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A highly selective synthesis of *N*-acetyllactosamine catalyzed by immobilised β-galactosidase from *Bacillus circulans*

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Abstract

Immobilisation of the β-galactosidase of *Bacillus circulans* on Eupergit C gave a highly selective and stable biocatalyst. The immobilised enzyme catalysed the transfer of the β-galactosyl residue of *p*-nitrophenyl β-D-galactopyranoside to *N*-acetylglucosamine with high selectivity for transfer to the 4-position of the glycosyl acceptor.

Keywords: β-Galactosidase; *Bacillus circulans*; Immobilisation; *N*-acetyllactosamine

1. Introduction

N-Acetyl-D-lactosamine (*N*-acetyllactosamine) is well known as a representative core structure in oligosaccharide components of glycoproteins and glycolipids [1,2]. Organic chemical methods for obtaining it have been developed [3,4], but they are characterized by various elaborate protection and deprotection procedures. On the other hand, enzymatic synthesis using glycosyl transferases has also been reported [5,6]. In this type of enzymatic synthesis, glycosyl transferases are widely used to perform regiospecific galactosylation and sialylation on a preparative scale [7], but these enzymes belong to the Leloir pathway and are difficult to obtain and have limited stability. Moreover, they require expensive cofactors as glycosyl donors. Glycosyl hydrolases (glycosidases) can also be used to synthesise oligosaccharides in a kinetically controlled reaction, where a glycosyl donor is used to transfer its glycosyl residue to a sugar acceptor present in the reaction medium [8–10]. In spite of the increased amount of work carried out with glycosyl hydrolases, their main drawback is a lack of regioselectivity, which limits their use for synthetic purposes. In an important contribution to glycosidase-catalysed oligosaccharide synthesis, Usui et al. [11,12] reported for the first time the use of a β-galactosidase from *Bacillus circulans* to synthesise some β-D-(1 → 4) galactosyl disaccharides bearing a GlcNAc or a GalNAc residue at the reducing end. Some β-(1–6) linkages were produced as well, but to the best of

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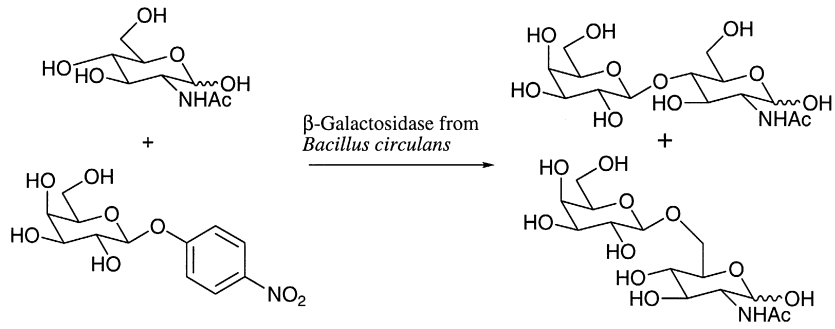


Scheme 1. Chemoenzymatic synthesis of *N*-acetyllactosamine based on the β-galactosidase from *B. circulans*.

our knowledge, it was the first time that a preparative scale β-D-galactosyltransfer has been shown to occur preferentially at the O-4 position using a galactosyl hydrolase. There is considerable synthetic interest in the use of galactosidase to perform this type of galactosyl transfer. Recently, it has been shown that the use of the thioethyl glycoside of *N*-acetylglucosamine as acceptor and *p*-nitrophenyl β-D-galactoside as donor gave complete selectivity for $1 \rightarrow 4$ transfer (Scheme 1) [13].

Immobilisation of the enzyme can offer several advantages such as enhancing operational stability, continuous operation, repeated usage of the derivative and retention of the biocatalyst in the reaction vessel [14].

Because of its synthetic usefulness, we have investigated the immobilisation of β-galactosidase from *B. circulans* on Eupergit C. We found that it can be usefully applied as a recoverable and reusable enzyme for the synthesis of *N*-acetyllactosamine with high regioselectivity.

