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Antarctic, cold-adapted β-galactosidase of *Pseudoalteromonas* sp. 22b as an effective tool for alkyl galactopyranosides synthesis

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ABSTRACT

The antarctic *Pseudoalteromonas* sp. 22b β-galactosidase was found to catalyze synthesis of alkyl galactopyranosides. The number of carbon atoms in C3–C6 alcohol molecules only slightly affected the yield of products. Reactions of transgalactosylation were conducted for 80 h and the maximum accumulation of their products was observed between 40 and 50 h. The highest and almost the same yields of alkyl galactosides were achieved at pH 6–9 and 10–30% water concentration. Like known mesophilic β-galactosidases, the antarctic enzyme more efficiently synthesized alkyl galactosides when reactions were carried out in mixtures of buffers and organic solvents (below 50%, v/v).

1. Introduction

The extraordinarily high regio- and enantioselectivity of enzymes make them excellent tools for synthesis of chiral building blocks, which are indispensable for manufacturing of pharmaceuticals and other useful products [1]. Many chiral compounds have been produced by chemical synthesis but usually the latter processes are tedious and have to be carried out under harsh conditions. Good examples of such compounds are alkyl glycosides [2–4]. Chemical synthesis of their pure anomers by Könnigs–Knorr method requires additional reactions of protection and further deprotection of functional groups. In nature, the glycosylated derivatives of alcohols, phenols and mercaptans are synthesized by highly specific glycosidases [5]. They occur, among others, in plant tissues but their concentration in plant materials is too low to make the extraction economically profitable.

Alkyl glycosides can be used as biosurfactants in manufacturing of cosmetics and household chemicals (e.g. washing powders) and as building blocks in pharmaceutical industry and molecular biology [2,5–8,10]. They are nonionic, bacteriocidal and easily biodegradable compounds [2]. Besides, they are more stable at basic pH than fatty acid sugar esters.

β-Galactosidase [EC 3.2.1.23] is one of alkyl galactopyranosides-producing enzymes. Its application for their synthesis instead of glycosyltransferases is more reasonable since the latter biocatalysts require supplementing with cofactors [6]. The principal substrate used for β-galactosidase-catalyzed synthesis of alkyl galactopyranosides is the cheap and easily available lactose, which is a by-product of milk processing. Apart from lactose also the modified lactose or galactose can be used for synthesis of alkyl galactopyranosides, but these substrates are relatively expensive [7].

β-Galactosidase is able to catalyze the hydrolysis of lactose into its component sugars, glucose and galactose. Two different mechanisms of this reaction have been postulated. According to Espinosa et al. [8] glucose and galactose are products of hydrolysis of allolactose, which is formed as an intermediate of lactose conversion. This is contradictory to the mechanism proposed by Juers et al. [9] who assume that lactose is directly hydrolyzed by β-galactosidase to glucose and galactose.

Modification of reaction conditions enables replacement of water, which participates in hydrolysis, with sugar or alcohol [2]. The low water content in reaction mixture favors synthesis of glycosidic bonds, which undergo hydrolysis when water concentration exceeds certain level.

Biosynthesis of alkyl glycosides is carried out by two different methods such as reversible hydrolysis, in which reaction equilibrium is kinetically controlled and the presence of water shifts the equilibrium in favor of hydrolysis, and transglycosylation. The latter

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reaction is also kinetically controlled and accumulation of reaction product can be significantly higher than in the former case if the substrate is more "attractive" for the enzyme than the product [5,7].

Not only monohydroxy alcohols but also prochiral diols [11], monoterpene alcohols (geraniol, cytronellol) [12], amino acids (Ser, Thr) [13,14], antibiotics (chloramphenicol) [15] and ergot alkaloids used in pharmacy [16,17] were applied as substrates for synthesis of sugar derivatives but in the majority of cases the yields of reactions were very low or the reactions did not occur at all.

We have applied a cold-adapted β-galactosidase produced by an antarctic marine bacterium *Pseudoalteromonas* sp. 22b for synthesis of alkyl galactopyranosides. Purification and characterization of this enzyme was described elsewhere [18,19,28].

2. Materials and methods

The studies were carried out by using the cold-adapted β-galactosidase synthesized by a gram-negative marine bacterium *Pseudoalteromonas* sp. 22b, isolated from alimentary tract of the Antarctic crustacean *Thysanoessa macrura*, populating shelf waters of Admiralty Bay (King George Island, South Shetlands, 62°10′S, 58°28′W). The enzyme was purified and characterized as described elsewhere [18,19,28].

