Immobilization of β-Galactosidase for Application in Organic Chemistry Using a Chelating Peptide

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The strong interaction of hexa-histidine fusion proteins with metal chelate adsorbents was utilized to immobilize β-galactosidase with a hexa-histidine peptide at the N-terminus to the Ni^{2+}-nitrilotriacetic acid adsorbent. The fusion protein was cloned and expressed in *Escherichia coli*. The purified soluble fusion protein showed the same specific activity as the purified β-galactosidase and retained 64 percent of its β-galactosidase activity when bound to the adsorbent. To demonstrate the potential of the immobilized β-galactosidase in organic chemistry, allyl-β-D-galactosidase was synthesized from lactose and allyl alcohol on a gram scale. The same enzyme preparation was reused in three subsequent batches to prepare the model compound with high yield.
Keywords: recombinant β-galactosidase fusion protein • chelating peptide • immobilized metal affinity chromatography • immobilized enzyme

INTRODUCTION

The synthesis and modification of carbohydrates are a difficult problem in classical synthetic chemistry. The application of enzymes in carbohydrate chemistry is attractive because of the high efficiency, substrate specificity, and stereospecificity of the biocatalysts.[20] β-Galactosidase has been used for the preparation of many glucose and galactose derivatives, e.g., allyl-β-D-galactopyranoside, a compound with a lot of utilities in chemistry and biology.[15]

Immobilized enzymes are easily separated from the reaction mixture, e.g., by filtration, and are reusable in subsequent preparations. In addition, immobilized enzymes render continuous production processes possible and may lead to more stable biocatalysts.[1,11] Recently reports of genetically engineered affinity tails to facilitate enzyme immobilization have been published.[13,16,17] Hybrid proteins were prepared by fusing the coding sequence of a protein of interest with the coding sequence for an affinity peptide. Such fusion proteins could be immobilized by taking advantage of the specific binding of the affinity peptide to an affinity adsorbent. Here we describe the production and subsequent immobilization of a β-galactosidase fusion protein comprising six histidine residues at the N-terminus to the Ni^{2+}-nitrilotriacetic acid (NTA) adsorbent. The interaction of the hexa-histidine peptide with immobilized Ni^{2+} ions at neutral or slightly basic pH has been found to be very stable.[6] Equilibrium binding analyses showed that the complex of fusion proteins bearing a hexa-histidine peptide extension with Ni^{2+} ions immobilized on the NTA resin had an apparent dissociation constant (K_d) of 0.7×10^{-6} M.[14]

Glycosidases have been known to catalyze the stereospecific formation of glycosidic bonds by transglycosidation. This specificity was used for the synthesis of allyl-β-D-galactopyranoside from lactose and allyl alcohol with soluble β-galactosidase.[15] To demonstrate the potential of the immobilized β-galactosidase in organic chemistry, the synthesis of allyl-β-D-galactopyranoside on a gram scale was used as a model reaction.

MATERIALS

Plasmid pMC-1871 containing the β-galactosidase gene (*lacZ* gene), restriction enzymes, and the *Escherichia coli* strain MC 1061 were purchased from Pharmacia LKB Biotechnology (Piscataway, NJ). The NTA adsorbent and the pQE-10 expression plasmid type IV were obtained from QIAGEN (Chatsworth, CA). Ampicillin and kanamycin were from Difco (Detroit, MI). Isopropyl-β-D-thiogalactopyranoside (IPTG) was from Senn Chemicals (Dielsdorf, Switzerland). β-Galactosidase from *E. coli* (grade VIII), *o*-nitrophenyl-β-D-galactopyranoside (ONPG), and silica gel (230–400 mesh) were from Sigma (St. Louis, MO). All other chemicals used were reagent quality.

METHODS

General Methods

Slab sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), employing the method of Laemmli,[10] was used to assess purity of the $(His)_6$-β-galactosidase. Samples were electrophoresed in 7.5% polyacrylamide under reducing conditions using a Mini-Protean II Dual Slab Cel (Bio-Rad Laboratories, Richmond, CA). Protein content was determined using the Bio-Rad protein microassay procedure and bovine serum albumin (BSA) as standard (protein assay manual, Bio-Rad Laboratories, Richmond, CA). β-Galactosidase activity

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was determined at 37°C using the synthetic substrate ONPG. The ONPG is hydrolyzed to galactose and *o*-nitrophenol in the presence of β-galactosidase, and the formation of *o*-nitrophenol is followed by its absorption at 410 nm (protocol for β-galactosidase enzyme assay; Sigma, St. Louis, MO). When immobilized enzyme (100-μL aliquots) was assayed, a magnetic stir bar was used to keep the resin particles suspended.