2. Materials and methods

2.1. Materials

The commercially available β-galactosidase (E.C. 3.2.1.23, from *B. circulans*, Biolacta) was a gift from Daiwa Kasei, Osaka, Japan. Eupergit C was also a gift from Röhm Pharma, Weiterstadt. All other chemicals were obtained from commercial sources.

2.2. Methods

2.2.1. Analytical methods

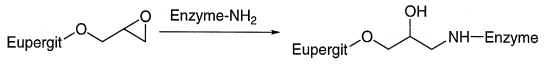
NMR spectra were recorded on Bruker AC-400 or 250 MHz spectrometers. The structure of the enzymatically synthesised disaccharides were assigned by proton–proton shift correlation, carbon–proton shift correlation and DEPT-experiments; $(1 \rightarrow 4)$ and $(1 \rightarrow 6)$ linkages were identified by the marked down-field shift of the C-4 and C-6 resonances. HPLC was performed with a graphitised carbon column (Hypercarb S, 4.6×100 mm^2) on a Gilson liquid chromatograph equipped with a light scattering detector (Sedex 55). Elution was effected with a gradient of H_2O–CH_3CN at a flow rate of 1 ml/min.

TLC was performed on 25-mm E. Merck silica gel plates (60F-254) with detection by spraying the plates with 10% aq. H_2SO_4 in methanol and heating.

The carbon–Celite column for the separation of the enzymatically synthesised disaccharide was prepared by mixing equal parts by weight of activated carbon (Darco G-60, Aldrich) and Celite (Celite 535, Fluka) in water. The mixture was packed into a glass column under pressure.

2.2.2. Immobilisation

Immobilisation of β-galactosidase from *B. circulans* on Eupergit C was carried out in 1 M potassium phosphate buffer of pH = 7.5 at 30°C for 10 h [15] (Scheme 2). The percentage of bound enzyme was determined by the difference



Scheme 2. Preparation of Eupergit C-β-Gal.

between the initial amount of protein in the native enzyme and that remaining in the filtrate (Scheme 2).

2.2.3. Enzyme assay

β-Galactosidase activity was assayed spectrophotometrically as follows: a sample of enzyme solution (0.01 ml) was added to 0.09 ml of 0.05 M McIlvain (citrate-phosphate) buffer (pH = 5.0) containing 5 mM *p*-nitrophenyl β-D-galactoside. The reaction mixture was incubated for 10 min at 30°C. Absorbency was measured at 400 nm upon addition of Na_2CO_3 (0.1 M, 3.9 ml).

One unit of enzyme activity is defined as that quantity of enzyme hydrolysing 1 μmol of *p*-nitrophenyl β-D-galactoside under the conditions stated above. The specific activity is expressed as units per mg of protein.

2.2.4. General methods for enzymatic galactosylation and disaccharide purification

The donor (*p*-nitrophenyl β-D-galactopyranoside, 100 mg, 0.33 M) and the acceptor (2-acetamido-2-deoxy-D-glucopyranose, 550 mg, 2.48 M) in citrate–phosphate buffer (50 mM, pH = 5.0, 1 ml) were incubated with the free and immobilised enzyme (50 mg, 3.1 U mg^{-1} for free enzyme and 62 mg, 2.5 U mg^{-1} for immobilised enzyme) at 30°C for 1 h. The progress of the reactions was monitored by HPLC. After all of the *p*-nitrophenyl β-galactopyranoside had been consumed, the reaction was quenched by heating for 5 min at 100°C in the case of free enzyme, and by filtration in the case of immobilised enzyme. The reaction mixture was then directly loaded onto a carbon–Celite column. The column was first eluted with water (200 ml) and then with a linear gradient of 0% to 15% (v:v) of ethanol.