2.1. Cultures and β-galactosidase isolation

The bacterium *Pseudoalteromonas* sp. 22b was maintained on agar slants containing 2.3% Bacto Nutrient Agar (Difco), 3.5% sea salt (Tropic Marin) and 0.5% (w/v) agar, and activated through inoculating fresh agar slants, which were further incubated for 5 days at 4 °C. Portions (4 dm^3) of liquid culture medium containing 1% lactose (Difco), 0.1% yeast extract (Difco), 0.2% bactopeptone (BD) and 3.5% sea salt (Tropic Marin) were inoculated with cells washed from 7 agar slants with a small portion of this liquid culture medium. Agitated cultures were incubated for 192 h at 6 °C and 130 rpm in Erlenmayer flasks (loading rate of 30%) using Unitron A 207 reciprocal shaker (Infos, HT, Switzerland). On completion of the culture, the cells were pelleted by centrifuging (40 min. 5000 × *g*, 4 °C) and the supernatant was discarded. The cells were suspended in 0.1 M sodium phosphate buffer pH 8.0 and the enzyme was extracted for 24 h at 4 °C with the same buffer (1:1, v/v) supplemented with 0.5% sodium cholate (Sigma), 1 mM PMSF (Sigma) and 2 mM EDTA (POCh). The solids remained after extraction were harvested by centrifuging (15 min, 3500 × *g*, 4 °C) and discarded and the supernatant was used as a crude preparation of β-galactosidase.

2.2. β-Galactosidase assay

β-Galactosidase activity in hydrolysis of ONPG (2-nitrophenyl-β-D-galactopyranoside; 10 mM) was determined at 30 °C and pH 8.0 [18]. One unit of enzyme activity (1 U) represented 1 μmol of product (2-nitrophenol) released from the respective substrate in 1 min under standard reaction conditions. Specific activity of soluble enzyme was expressed in U mg^{-1} of protein.

2.3. Alkyl galactopyranoside synthesis

All the chemicals used in experiments were analytical grade. Prior to reactions, the liquid alcohols and organic solvents were dried over the molecular sieve 4 Å and the solids were dried for 48 h in a desiccator using calcium chloride. Enzymatic reactions were conducted at 30 °C at 200 rpm (a reciprocal shaker). The standard time of reaction was 48 h and reaction mixtures contained the crude enzyme preparation with activity of 3.5 U ml^{-1}. The blanks contained thermally denatured enzyme.

2.3.1. Determination of β-galactosidase selectivity for alcohols

The water-immiscible alcohols were saturated with 0.1 M sodium phosphate buffer pH 8.0, and water-miscible alcohols (2-propanol and 2-butanol) were mixed with this buffer at alcohol:buffer ratio of 10:6 (v/v). Reaction mixtures contained 0.7 U of enzyme activity (0.2 ml of crude preparation), 1 ml of alcohol and 0.2 g lactose. Molar concentration of alcohol was 1.5-fold higher than that of lactose.

2.3.2. Identification of sugar moiety contained in alcohol derivatives

Reactions were carried out as described in Section 3.2 using 1-hexanol as a model alcohol and lactose, glucose and galactose (separately, 0.1 g each) as glycosyl donors.

2.3.3. Effect of water content in reaction mixture on the yield of alkyl galactopyranoside synthesis

Water content in reaction mixtures ranged from 5 to 50% (it was contained in a solution of the crude enzyme preparation in 0.1 M sodium phosphate buffer, pH 8.0). Each reaction mixture contained 50 mg lactose, an excess of alcohol (molar concentration of alcohol was 1.5-fold higher than that of lactose) and 0.08 U of enzyme activity. Reactions were conducted for 48 h and terminated by thermal denaturation of enzyme. In the next step portions of water were added to reaction mixtures (to achieve the same total volume) and their components were determined quantitatively by TLC. Alcohols used for syntheses were as follows: 2-propanol, 1-butanol, t-butanol, 1-pentanol, 2-pentanol, 1-hexanol, cyclohexanol, 1-octanol, 1-nonanol, phenol, benzyl alcohol, 1-phenylethanol, 2-ethyl-1-hexanol and 2-ethoxyethanol.