Cloning and Expression of $(His)_6$-β-Galactosidase

To construct the plasmid for expression of the $(His)_6$-β-galactosidase fusion protein, the *lacZ* gene was first excised from the plasmid pMC-1871 by digesting with BamHI. The 3.1-kb fragment corresponding to *lacZ* was isolated by agarose gel electrophoresis. After digesting the pQE-10 expression vector type IV with BamHI, the isolated *lacZ* gene was ligated into the BamHI site using a 3 : 1 ratio of *lacZ* insert to expression vector.

The resulting plasmid p$(His)_6$-*lacZ* (see Fig. 1) was transformed into *E. coli* MC 1061 cells harboring *lac* repressor plasmid pDMI, 1.[5] The *E. coli* transformant was grown at 37°C in Luria-Bertani (LB) medium containing 10 μg/mL ampicillin and 25 μg/mL kanamycin. At an optical density of (OD) 500 nm, induction was begun by adding IPTG to a final concentration of 1 m*M*. The cells were incubated for an additional 3 h before being harvested by centrifugation.

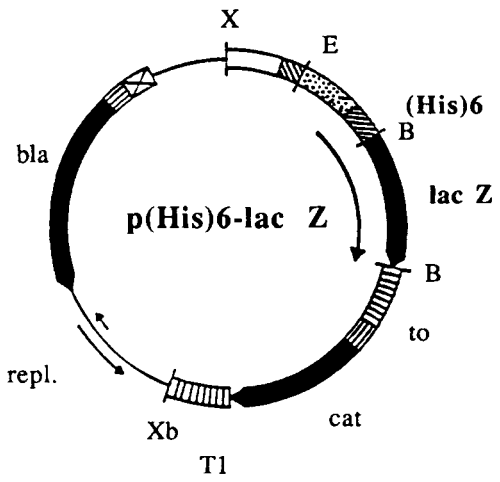


Figure 1. Plasmid p$(His)_6$-*lacZ* used for the expression of $(His)_6$-β-galactosidase. For the construction of plasmid p$(His)_6$-*lacZ*, the *lacZ* gene was excised from plasmid pMC-1871 by digesting with BamHI and then ligated into the BamHI site of the expression plasmid pQE-10 Type IV. The region encoding six histidine residues, the genes for β-galactosidase, β-lactamase, and chloramphenicol acetyltransferase are denoted $(His)_6$, *lacZ*, *bla*, and *cat*. Transcriptional terminator of phage lambda and T1 of *rrnB* operon of *Escherichia coli* are indicated by to and T1. B, E, X, and Xb denote cleavage sites for restriction enzymes BamHI, EcoRI, XhoI, and XbaI respectively. The coding region under control of the promoter/operator N250PSN250P29 and the ribosomal binding site RBSII is indicated by an arrow.

Purification of $(His)_6$-β-Galactosidase

A 0.35-g sample of *E. coli* cells containing β-galactosidase with six histidine residues at the amino terminus [$(His)_6$-β-gal] was extracted by sonication in 3 mL 50 m*M* Tris, pH 7.4, containing 10% sucrose. Sonication was performed with a 0.5-in. probe for 3 min in 30-s bursts. After centrifugation (10,000×*g*, 45 min, 4°C), the supernatant was directly pumped on a NTA–chelate column (0.7 × 30 cm, 6 mL resin) charge with Ni^{2+} prepared as previously described.[7] The column was pre-equilibrated with 150 mL of equilibration buffer (0.10 *M* Na–P, 0.10 *M* NaCl, 0.1% Tween-20, pH 7.4, containing 5 m*M* β-mercaptoethanol and 2 m*M* $MgSO_4$). The loaded column was washed with equilibration buffer, checked for β-galactosidase enzymatic activity, and rewashed with equilibration buffer until UV absorbance (280 nm) returned to baseline. The column was eluted with 0–100 m*M* imidazole gradient in equilibration buffer. Fractions (5 mL) were collected and analyzed for protein concentration, β-galactosidase activity, and purity (SDS–PAGE). The fractions were stored at 4°C.