3. Results and discussion

3.1. Influence of immobilisation of the enzyme on the selectivity of the process

The β-galactosidase from *B. circulans* has been proven to be a valuable biocatalyst for

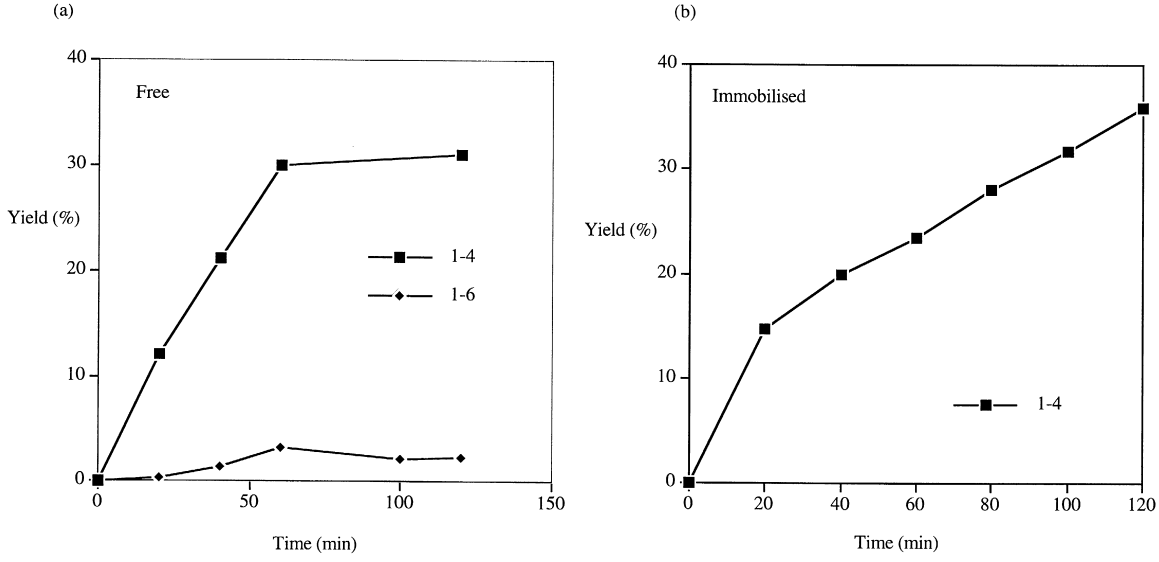
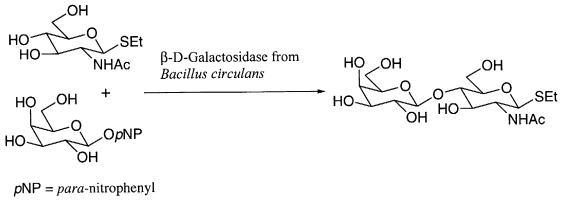


Fig. 1. Time course of the formation of *N*-acetyllactosamine and of its 1 → 6 isomer by the action of the β-galactosidase from *B. circulans* in free (a) and immobilised (b) form. Experimental conditions: *p*-nitrophenyl β-D-galactopyranoside (0.33 M), 2-acetamido-2-deoxy-D-glucopyranose (2.48 M) in sodium acetate buffer (50 mM, pH = 5.0, 1 ml), 30°C, 150 rpm.

galactosyl transfer from suitable donors onto a variety of substrates. With *N*-acetylglucosamine or *N*-acetylglucosamine glycosides as acceptors, β-D-(1 → 4) transfer product predominates giving *N*-acetyllactosamine or *N*-acetyllactosamine glycosides, respectively. Some β-(1–6) galactosyl transfer was also observed [13,16,17].

Commercially available crude β-D-galactosidase, free and immobilised on Eupergit C, was used for the preparation of *N*-acetyllactosamine without purification. The desired compound was readily synthesized on a mmol scale, and conveniently isolated by chromatography on a column of charcoal–Celite. Fig. 1 shows the transglycosylation profile of the reaction of *B. circulans* β-D-galactosidase with *p*-nitrophenyl β-D-galactopyranoside and GlcNAc in acetate buffer (pH = 5.0) (Scheme 3). The amount of *N*-acetyllactosamine and its 1 → 6 isomer produced as a function of time were examined. The samples (30 μl) were taken at intervals during the incubation and were then diluted with 7 vol. of acetonitrile for analysis by HPLC.

