wang, 2007).

As an alternative, biocatalytic approaches employing stereo- and regio-selective enzymes, such as glycosyltransferases (EC 2.4) and glycosidases (EC 3.2.1), have been found to be powerful strategies that can complement existing chemical methods (Palcic, 1999; Murata and Usui, 2006). Enzymatic synthesis using glycosyltransferases, the biocatalysts responsible for the construction of oligosaccharides *in vivo*, is efficient, but the necessary enzymes and sugar nucleotides are too costly to be practical for use in large-scale synthesis. On the other hand, glycosidases are proving to be good catalysts for oligosaccharide synthesis, as these enzymes are readily available, inexpensive, highly stable, and easy to handle. They can also accommodate much simpler substrates and require no cofactors (Scigelova et al., 1999; Bojarová and Kren, 2009).

β-Galactosidases are among the most promising glycosidases. They are useful in synthesizing prebiotic galacto-oligosaccharides (Cruz et al., 1999; Li et al., 2008, 2009b), as well as galactose containing chemicals (GCCs), which include diverse oligosaccharides, alkyl-glycoside, glycoconjugates, and others that play important roles in the industries of food additives, cosmetics, and medicines.

Using β-galactosidases from various sources, Yoon and Ajisaka (1996) synthesized Galβ(1 → 3)GalNAc, the representative unit of mucin-type sugar chains, as well as Galβ(1 → 3)GlcNAc and Galβ(1 → 4)GlcNAc, the components of blood group determinants of the ABO system. Murata et al.'s (2001) group utilized *Bacillus circulars* β-galactosidase for the synthesis of 6′-sulfo-*N*-acetyllactosamine and its isomer, both of which have been shown to play various roles in biological events such as selectin binding, laminin binding, neural cell migration, bacteria binding, and the activation of macrophages. Jia and Wang (2007) applied *Aspergillus oryzae* β-galactosidase in the preparation of β-D-galactosyl-L-lactic acid ethyl ester, which was subsequently polymerized to form a novel and promising biomaterial, poly (β-D-galactoside-co-L-lactic acid). At present, it is worth noting that the synthetic capabilities of β-galactosidases are continuously expanding. Recent reports show that more acceptors such as sorbitol and trehalose have also been found to be glycosylated by these enzymes to form novel chemicals (Robert, 2007; Kim et al., 2007).

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In our previous work, a novel β-galactosidase from *Enterobacter cloacae* B5 was purified and found to have the potential to transfer glycosyl to various acceptors, including hexose, pentose, disaccharide, hexahydroxy alcohol, cyclitol, and aromatic glycosides (Lu et al., 2009). In this work, transglycosylations using three novel acceptors which represent alcohol, sugar, and aromatic glycoside in the chemical structures were investigated in detail.

2. Methods

2.1. β-Galactosidase preparation

The β-galactosidase from *E. cloacae* B5 has been successfully expressed in *Escherichia coli* BL21 (DE3) using the vector pET-15b (Lu et al., 2009). The enzyme used in this work was prepared from recombinant *E. coli*.

2.2. Enzyme assays

β-Galactosidase activity was measured by adding 50 μl of the enzyme solution to 450 μl of 2 mM *o*-nitrophenyl-β-D-galactopyranoside (*o*NPGal) in 50 mM phosphate buffer (pH 7.0). The reaction was performed at 35 °C for 10 min and then stopped by adding 1 ml of 500 mM sodium carbonate (Na_2CO_3). The amount of *o*-nitrophenol released was measured at 400 nm. One unit of enzyme activity (U) was defined as the amount of enzyme required to liberate 1 μmol of *o*-nitrophenol per minute under the assay conditions.

2.3. Synthesis of GCCs by β-galactosidase

The synthesis of GCCs was performed by incubating *E. cloacae* B5 β-galactosidase with three different acceptors using *o*NPGal as the glycosyl donor. The process was performed according to the recommended time and temperature, and was stopped by heating at 100 °C for 10 min. The products obtained from D-mannitol, L-sorbose, and salicin were designated as GCC-I, GCC-II, and GCC-III, respectively.

The influence of variations in the process parameters on GCC-I synthesis were investigated in detail using 40 U of enzyme in a 300 μl reaction mixture (pH 7.0). The effects of variations in initial *o*NPGal concentrations were determined by incubating the enzyme with 80 mM mannitol at 50 °C for 8 h. Ten *o*NPGal concentrations were used (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 mM). The effects of variations in reaction time were determined using a solution of 30 mM *o*NPGal and 80 mM mannitol at 50 °C. Aliquots were serially analyzed after reacting for 5, 10, 30, 60, and 120 min. To study the effects of variations in temperature, reactions were performed at 35, 40, 45, 50, 55, and 60 °C, with samples periodically analyzed within 180 min. The effects of variations in acceptor concentrations were determined in the presence of 30 mM *o*NPGal at 50 °C for 10 min, with mannitol concentrations at 10, 20, 40, 60, 80, 100, 150, 200, 300, and 400 mM.

The reaction conditions for GCC-II and GCC-III synthesis followed the optimized conditions for GCC-I, except for the acceptor concentration. The ideal acceptor concentration was determined by incubating the enzyme with sorbose or salicin at concentrations from 10 to 400 mM in the presence of 30 mM *o*NPGal at 50 °C for 10 min.

Larger scale synthesis of GCC amounting to 10 ml was performed under optimal conditions. The results reported for all the experiments are the mean of triplicate experiments.

