Characterization of the Immobilized β-Galactosidase C from *Bacillus circulans* and the Production of β(1→3)-linked Disaccharides

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Received December 24, 1997

A recombinant β-galactosidase, which was obtained from the β-galactosidase C gene of *Bacillus circulans* and cleaves the non-reducing end galactosyl residue of β(1→3)-linkages selectively, was immobilized using CNBr-Sepharose. Although the effect of pH was not changed by the immobilization, the thermostability and stability in the presence of DMF were increased. Optimization of the transglycosylation using *para*-nitrophenyl β-D-galactopyranoside as a donor and benzyl-α-D-N-acetylgalactosaminide as an acceptor afforded a β(1→3)-linked disaccharide derivative with 62% molar yield in a gram scale synthesis. Using the methyl-analogue as an acceptor, 53% of the acceptor was converted to the respective β(1→3)-disaccharide.

Keywords: β-galactosidase; *Bacillus circulans*; immobilization; transglycosylation; Gal-β(1→3)-GalNAc

Glycosidases, like proteases and lipases, show some advantages in enzymatic syntheses as to be their availability, stability, easy handling (no special technical equipment), and large quantities of inexpensive substrates.1,3) This make glycosidases of interest for large-scale synthesis of oligo- and poly-saccharides. A great number of glycohydrolases including β-galactosidases of different origins have been isolated and their substrate specificity characterized.4,5) Using lactose or nitrophenyl β-galactoside as a donor in the transfer reaction, various galactosylated oligosaccharides were prepared, therefore avoiding laborious protection and deprotection procedures common in chemical carbohydrate synthesis.6,7)

The sequence Gal-β(1→3)-GalNAc, commonly found in mucin-type glycopeptide side chains,8,9) could be obtained with the β-galactosidase from bovine testes and molar yields up to 36% were reported by protecting the product in situ against the hydrolytic activity of the same enzyme.10) However, due to its biological source, this enzyme seems to be of limited availability. Recently we have described a new β-galactosidase from *B. circulans* showing an high specificity for the sequence of Gal-β(1→3)-GalNAc-α-OR.11) Because all β-galactosidases from *B. circulans* were screened by the same nitrophenol-assay, it has shown to be difficult to separate this new β-galactosidase from the major fraction of β-galactosidases I and II.12,13) Ito and Sasaki were able to overcome this drawback by engineering the gene, bgαC, of this new β-galactosidase in *E. coli*.14)

In spite of the advantages noted above, this new β-galactosidase is still lacking stability, especially in the presence of organic cosolvent, and we store it in a freezer until use. We now want to report on the immobilization of this enzyme to increase its stability. Furthermore we focused on the conversion rates of the GalNAc-derivatives, since availability of the enzyme or glycosyl donor do not hamper the oligosaccharide synthesis at all. We will take as our main example the disaccharide formation of the known compound Gal-β(1→3)-GalNAc-α-OBn.

Materials and Methods

General. TLC analysis was done using silica gel plates (Merck, 60 F345) with a solvent system of ethyl acetate/isopropanol/water, 40:50:10. The spots were detected by UV and/or spraying a reagent of 3% *para*-anisaldehyde in ethanol containing 5% sulfuric acid followed by heating. Analytical HPLC of the benzyl-derivatives was done using a LiChrospher RP-18 (4 × 250 mm, 0.8 ml/min, acetonitrile/water, 8:92) and monitored at 215 nm. The methyl analogues were measured on a LiChrospher NH2 (10 × 250 mm, 5.0 ml/min, acetonitrile/water, 84:16).

β-Galactosidase. The enzyme used in this study is a recombinant β-galactosidase C from *B. circulans* expressed in *E. coli* by Ito and Sasaki.14) Its substrate specificity was characterized by Fujimoto et al.15) Most of the assays have been done in a sodium phosphate buffer (pH = 6.0) at 37°C using Gal-β-pNP (Sigma) as the substrate. One unit of enzymatic activity is defined as the amount of enzyme that liberates 1 μmol of *para*-nitrophenol per min at 37°C.