A key objective was to compare the regioselectivity of the free and immobilised enzyme, which could be changed by the effect of the immobilisation. The experiments were performed in sodium acetate buffer (50 mM, pH = 5.0) at 30°C. The amounts of *N*-acetyllactosamine and its 1 → 6 isomer were examined as a function of time, and samples were analysed by HPLC. Formation of the 1 → 6 isomer was much slower than formation of the 1 → 4 isomer. With the free enzyme, production of *N*-acetyllactosamine reached a maximum of 30% at 60 min at which point 3% of the 1 → 6 isomer was present. In the case of the immobilised enzyme, the reaction was somewhat slower, but after 120 h, a somewhat higher conversion had been achieved (36% *N*-acetyllactosamine) and the reaction was more selective (none of the 1 → 6 isomer was detected). This suggests that the immobilisation process produced a change in the behaviour of the enzyme.



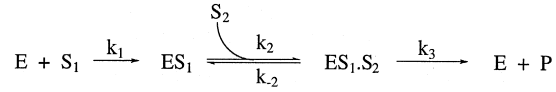
Scheme 3.

Formation of *N*-acetyllactosamine was examined as a function of time. As shown in Fig. 1, the rate of the reaction (as measured by the yield, which is based on the donor) was faster during the first 20 min than afterwards. For this reason, the synthesis of *N*-acetyllactosamine with free and immobilised enzyme was carried out in the presence of added *N*-acetyllactosamine to determine if there was inhibition by the product. In the presence of 0.2 M *N*-acetyllactosamine, no inhibition of the reaction with either free or immobilised enzyme was apparent within experimental error (data not shown).

3.2. Influence of the concentration of donor and acceptor

The influence of the concentration of donor and acceptor were studied, and the experiments were performed with the enzyme supported on Eupergit C. Thus, the supported galactosidase (62 mg, 2.5 U mg^{-1}) was added to a mixture of donor (0.083, 0.166, 0.33 and 0.5 M) and acceptor (0.62, 1.24, 2.48 and 3.73 M), but keeping the same molar ratio. The reaction mixtures were stirred at 30°C in acetate buffer (50 mM, pH = 5.0), after which they were filtered and analysed by HPLC. The amount of *N*-acetyllactosamine formed as a function of time was determined. The samples were taken at intervals of 20 min. The results are shown in Fig. 2, and are presented as % yield vs. time.

For a glycosidase-catalysed reaction, we can write:



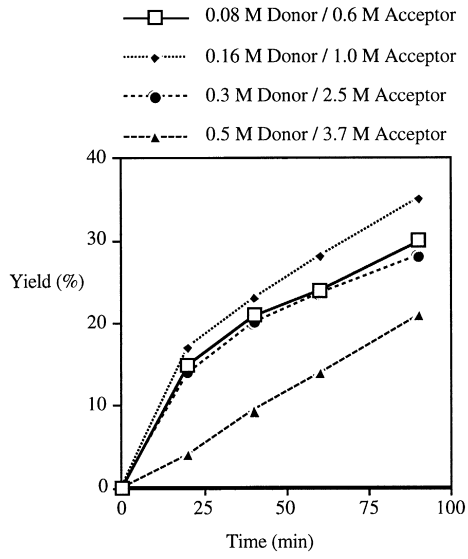


Fig. 2. Time course of *N*-acetyllactosamine formation at different concentrations of donor and acceptor catalysed by the β-galactosidase from *B. circulans* immobilised on Eupergit C. Experimental conditions: sodium acetate buffer (50 mM, pH = 5.0, 1 ml), 30°C, 150 rpm.

where E = enzyme, S_1 = glycosyl donor, S_2 = acceptor, ES_1 = glycosyl–enzyme intermediate, $ES_1 \cdot S_2$ = glycosyl enzyme–acceptor complex.

With a *p*-nitrophenyl glycoside as donor, the first step is fast and essentially irreversible, as indicated. Assuming steady state kinetics, it is readily shown that the velocity, v, of the reaction is given by:

$$v = \frac{k_3[E]_0[S_1][S_2]}{\frac{k_3}{k_1} + \frac{(k_{-2} + k_3)}{k_2}[S_1] + [S_1][S_2]},$$

where $[E]_0$ = total enzyme concentration.