Immobilization of $(His)_6$-β-Galactosidase

A 0.42-g sample of *E. coli* cells containing $(His)_6$-β-gal was extracted by sonication in 3 mL 50 m*M* Tris, pH 7.4, containing 10% sucrose as described above. After centrifugation, the supernatant was added to 5.5 mL wet Ni^{2+}–NTA suspended in 50 mL of equilibration buffer. The suspension was shaken on a rotary platform for 2 h at 4°C, filtered, then transferred to 50 mL equilibration buffer. The immobilized $(His)_6$-β-galactosidase was stored at 4°C. Purity (SDS–PAGE), protein, and activity were determined in order to show that no $(His)_6$-β-galactoosidase was leaking from the resin.

Synthesis of Allyl-β-D-Galactopyranoside

Following the method of Nilsson,[15] 16.6 g lactose and 8.3 mL allyl alcohol were added to the 5.5 mL of immobilized $(His)_6$-β-galactosidase suspended in 50 mL equilibration buffer. The reaction mixture was stirred using a magnetic stirrer and the reaction continued for 48 h. The reaction was monitored by thin-layer chromatography (TLC) at various time points (0, 24, and 48 h). The TLC was performed on Kieselgel 60 F_{254} applying 1 μL of a 1 : 25 dilution of the reaction mixture. The mobile phase contained ethylacetate–acetic acid–methanol–water in a ratio of 60 : 15 : 15 : 10. Carbohydrates were detected by spraying the TLC plate with naphthoresorcinol–sulfuric acid contained in an aerosol sprayer and then heating for 5–10 min at 100°C.[12] After 48 h the reaction was stopped by spinning down the gel and collecting the supernatant. This reaction was repeated twice more using he same immobilized β-galactosidase. After each reaction the resin was first removed by centrifugation, washed twice

with 50 mL equilibration buffer, and then reassayed for β-galactosidase activity.

After removing solvents with a rotary evaporator (Buchi RE 121), the product, allyl-β-D-galactopyranoside, was isolated by column chromatography on silica gel (230–400 mesh) with dichloromethane–methanol–ethanol–water, 6 : 3.5 : 1 : 0.8, as eluents. After eluting the first 200 mL, 9-mL fractions were collected. Each fraction was monitored for product by TLC (25 μL/fraction). The fractions containing allyl-β-D-galactopyranoside were pooled and solvents removed by rotary evaporation. The structure of the final product was confirmed by the melting point and ^{1}H nuclear magnetic resonance (NMR) spectroscopy (D_2O as solvent).

RESULTS

Cloning and Expression of the $(His)_6$-β-Galactosidase

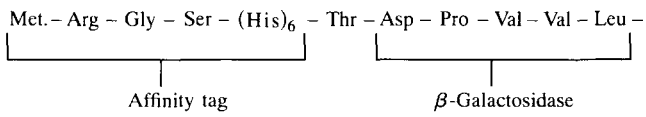
The plasmid pQE-10 type IV, which has been developed for the production of fusion proteins containing an N-terminal hexahistidine peptide extension,[6,19] was used for the construction of the plasmid directing the expression of $(His)_6$-β-galactosidase. This plasmid, belonging to the pDS family of plasmids,[3] contains the following elements: (1) the regulatable promoter/operator element N250PSN250P29 (M. Lanzer and H. Bujard, unpublished) which is repressed in the presence of the *E. coli lac* repressor but can be induced by addition of IPTG; (2) the synthetic ribosomal binding site RBSII; (3) the region encoding six histidine residues; (4) the transcriptional terminator "t_0" of phage lambda;[18] (5) the promoter-free gene for chloramphenicol acetyltransferase with its own ribosomal binding site; (6) the transcriptional terminator T1 of *E. coli rrnB* operon;[2] and (7) the replication region and the gene for β-lactamase present in plasmid pBR322. Due to the high efficiency of the expression signals, the vector can only be stably maintained when the plasmid pDMI, 1,[5] which expresses elevated levels of *lac* repressor, is present in the cells.

