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Immobilisation of β-D-galactosidase from *Escherichia coli* on cellulose beads and its use for the synthesis of disaccharide derivatives

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ABSTRACT

β-D-Galactosidase, isolated from cloned *E. coli*, was immobilised on cellulose beads *via* oxidation with sodium periodate, activation by cyanuric chloride, or diazotisation. β-D-Galactosidase immobilised *via* azo bonds showed the highest relative activity and thermostability, and was used for synthesis of disaccharide methyl glycosides.

INTRODUCTION

There is a growing interest in the carbohydrate components of glycoconjugates[1,2]. Chemical syntheses of the glycosidic parts of glycoconjugates are complex and require selective protection of the hydroxyl groups[3]. Therefore, biotechnological approaches that use enzymes to synthesise oligosaccharides are valuable[4–7]. Enzymes which are useful for this purpose are glycosidases and glycosyltransferases[7]. The former are available commercially and one of the most useful is β-D-galactosidase isolated from *E. coli*, which has been used[6–10] for the semi-preparative synthesis of disaccharides. However, the use of immobilised β-D-galactosidase appears not to have been described in detail.

We now report on the activity and thermostability of β-D-galactosidase from *E. coli* (genetically manipulated to overproduce the enzyme; 20% of the total protein) immobilised on cellulose beads.

EXPERIMENTAL

β-D-Galactosidase (19.4 U/mg; 1 unit catalyses the hydrolysis of 1 μmol of 4-nitrophenyl β-D-galactopyranoside per min at 30° and pH 7.0), isolated from cloned bacterial strain *E. coli*, was obtained from the Institute of Biochemistry and Biotechnology, Commenius University, Bratislava. Beaded cellulose (Spolchemie, Ústí nad

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Labem, Czechoslovakia) was used in unmodified (OSTSORB) and modified (4-aminophenylsulfonyl) forms (OSTSORB AV).

Immobilisation of the β-D-galactosidase. — (*a*) OSTSORB AV (5 g) was added to 0.1M HCl (10 mL), cold (+4°) M sodium nitrite (1 mL) was added, and the mixture was stirred for 4 min. The carrier was then washed with ice-cold 0.1M sodium carbonate (pH 9.0), and a suspension in a solution of β-D-galactosidase (1 mg/mL) in the same buffer (8 mL) was shaken at ambient temperature for 2 h. The carrier was then washed with M sodium chloride and 0.1M phosphate buffer (pH 7.0) and stored at +4°.

(*b*) Cellulose beads (5 g) were washed with distilled water and then suspended in 2M sodium hydroxide (10 mL). After 30 min, the carrier was collected, and a suspension in 0.05M cyanuric chloride in acetone was stirred for 30 s, then washed with aqueous 20% acetic acid, acetone, and water. The activated carrier was then suspended in a solution (8 mL) of β-D-galactosidase (1 mg/mL) as in (*a*).

(*c*) Cellulose beads (5 g) were washed with distilled water and a suspension in 0.05M sodium periodate (10 mL) was shaken for 30 min. The beads were collected, washed with distilled water, and added to a solution of β-D-galactosidase as in (*a*). After incubation for 20 min, sodium borohydride (5 mg) was added, and, after 20 min, more sodium borohydride (5 mg) was added. The mixture was stirred for another 20 min and then processed as in (*a*).

Determination of β-D-galactosidase activity. — The method of Kuby and Lardy[11] was used. 4-Nitrophenyl β-D-galactopyranoside (0.2 mL, 20mM) was mixed with 0.1M phosphate buffer (0.9 mL, pH 7.0), and a solution of β-D-galactosidase (2 μL) or immobilized enzyme (10 mg) was added. After incubation for an appropriate time at 30°, the reaction was stopped by the addition of aqueous 4% sodium carbonate (2 mL), and the absorption of the liberated 4-nitrophenolate was measured at 420 nm.

Determination of the amount of immobilised protein. — Samples of immobilised enzyme were washed with ethanol and acetone, dried at 25° to constant weight, then hydrolysed with 6M hydrochloric acid for 20 h at 105°. The amount of immobilised protein was calculated from the amino acid content of the hydrolysates determined using an amino acid analyser T-339 (Microtechna, Prague).

Oligosaccharide syntheses. — Each mixture contained 4-nitrophenyl β-D-galactopyranoside (0.55 g), methyl α-D-galactopyranoside (1 g), mM $MgCl_2$, 5mM 2-mercaptoethanol, 0.05M sodium phosphate (7 mL, pH 6.8), *N,N*-dimethylformamide (3 mL), and immobilised (5 g, 51 U) or soluble (10 mg, 194 U) β-D-galactosidase, and was stirred at ambient temperature until the acceptor had been consumed. The reaction was then stopped by heating at 75° for 10 min. The products were analysed by t.l.c. on Silufol (Kavalier, Votice) with chloroform–methanol–water (6:5:0.5) and detection by charring with sulfuric acid. The products were isolated by chromatography on a column (2.5 × 100 cm) of Silica Gel L-100/160 (Lachema, Brno), using the above solvent. ^{13}C-N.m.r. spectra of the products were measured at 75° with a Bruker AM-300 (75.46 MHz) spectrometer on solutions in D_2O (internal MeOH, δ 50.15).