Immobilization on CNBr-Sepharose. CNBr-Sepharose (Pharmacia, 0.10 g) was washed with 40 ml ice-cold 1 mM HCl (the final volume will be 0.35 ml). To the gel was added 1.2 ml of enzyme solution with 1.75 mg of protein (5.0 mg per ml gel) in a 100 mM sodium ci-

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Abbreviations: Gal-β-pNP, *para*-nitrophenyl β-D-galactopyranoside; GalNAc-α-OBn, benzyl α-D-N-acetylglactosaminide; GalNAc-α-OMe, methyl α-D-N-acetylglactosaminide; DMF, N,N-dimethylformamide.
trate buffer (pH=6.1) containing 0.4 M NaCl, and the mixture was incubated at room temperature for 2 h under gentle stirring (head-over-head rotation). The gel was transferred into 1.2 ml of 100 mM Tris-HCl buffer (pH = 8.0) containing 0.4 M NaCl, and incubated for additional 2 h. The gel was washed with 2 cycles of altering pH with 100 mM citrate (pH = 5.0) and 100 mM Tris buffer (pH = 8.0) each containing 0.4 M NaCl. All solutions and washings were kept for measuring of the coupling yield. The immobilized enzyme was stored at +4°C in a 100 mM sodium phosphate buffer (pH = 6.0) containing 0.02% sodium azide as a bacteriostatic agent. 'Coupling yield on protein' was calculated from the difference of 'initial protein amount' and 'protein amount in the washings'. Protein was measured by the Bradford assay using a stock solution from Bio-Rad. The percentage of 'bound enzymatic activity' is given by the ratio of 'activity immobilized enzyme' and 'initial enzymatic activity'.

In the case of the coupling at pH 8, bicarbonate buffer (pH = 8.3) was used instead of the citrate buffer (pH = 6.1). The other parts were as described above.

Effects of temperature, pH, and cosolvent. The effects of pH on the stability were examined by incubating the enzyme at various pHs using a glycine, acetate, or phosphate buffer for 20 h at room temperature. After this incubation, the enzyme activity was measured in a 100 mM sodium phosphate buffer (pH = 6.0) at 37°C for 5 min.

The thermal stability of the enzyme was examined by incubation for 30 min in a 100 mM phosphate buffer (pH = 6.0) at elevated temperature. The enzymatic activity was measured afterwards by the nitrophenol assay at 37°C for 5 min.

The stability against treatment with organic cosolvent was measured for 20% DMF, conditions used also in preparative scale synthesis. Free enzyme was kept at 37°C in phosphate buffer with or without DMF, and from these mixtures a sample was taken at various times to measure enzymatic activity. For the immobilized enzyme, several portions of a specific amount of moist gel was incubated in phosphate buffer containing 20% DMF at 37°C in a shaker. At various times, substrate in phosphate buffer was added, the enzymatic reaction was quenched after 5 min by the addition of 0.2 M NaHCO3, and the mixture was filtered over a hydrophilic membrane (Millipore) with a cut-off of 0.2 μm.

General procedure in the optimization of the transglycosylation reaction. A 100 mM sodium phosphate buffer solution (pH = 6.0, 0.6 ml) containing 10-125 μmol GalNAc-α-Obn or GalNAc-α-OMe, 50 μmol Gal-β-pNP, 20% DMF and 0.28 units of immobilized or free β-galactosidase was incubated at 37°C. After appropriate times, a sample was passed through a membrane filter of molecular cut-off 10 kDa by centrifugation, and treated with Dowex 1 (Cl-') to remove phosphate and para-nitrophenol. The filtrate was analyzed by HPLC.

Preparative-scale synthesis of Gal-β(1→3)-GalNAc-α-OBn. GalNAc-α-OBn (1037 mg, 3.33 mmol), Gal-β-pNP (1506 mg, 5.0 mmol, 1.5 eq) and 20 units of immobilized β-galactosidase (or 28 units of free β-galactosidase) were incubated for 3 h at 37°C in a mixture of sodium phosphate (100 mM, pH = 6.0, 28 ml) and DMF (7.0 ml, 20%). Enzyme was filtered off on a 0.2 μm membrane, phosphate and para-nitrophenol were removed by passing two times over Dowex 1 (Cl−, 1×10 cm, eluent water), and the main part of DMF was evaporated off. The residue was put on an amino-derivatized silica-gel column (4×18 cm, Fuji Silysia) and eluted with a step-wise gradient of 5–20% water in acetonitrile. Fractions were monitored by TLC and/or HPLC. A subsequent polishing step on Sephadex G-10 (2.6×80 cm, 0.5 ml/min) and lyophilization gave 987 mg (62%) Gal-β(1→3)-GalNAc-α-OBn in >99% purity. 1H- and 13C-NMR were consistent with data from the literature.