For construction of plasmid p$(His)_6$-*lacZ* (Fig. 1), the *lacZ* gene, encoding β-galactosidase, was inserted into the BamHI site of plasmid pQE-10 type IV. The *E. coli lacZ* gene was isolated as a BamHI 3.1-kb fragment from the plasmid pMC1871.[4] *Escherichia coli* MC 1061 cells containing plasmid pDMI,1 were transformed with plasmid p$(His)_6$-*lacZ* and subsequently grown in medium containing ampicillin and kanamycin to select for the presence of the expression plasmid (ampicillin) and pDMI,1 (kanamycin). The synthesis of $(His)_6$-β-galactosidase was induced with IPTG.

Purification and Immobilization of the $(His)_6$-β-Galactosidase

$(His)_6$-β-galactosidase produced in *E. coli* cells was extracted by sonication and purified by chromatography on the Ni^{2+}–NTA column (Fig. 2). The SDS–PAGE analysis showed that $(His)_6$-β-galactosidase was retained on the Ni^{2+}–NTA column at a pH of 7.4 even after being extensively washed with a phosphate buffer containing 0.1 *M* NaCl, 0.1% Tween-20, 5 m*M* β-mercaptoethanol, and 2 m*M* $MgSO_4$. The contaminating *E. coli* proteins were eluted from the column in the flow-through material, while no protein band corresponding to β-galactosidase was observed (Fig. 2, lane 3). Enzyme activity analysis of the NTA resin demonstrated that the $(His)_6$-β-galactosidase remained active while still on the column. The $(His)_6$-β-galactosidase was eluted from the column in the above phosphate buffer containing a 0–100 m*M* imidazole gradient and yielded a product with a purity of at least 95% as shown by SDS–PAGE (Fig. 2, lanes 5–8). Once eluted, enzyme activity analysis demonstrated a highly active enzyme (466 U/mg protein) which remained stable for at least 4 months when stored at 4°C. When β-mercaptoethanol and $MgSO_4$ were not included in the elution buffer, there was a rapid loss in enzymatic activity within 24 h (data not shown).

After column elution an N-terminal amino acid sequence analysis (Edman degradation) was performed on the $(His)_6$-β-galactosidase. The N-terminal analysis confirmed the sequence



of the purified fusion protein as predicted from the DNA sequence (data not shown).

A control experiment was performed in which β-galactosidase from *E. coli*, which lacks the six histidines, was applied to the Ni^{2+}–NTA column. The β-galactosidase was not retained on the resin and was eluted in the flow-through.

Enzymatic Synthesis of Allyl-β-D-Galactopyranoside

Immobilized β-galactosidase was used to synthesize allyl-β-D-galactopyranoside from lactose and allyl alcohol (Fig. 3). In this reaction, lactose acts as the glycosyl donor and allyl alcohol as the acceptor, and glucose is formed as a by-product. The same immobilized enzyme preparation was used in three subsequent batches. Table I compares the hydrolysis activity of the immobilized β-galactosidase after each batch to the original immobilized enzyme as well as to the soluble fusion protein and a commercially available β-galactosidase from *E. coli*. As shown, the purified fusion protein was found to be equally active to the β-galactosidase without the affinity tag. After immobilization onto the Ni^{2+}–NTA resin, the immobilized enzyme retained 64% of its original activity. At the conclusion of the first batch, the hydrolysis activity

was still about 20% of the activity for the soluble purified enzyme. There was no additional loss of activity after the subsequent batches. The decrease in hydrolysis activity did not affect the outcome of the synthetic reaction. Thin-layer chromatography analysis of each batch indicates that the reaction had reached equilibrium in 24–48 h. Figure 4 compares the TLC results of the reaction mixtures of the first to the third batch. No differences resulted in product formation, indicating that the immobilized β-galactosidase is reusable.

After each batch 2.2 g of product was recovered. The melting point of the isolated product, allyl-β-D-galactopyranoside, was 98–99°C, slightly lower than the reported literature value of 101–102°C.[9,15] The TLC analysis of allyl-β-D-galactopyranoside (Fig. 4) as well as further characterization by ^{1}H NMR spectroscopy indicates that the product is pure.