2.3.4. Effect of molar ratio alcohol: lactose on the yield of alkyl galactopyranoside synthesis

Molar ratios hexanol:lactose in reaction mixtures were as follows 10:1, 5:1, 2:1 1:1 and 1:2. Water content in reaction mixtures (contained in the solution of crude enzyme preparation with total activity of 0.8 U in 0.05 M sodium phosphate buffer, pH 8.0) was 30%. Reactions were carried out for 48 h and products were quantitatively determined by TLC.

2.3.5. Effect of pH on the yield of hexyl galactopyranoside synthesis

Enzymatic extract was diluted 10-fold in Britton–Robinson buffer solutions with pH ranging from 4 to 12 and pH of each solution was checked (using pH-meter) prior to synthesis reactions. The reference sample contained enzymatic extract diluted 10-fold with sodium phosphate buffer pH 8.0. Aliquots of enzymatic extract (0.86 ml, 0.3 U) were supplemented with 1.64 g of hexanol (dried over molecular sieve 4 Å) and 2.7 g of lactose. Reactions of synthesis were carried out with shaking at 30 °C for 24 h and their products were determined by TLC and HPLC.

2.3.6. Kinetics of alkyl galactopyranoside synthesis

Concentration of alcohol was 2-fold higher than that of lactose. The activity of enzymatic extract was 2.4 U/ml. Reaction progress was followed by HPLC (samples of reaction mixtures were withdrawn at intervals).

2.3.7. Effect of organic solvents on activity of antarctic β-galactosidase

The crude enzymatic extract was mixed with organic solvents (10, 20 and 50%, v/v) and incubated for 24 h at 4 °C. The residual activity in ONPG hydrolysis was assayed as described in Section 2.2.

2.3.8. Effect of organic solvents on the yield of hexyl galactopyranoside synthesis

Reaction mixtures contained 100 mg lactose, the crude enzymatic extract (1 U) diluted in 0.1 M sodium phosphate buffer pH 8.0 (total volume of 300 μl) and (a) 100 μl of organic solvent and 600 μl of hexanol when the concentration of organic solvent was 10%, or (b) 200 μl of organic solvent and 500 μl of hexanol when the concentration of organic solvent was 20%. The reaction mixtures were incubated for 48 h and analyzed by HPLC. The reference samples contained 10 and 20% 0.1 M sodium phosphate buffer pH 8.0 instead of the organic solvent, respectively.

2.3.9. Preparative synthesis and crystallization of alkyl galactopyranosides

The volume of alcohol was 100 ml. Amounts of lactose and crude enzymatic extract were proportionally increased to maintain the water content of 30% and alcohol:lactose molar ratio of 5:1.

Products of 48 h reactions were purified according to Das–Bradoo [2]. Water was evaporated under reduced pressure and 3 volumes of ethyl acetate were added. The mixture was filtered to discard precipitated lactose and reaction products were crystallized at −20 °C. The crystals were harvested, re-dissolved in ethyl acetate and re-crystallized. The latter crystals were dried and analyzed by NMR and LC–MS. They were further used as standards for HPLC and TLC.

2.3.10. HPLC conditions

Reaction mixtures were dissolved in deionized water, filtered through Millipore membranes (10 kDa) for 20 min at 10,000 rpm and separated by liquid chromatography using Beckman Gold System, equipped with HPX 87H BioRad column. The adsorbed compounds were eluted with 0.005 M H_2SO_4 (flow rate of 0.5 ml/min) and detected using the refractometric detector (RI). Alkyl galactopyranosides, which were earlier crystallized (structure confirmed by NMR H and C spectroscopy) were used as standards for qualitative and quantitative determination of individual compounds.

2.3.11. TLC conditions

Aliquots (1 or 2 μl) of reaction mixtures were applied on TLC 60 Å plates (Merck), resolved in a system A butanol/acetone/water (4:1:1), dried, resolved in a system B isopropanol/ethyl acetate/water (2:2:1), dried again and stained with 0.5% α-naphtol in methanol supplemented with 5% H_2SO_4. When reaction mixtures were too dense to apply them directly on TLC plates, they were dissolved in deionized water prior to TLC.

2.3.12. LC–MS analysis

The samples were analyzed by +/− electro-spray technique using Waters 2695 apparatus equipped with C18 column (50 mm, 3.5 μm mesh).