Preparative-scale synthesis of Gal-β(1→3)-GalNAc-α-OMe. GalNAc-α-OMe (118 mg, 0.5 mmol), Gal-β-pNP (301 mg, 1.0 mmol, 2.0 eq) and 4.0 units of immobilized β-galactosidase (or 5.6 units of free β-galactosidase) were incubated for 3 h at 37°C in a mixture of sodium phosphate (100 mM, pH = 6.0, 4.8 ml) and DMF (1.2 ml, 20%). After filtration through a 0.2-μm membrane, passing over Dowex 1 (Cl−, 10×1 cm, eluent water), and evaporation of DMF, the residue was put on an activated carbon column (2.5×16 cm, 15 g carbon). The column was developed with a gradient of 0–25% ethanol in water and fractions monitored by TLC. A subsequent polishing step on Sephadex G-10 (2.6×80 cm, 0.5 ml/min) and lyophilization gave 106 mg (53%) Gal-β(1→3)-GalNAc-α-OMe in >99% purity. 1H- and 13C-NMR were consistent with data from the literature.

Results and Discussion

Imobilization of β-galactosidase

Although we have tested a variety of commercially available resins and activated supports, we obtained acceptable recovery rates for enzymatic activity only with highly hydrophilic matrices such as hydroxyapatite and Sepharose (data not shown). This is consistent with the observation that the free β-galactosidase is unstable in the presence of organic cosolvent. Immobilization via adsorption was tested with hydroxyapatite (Bio-Rad) and the anion exchanger Q Sepharose (Pharmacia). However, for some unknown reason a dramatic decrease in selectivity occurred, showing a ratio β(1→3):β(1→6) = 1.5–2.0:1 in the synthesis of disaccharides by transglycosylation. So we ended up with immobilization via covalent binding on CNBr-Sepharose (Pharmacia) being the best choice for this enzyme. Using the standard coupling procedure (bicarbonate buffer, pH = 8.3) for CNBr-Sepharose, the coupling yield on protein was always in the range of 80–90%, but bound enzymatic activity was only about 60%. In one experiment we did not stop the coupling reaction in time (more than 2 h) and in this case the loss on enzymatic activity was much larger. So we supposed that the inactivation of the enzyme would be caused for example by multi-point at-
tachment or steric hindrance of the binding site. Therefore we compared coupling at pH=8.3 in a bicarbonate buffer and coupling at pH=6.1 in a citrate buffer. For both incubations, the coupling yield on protein was close to 90%. But the bound enzymatic activity was found to be 64% at pH=8.3 and about 89% at pH=6.1. Based on this results, immobilization on CNBr-Sepharose was performed at a pH of about 6.

Characterization of the CNBr-Sepharose immobilized β-galactosidase

The free enzyme is stable in the range of pH 5 and 9. Its optimum pH for hydrolytic activity is between 5 and 6.15) Optimum pH and pH stability (data not shown) of the immobilized enzyme are very similar to the free enzyme. Figure 1 shows the effects of temperature on the enzymatic activity and stability. Although the free enzyme was stable below 50°C and its optimum temperature was observed at 55°C, the immobilized enzyme was stable below 55°C and the optimum temperature was observed at 65°C. The stability seems to be higher than in the case of the free enzyme15 and the optimum temperature has also been shifted to higher temperatures. The immobilization procedure allows an increase in the thermostability of the enzyme.

This increased thermostability also benefits the stability when stored in a refrigerator. It was noted that the free enzyme is not very stable at +4°C. A 35% loss of enzymatic activity was observed after one month of storage. We therefore freeze it and store it at −80°C until use. The loss induced by freezing was estimated to be in the range of 2–10%. For the immobilized enzyme kept at +4°C in a buffer containing 0.02% sodium azide as a bacteriostatic agent, no loss of enzymatic activity was detectable after 2 months.

Figure 2 shows the effects of 20% DMF on the thermostability of the β-galactosidase. Incubation at 37°C of free and immobilized enzymes in buffer alone caused a loss on enzymatic activity in the range of 2–5% per day. For the free enzyme, the half life time was estimated to be \( t_{1/2} \approx 1 \) h when treated with 20% DMF. The same enzyme immobilized on Sepharose shows an increased stability with \( t_{1/2} \approx 7 \) h. Recovered activity after 3 h incubation was found to be in the range of 80–85%. The immobilization of the enzyme allows its reuse and would be a benefit in other applications like its use in a continuous process or easy separation of enzyme and products.