DISCUSSION

We have shown that β-galactosidase having a hexa-histidine tag at the N-terminus can be cloned, expressed in *E. coli,* and purified using a nitrilotriacetic acid adsorbent charged with nickel ions (Ni^{2+}–NTA adsorbent). The fusion protein is enzymatically active when bound to the Ni^{2+}–NTA adsorbent, and once eluted, the $(His)_6$-β-galactosidase retains its activity when stored at 4°C. This is in direct contrast to a study by Ljungquist et al.[13] in which a β-galactosidase fusion protein bearing a $(Ala–His–Gly–His–Arg–Pro)_n$ peptide extension (n = 4, 8) at the N-terminus was purified using the iminodiacetic acid (IDA) adsorbent charged with Zn^{2+} ions (Zn^{2+}–IDA adsorbent). The fusion protein retained some activity when bound to the Zn^{2+}–IDA adsorbent. However, once eluted, most of the enzymatic activity was lost. The authors indicated that contaminating Zn^{2+} ions leaking from the adsorbent were poisoning the β-galactosidase. The present study indicates that, by using the NTA adsorbent for the purification and immobilization of metal-binding proteins, the NTA adsorbent binds metal ions much tighter than the IDA adsorbent,[7] minimizing product contamination with metal ions.

We have taken advantage of the strong interaction of hexa-histidine fusion proteins with metal chelate adsorbents for immobilization of $(His)_6$-β-galactosidase to the Ni^{2+}–NTA adsorbent. The purification and immobilization of the $(His)_6$-β-galactosidase was done in one operation. Since the $(His)_6$-enzyme binds preferentially to the metal chelate adsorbent, the immobilized enzyme was prepared

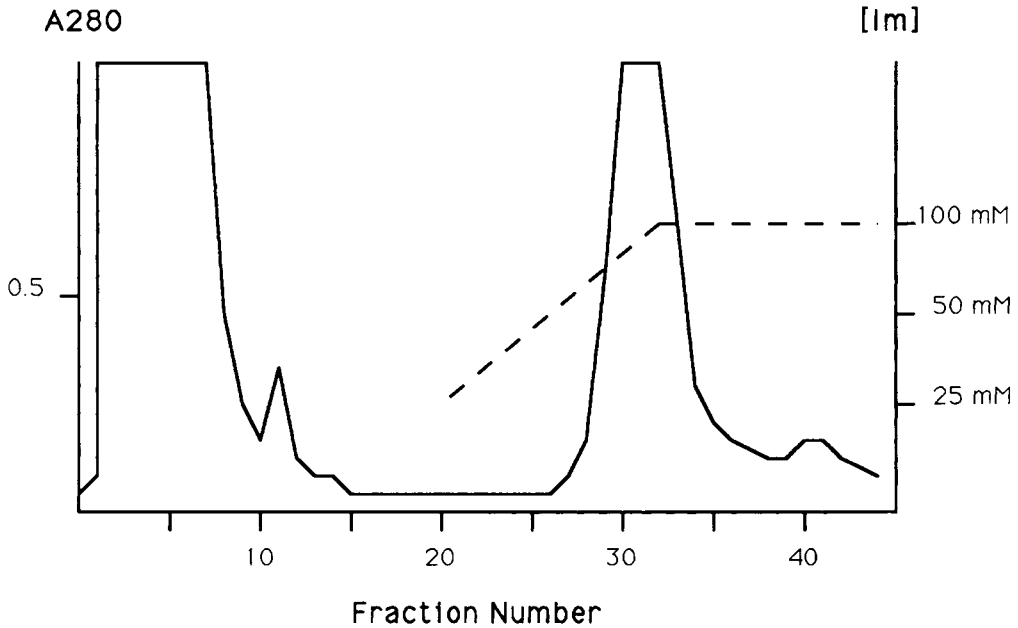


Figure 2. Chromatography of $(His)_6$-β-galactosidase. Crude extract was loaded at a flow rate of 1 mL/min onto a Ni^{2+}–NTA column, equilibrated with phosphate buffer (0.1 *M* Na–P, 0.1 *M* NaCl, 0.1% Tween-20, pH 7.4, containing 5 m*M* β-mercaptoethanol and 2 m*M* $MgSO_4$). The column was washed with equilibration buffer and then $(His)_6$-β-galactosidase was eluted with a 0–100 m*M* imidazole gradient [Im] in equilibration buffer. Fractions were analyzed by SDS–PAGE. (1) Molecular weight markers. (2) β-galactosidase from *E. coli.* (3) Column flow-through (fractions 1–13). (4) Crude *E. coli* extract. (5–8) Purified $(His)_6$-β-galactosidase, fractions 28–31. In a control experiment β-galactosidase, without the affinity tag, was applied to the same column and eluted entirely in the flow-through.