2.4. HPLC analysis

The GCC synthesis reactions described above were analyzed by high performance liquid chromatography (HPLC) via the Agilent 1200 Series Rapid Resolution LC System under the following conditions: detector = 1200 refractive index detector; software = Agilent Chemstation B.04.01; column = ZORBAX carbohydrate analysis column (4.6 × 250 mm); solvent = 70% acetonitrile; flow rate = 1 ml/min; and column temperature = 30 °C.

2.5. Isolation of GCC-I, GCC-II and GCC-III

Reaction products in a 10 ml mixture were concentrated, applied to a Bio-Gel P2 (Bio-Rad, US) column (1.6 × 100 cm), and then eluted with water. Collected fractions were subjected to sugar determination by thin layer chromatography using butanol–ethanol–water (5:3:2, v/v/v) as the mobile phase. Detection was achieved by spraying with 0.5% (w/v) 3,5-dihydroxytoluene in 20% (v/v) sulfuric acid and heating for 5 min at 120 °C. Identical purified fractions were combined and concentrated to dry powder.

2.6. MS and NMR analysis

Mass spectra were recorded with an API4000 TQ Mass Spectrometer (Applied Biosystems, American) using the ESI technique to introduce the sample. ^1H and ^{13}C NMR spectra were recorded at 25 °C on a Bruker DRX Avance-400 spectrometer (Switzerland) at 400 MHz for ^1H and 100 MHz for ^{13}C. Chemical shifts in parts per million (ppm) were reported relative to the internal standard 2,2-dimethyl-2-silapentane-5-sulfonate. Chemical shifts and coupling constants were obtained from a first order spectra analysis. Assignments were fully supported by homo- and hetero-nuclear correlated 2D techniques, including COSY (Correlation Spectroscopy), HSQC (Hetero-nuclear Single Quantum Coherence), and HMBC (Hetero-nuclear Multiple Band Correlation) experiments, using standard Bruker pulse programs.

2.7. Spectrometric identification of isolated GCC

β-D-Galactopyranosyl-(1 → 1′)-D-mannitol (GCC-I). ESI-MS: $[M + Na]^+$ 367.3. ^1H NMR (400 MHz, D_2O): δ 4.38 (1H, d, $J_{1,2}$ = 7.68 Hz, H-1), 4.12 (1H, H-$1'_a$), 3.86 (1H, H-4), 3.80 (1H, H-$6'_a$), 3.78 (1H, H-$1'_b$), 3.72 (1H, H-6_a), 3.68 (1H, H-6_b), 3.65 (1H, H-5), 3.61 (2H, H-3, H-$6'_b$), 3.51 (1H, H-2). ^{13}C NMR (100 MHz, D_2O): δ 103.44 (C-1), 75.13 (C-5), 72.61 (C-3), 71.76 (C-1′), 70.88 (C-2), 68.99–70.80 (4C, C-2′, C-3′, C-4′, C-5′), 68.64 (C-4), 63.18 (C-6′), 60.99 (C-6).

β-D-Galactopyranosyl-(1 → 1′)-L-sorbose (GCC-II). ESI-MS: $[M + Na]^+$ 365.4. ^1H NMR (400 MHz, D_2O): δ 4.31 (1H, d, $J_{1,2}$ = 7.80 Hz, H-1), 3.82 (2H, H-4, H-$1'_a$), 3.71 (1H, H-$1'_b$), 3.68 (1H, H-6_a), 3.62 (1H, H-6_b), 3.61 (1H, H-5), 3.57 (1H, H-3), 3.48 (1H, H-2). ^{13}C NMR (100 MHz, D_2O): δ 103.44 (C-1), 97.38 (C-2′), 75.25 (C-5), 73.65 (C-3), 71.76 (C-1′), 71.13 (C-2), 69.52 (C-4), 61.16 (C-6).

2-(hydroxymethyl) Phenyl β-D-galactopyranosyl-(1 → 6′)-β-D-glucopyranoside (GCC-III). ESI-MS: $[M + Na]^+$ 471.4. ^1H NMR (400 MHz, D_2O): 7.31 (1H, H-5″), 7.28 (1H, H-3″), δ 7.16 (1H, H-2″), 7.06 (1H, H-4″), 5.07 (1H, d, $J_{1',2'}$ = 7.24 Hz, H-1′), 4.61(1H, H-7″), 4.30 (1H, d, $J_{1,2}$ = 7.52 Hz, H-1), 4.10 (1H, H-$6'_a$), 3.81(1H, H-$6'_b$), 3.77 (1H, H-4), 3.71 (H-5′), 3.63 (1H, H-6_a), 3.57 (1H, H-6_b), 3.53 (H-2′), 3.52 (H-3′), 3.49 (H-4′), 3.45 (1H, H-3), 3.43 (1H, H-5), 3.41 (1H, H-2). ^{13}C NMR (100 MHz, D_2O): δ 154.46 (C-1″), 129.75 (C-5″), 129.69 (C-3″), 129.46 (C-6″), 123.37 (C-4″), 115.33 (C-2″), 103.11 (C-1), 100.28 (C-1′), 75.50 (C-3′), 75.39 (C-5′), 75.11 (C-5), 72.92 (C-2′), 72.65 (C-3), 70.75 (C-2), 69.24 (C-4′), 68.64 (C-4), 68.11 (C-6′), 60.95 (C-6), 59.25 (C-7″).