The β-galactosidase bound to Sepharose shows a nonlinear Lineweaver-Burk plot, as known for immobilized enzymes.19) Only for higher substrate concentrations (here above 1.5 mM) the enzymatic reaction is mainly kinetically controlled and an apparent \( K_m = 0.95 \) mM and \( V_{max} = 7.8 \) μmol/min/mg protein was estimated. For the free enzyme, using the same batch as in the immobilization experiments, we estimated a \( K_m = 0.93 \) mM and a \( V_{max} = 8.2 \) μmol/min/mg protein. The kinetics of the enzymatic reaction, especially for higher substrate concentrations as in the preparative synthesis, seems to be not very much affected by the immobilization. Due to the increased stability in the presence of 20% DMF, a smaller amount of immobilized β-galactosidase C (60–70% of free β-galactosidase) resulting the same yields in preparative synthesis.

Optimization of the transglycosylation reaction

To improve the yield of the β(1→3)-disaccharide by considering the higher prices of α-configured GalNAc-derivatives compared to the availability of the galactosyl-donor and the recombinant β-galactosidase, we have focused on the conversion rates of the GalNAc-acceptor. Figure 3 shows the influence of the molar ratio donor: acceptor on the β(1→3)-linkage formation after 3 h when more than 90% of the Gal-β-pNP is cleaved by the enzyme. It might appear that increasing the ratio of

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**Fig. 1.** Effects of Temperature on the Activity and Stability of the Immobilized β-Galactosidase.

○, activity; ●, stability.

**Fig. 2.** Courses of the Stability of Free and Immobilized β-Galactosidase in the Presence of Cosolvent.

○, 100 mM sodium phosphate buffer alone (pH 6.0); ●, free enzyme in buffer containing 20% DMF; ▲, immobilized enzyme in buffer containing 20% DMF.

**Fig. 3.** Yield of Gal-β(1→3)-GalNAc-α-OBn (%) as a Function of the Molar Ratio of Donor and Acceptor.
donor would increase the yield of \( \beta(1\rightarrow3) \)-disaccharide, but we found that the yield was not improved and numerous unwanted tri- or tetrasaccharides were produced. Also an increasing amount of several oligo-galactosyl derivatives has to be separated afterwards. In the case of GalNAC-\( \alpha \)-OBn used as an acceptor, the \( \beta \)-galactosidase C transfers mainly to the GalNAC-\( \alpha \)-OBn and a molar ratio donor/acceptor = 1.5 gave a relatively uncomplicated mixture of products after enzymatic incubation. For the methyl analogue disaccharide formation, a molar ratio of donor/acceptor = 2.0 has been shown to be sufficient.

Varying the amount of enzyme in relation to the amount of galactosyl donor resulted only in a shift on the time scale but did not influence the maximum yield of \( \beta(1\rightarrow3) \)-product. We have chosen reaction conditions where the maximum yield occurs after 3 h. Figure 4 shows the course of a typical experiment using a molar ratio of Gal-\( \beta \)-pNP/GalNAC-\( \alpha \)-OBn = 1.5. From the initial amount of Gal-\( \beta \)-pNP, 90% was cleaved after 2.5 h, 95% after 3.5 h and after 6 h Gal-\( \beta \)-pNP was no longer detectable. The maximum yield of the desired \( \beta(1\rightarrow3) \)-linked product occurred after 2 h with a yield of 68% (calculated as a percentage of all eluted GalNAC-derivatives). As long as the concentration of Gal-\( \beta \)-pNP was kept high, the ratio of \( \beta(1\rightarrow3)/\beta(1\rightarrow6) \)-linked disaccharides was in the range of 15:1. When the concentration on Gal-\( \beta \)-pNP (initial concentration 167 nm) dropped below 15–20 nm, the reaction resulted in higher production of the unwanted \( \beta(1\rightarrow6) \)-linked disaccharide and in more hydrolysis of the \( \beta(1\rightarrow3) \)-linked disaccharide. However, we have not estimated the kinetic parameters for substrates other than Gal-\( \beta \)-pNP, for example Gal-\( \beta(1\rightarrow3) \)-GalNAC-\( \alpha \)-OBn. After incubation with the immobilized enzyme for 24 h, about 15% of the enzymatic activity remained and the yield of \( \beta(1\rightarrow3) \) disaccharide was 24%. The yield of \( \beta(1\rightarrow6) \)-linked disaccharide increased to 12.1% (compared to 4.2% after 3 h). Therefore we assume that this \( \beta \)-galactosidase shows not only hydrolytic activity toward the \( \beta(1\rightarrow3) \)-linked disaccharide but also transglycosylation activity toward the \( \beta(1\rightarrow6) \)-linked disaccharide. And indeed we found that, when we incubated the purified Gal-\( \beta(1\rightarrow3) \)-GalNAC-\( \alpha \)-OBn with enzyme, Gal-\( \beta(1\rightarrow6) \)-GalNAC-\( \alpha \)-OBn was formed together with monosaccharides and trisaccharides.