If, as would be expected, $k_3 \ll k_1$, k_2, k_{-2}, then

$$v = \frac{k_3[E]_0[S_2]}{\frac{k_{-2}}{k_2} + [S_2]}.$$

Thus, with a highly activated donor, the velocity of the reaction is independent of the rate of formation of the glycosyl–enzyme intermediate. The reaction shows simple Michaelis–Menten kinetics, the velocity of which can be expressed as:

$$v = \frac{V_{max}}{1 + K_m/([S_0] - [P])},$$

where v = velocity of the reaction, V_{max} = maximum rate, K_m = Michaelis constant, $[S_0]$ = initial substrate concentration, $[P]$ = product concentration. Integration of this equation [18] leads to the following equation:

$$V_{max}t = [P] + K_m \ln\left\{\frac{[S_0]}{([S_0] - [P])}\right\}.$$

If Y is the yield of the reaction (expressed as a fraction) then:

$$Y = \frac{[P]}{[S_0]}.$$

From the above two equations, we obtain the following:

$$V_{max}t = Y[S_0] + K_m \ln\left\{\frac{1}{1 - Y}\right\}.$$

Calculated plots of this equation are shown in Fig. 3. V_{max} and K_m were arbitrarily set at 1, and $[S_0]$ was varied between 0.3 and 10.0, i.e., between $[S_0] = 0.3K_m$ and $10.0K_m$, all in arbi-

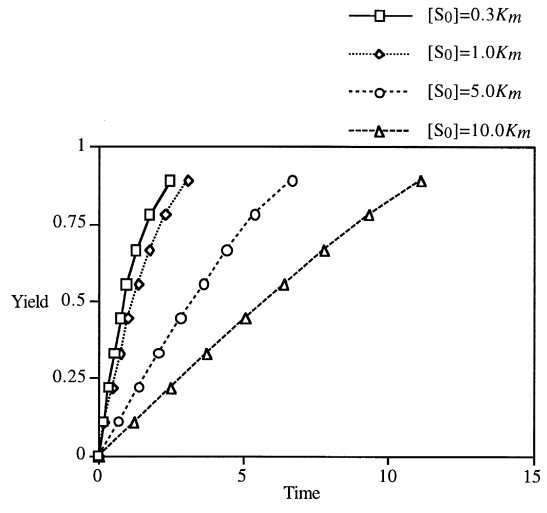


Fig. 3. Theoretical curves showing the relationship between yield and time for a glycosidase-catalysed reaction with an activated donor and for varying initial concentrations of acceptor.

trary units. The figure shows that plots of yield vs. time are very similar as long as $[S_0]$ is equal to or less than K_m, but fall off sharply as $[S_0]$ becomes larger than K_m. The theoretical curves of Fig. 3, and the experimental data shown in Fig. 2 indicate that for highest yields in a given time interval, the initial concentration of acceptor should not exceed the value of K_m. The experimental data of Fig. 2 would suggest that the effective K_m value was $\sim$2.5 M. It is known that maximisation of yields in glycosidase-catalysed reactions requires high concentrations of acceptors. The value of 2.5 M for the apparent K_m probably also reflects diffusional problems when the concentration of substrates in reactions catalysed by immobilised enzymes are increased [19].

3.3. Re-use of the immobilised enzyme

The potential for the re-use of the supported enzyme was investigated. After the first assay, the immobilised biocatalyst was recovered, washed and re-assayed with fresh substrate mixture under the same conditions as in the first experiment, and then the process was repeated three times. The immobilised enzyme retained 90% of its initial activity after the second re-use and had lost only 20% of this original activity after the third re-use. However, the selectivity with respect to *N*-acetyllactosamine formation remained after re-use. Similar stability data have been obtained using the β-galactosidase from *B. circulans* immobilised on Duolite ES-762 for the hydrolysis of lactose [14].

4. Conclusions

All results presented in this work suggest that β-galactosidase from *B. circulans* immobilised on Eupergit C could be usefully applied as a recoverable and reusable enzyme for the synthesis of *N*-acetyllactosamine with high regioselectivity.

Acknowledgements

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