RESULTS AND DISCUSSION

β-D-Galactosidase, isolated from genetically manipulated *E. coli*, was immobilised on cellulose beads by azo-bond formation, activation with cyanuric chloride, or periodate oxidation. Bonds between amino groups of the enzyme and the activated carrier are created by the last two methods. The azo bonds involved the tyrosine and histidine residues of the enzyme.

The data in Table I show that the bonding of β-D-galactosidase *via* amino groups led to a greater loss of activity than for the formation of the azo bonds. The highest efficiency of immobilisation was achieved by the first technique. Table I also shows the amount of the immobilised protein and also the efficiency of the β-D-galactosidase immobilisation *via* azo bonds and by other techniques.

TABLE I

Bound protein (determined by amino acid analysis) and the efficiency of the immobilisation of β-D-galactosidase from *E. coli* coupled to the cellulose beads *via* azo bonds (A), cyanuric chloride-activation (B), and periodate oxidation (C)

Immobilisation technique	*Immobilised protein (mg/g)*	*Total activity of the carrier (U/g)*	*Efficiency of immobilisation*[a] *(%)*
A	0.523	10.3	33.1
B	0.402	3.5	11.2
C	0.196	2.8	9.0

[a] Percentage of original β-D-galactosidase activity.

The β-D-galactosidase immobilised *via* amino groups is less active than that immobilised *via* azo bonds (Table I). However, Fig. 1 shows that immobilisation of β-D-galactosidase on cyanuric chloride-activated cellulose increased the thermostability

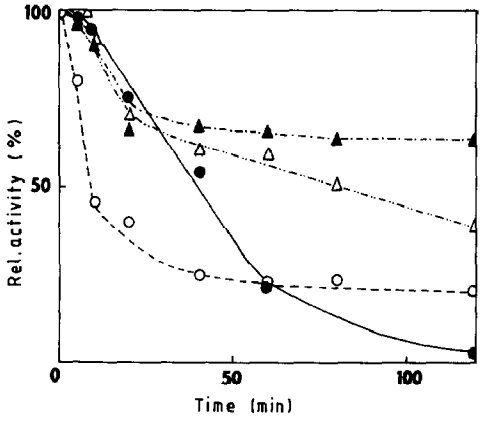

Fig. 1. Thermostability of the native (—) and immobilised β-D-galactosidase on cellulose beads activated by diazotisation (–·–), cyanuric chloride (–··–), and periodate oxidation (– – –) at 40° (2 U/mL in 0.1M phosphate buffer, pH 7.0).

of the enzyme in comparison to that of the enzyme immobilised *via* periodate activation, which may be due to the increased hydrophobicity[12] of the carrier.

β-D-Galactosidase in soluble form and immobilised *via* azo bonds was used in a transglycosylation reaction with methyl α-D-galactopyranoside as the acceptor and 4-nitrophenyl β-D-galactopyranoside as the galactosyl donor. The products were isolated by chromatography and identified by ^{13}C-n.m.r. spectroscopy. The data in Table II indicate that three main products were formed, the relative amounts of which are shown in Table III. Each reaction was allowed to proceed until the galactopyranosyl donor was consumed. Methyl 6-*O*-β-D-galactopyranosyl-α-D-galactopyranoside was the main product with each form of the β-D-galactosidase. Other di- and tri-saccharide derivatives were formed, but were not isolated by the chromatographic method used, and were identified by ^{13}C-n.m.r. spectroscopy and comparison with published data[13]. The results in Table III also show that the method of immobilisation of the enzyme had little influence on the composition of the mixture of products and the yields were comparable with those published by Ajisaka *et al.*[4], where 2-acetamido-2-deoxy-β-D-galactopyranosyl-D-glucoses were synthesised in a reaction catalysed by β-D-galactosidase from *E. coli* immobilised on Eupergite C.

TABLE II

^{13}C-N.m.r. data (δ in p.p.m.) of the products formed from methyl α-D-galactopyranoside and 4-nitrophenyl β-D-galactopyranoside by the action of soluble and immobilised β-D-galactosidase from *E. coli*

Product	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*OMe*
β-Gal-(1→6)-	104.6	72.0	73.9	70.7	76.4	62.3	—
-α-Gal-OMe	100.8	69.4	69.9	70.4	70.7	70.5	56.7
β-Gal-(1→3)-	105.6	72.2	73.9	70.5	76.4	62.2	—
-α-Gal-OMe	100.6	69.9	80.7	68.7	71.4	62.3	56.7
β-Gal-(1→3)-	105.6	72.3	73.8	69.9	76.3	62.2	—
-β-Gal	97.4	70.5	83.7	69.9	76.0	62.2	—

TABLE III

Percentages of the products formed from methyl α-D-galactopyranoside and 4-nitrophenyl β-D-galactopyranoside by the action of soluble and immobilised β-D-galactosidase calculated from the weights of the products isolated by chromatography

Product[a]	*I*	*II*	*III*	*IV*	*V*
Soluble enzyme	67.0	20.0	9.4	1.8	1.6
Immobilised enzyme	71.3	16.9	7.3	2.1	2.1

[a] Key: I, methyl α-D-galactopyranoside; II, D-galactose; III, methyl 6-*O*-β-D-galactopyranosyl-α-D-galactopyranoside; IV, methyl 6- and 3-*O*-β-D-galactopyranosyl-α-D-galactopyranoside, and 6-*O*-β-D-galactopyranosyl-α-D-galactopyranoside; V, mixture of trisaccharide derivatives.

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