**Preparative scale synthesis**

The enzymatic reaction was monitored by analytical HPLC using a reversed phase column (Fig. 5), but the workup for preparative-scale synthesis using the same type of column with a larger bore size seemed to be not very attractive. When incubating a molar ratio of Gal-\( \beta \)-pNP/GalNAC-\( \alpha \)-OBn = 1.5, we obtained a relatively uncomplicated mixture of products and therefore tested a separation on gels like Toyopearl HW-40, Biogel P-2, Sephadex G-10, and G-15. Among these materials, only Sephadex G-10 allows for the separation of disaccharides and monosaccharides and additionally separates the \( \beta(1\rightarrow3) \) and \( \beta(1\rightarrow6) \)-regioisomers. Figure 6 shows a 50-µg scale run on Sephadex G-10 (2.6 × 80 cm, 0.5 ml/min) observed with a UV monitor at 254 nm. The separation worked surprisingly well up to a 50-µg scale and

**Fig. 5.** HPLC Pattern of the Reaction Mixture in the Synthesis of Gal-\( \beta(1\rightarrow3) \)-GalNAC-\( \alpha \)-OBn.

Thirty-three µmol GalNAC-\( \alpha \)-OBn and 50 µmol Gal-\( \beta \)-pNP were incubated in 20% DMF in the Presence of 0.28 Units of the Immobilized \( \beta \)-Galactosidase C. Column, LiChrospher RP-18; Solvent, CH\(_3\)CN/H\(_2\)O = 8:2; Detection, UV\(_{254}\); A, Gal-\( \beta \)-pNP; B, Gal-\( \beta \)-pNP; C, Gal-\( \beta \)-pNP; D, Gal-\( \beta \)-pNP; E, GalNAC-\( \alpha \)-OBn; F, pNP.

**Fig. 6.** Gel Filtration of the Reaction Mixture for the Preparative Synthesis of Gal-\( \beta(1\rightarrow3) \)-GalNAC-\( \alpha \)-OBn.

Reaction mixture (50 mg scale) put on Sephadex G-10 (2.6 × 80 cm, 0.5 ml/min) monitored at 254 nm, and fractions of 5 ml collected: A, tri- and higher saccharides; B, Gal-\( \beta(1\rightarrow6) \)-GalNAC-\( \alpha \)-OBn; C, Gal-\( \beta(1\rightarrow3) \)-GalNAC-\( \alpha \)-OBn; D, GalNAC-\( \alpha \)-OBn and pNP-derivatives.
the product (fractions 51–65) showed about 95% purity in $^1$H-NMR after a single run. However, for the gram-scale synthesis we did not use a 20-fold larger gel column, but first purified it on amino-derivatized silica gel and then used Sephadex G-10 in a subsequent polishing step. For the methyl analogues of the GalNAc-derivatives, a separation by an activated carbon column was successful. The order of elution was at first galactose and oligo-galactosyl-derivatives, then unconverted GalNAc-α-OMe, β(1→3)-linked product, and finally β(1→6)-linked disaccharide together with higher oligosaccharides. The yields in a gram-scale synthesis were 62% using GalNAc-α-OBn as an acceptor and 53% in a 100-mg scale using GalNAc-α-OMe as an acceptor. We believe these isolated yields are very close to the conversion rates obtained with galactosyl-transferases.17,20,21 The reason for this seems to be the relatively low $K_m$ (0.93 mM for Gal-β-pNP) of our enzyme compared to other β-galactosidases and the high selectivity toward Gal-β(1→3)-GalNAc-α-OR formation.

Acknowledgment

This work was done as a part of the Research and Development Projects of Industrial Science and Technology Program supported by NEDO (New Energy and Industrial Technology Development Organization).

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