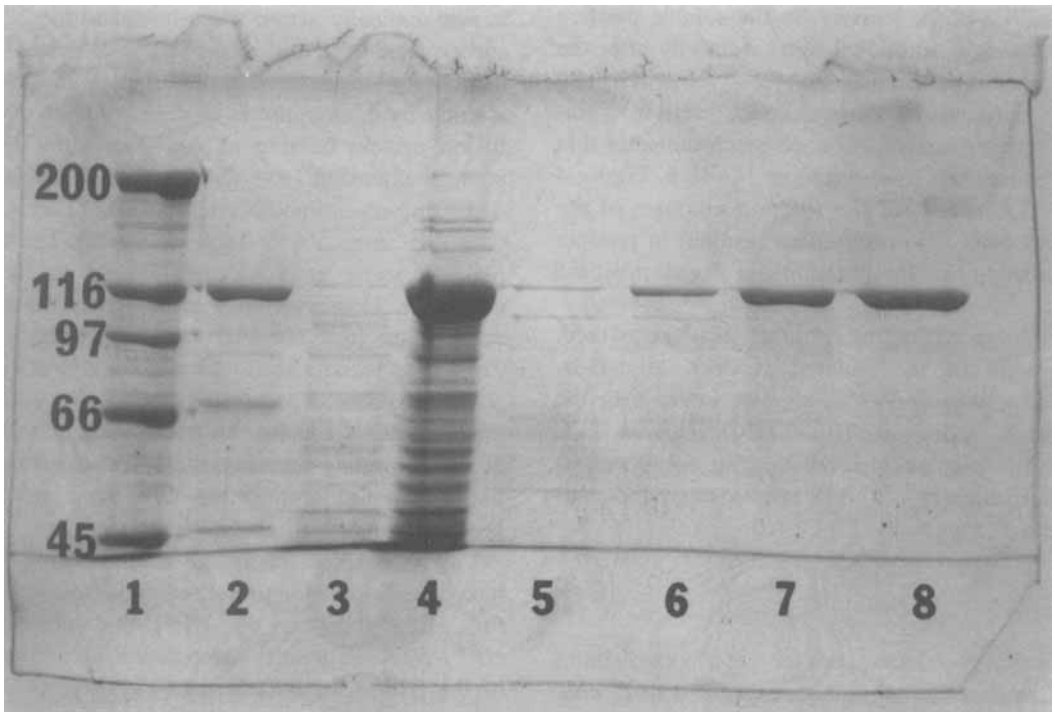


Figure 2. *(continued)*

by loading the bacterial crude extract directly on the adsorbent. After washing out the contaminating proteins the resulting biocatalyst was ready to be used for the synthesis of allyl-β-D-galactopyranoside from lactose and allyl alcohol. The reaction was run with 5.5 mL biocatalyst (296 β-galactosidase units), 16.6 g lactose, and 8.3 mL allyl alcohol. The results are comparable to those of Nilsson,[15] who used 1550 units of soluble β-galactosidase (grade VIII, Sigma) to catalyze the same reaction, starting with 100 g lactose and 50 mL allyl alcohol in 300 mL buffer. Nilsson reported a yield of 16 g, whereas we got in each batch 2.2 g from six times smaller batches using the same 5.5 mL of immobilized enzyme in every cycle. The decrease of β-galactosidase activity measured after the first preparative cycle seems not to affect the outcome of the synthetic reaction. Synthesis rates for allyl-β-D-galactopyranoside would give more accurate information about the synthesis activity of the enzyme, but to measure transglycosidation or reversed hydrolysis reaction rates[15] would be most complicated. On the other hand, in repeating the synthesis of allyl-β-D-galactopyranoside with soluble β-galactosidase, following the method of Nilsson, we have found that at the end of the reaction the enzyme was completely inactive (data not shown). By using the

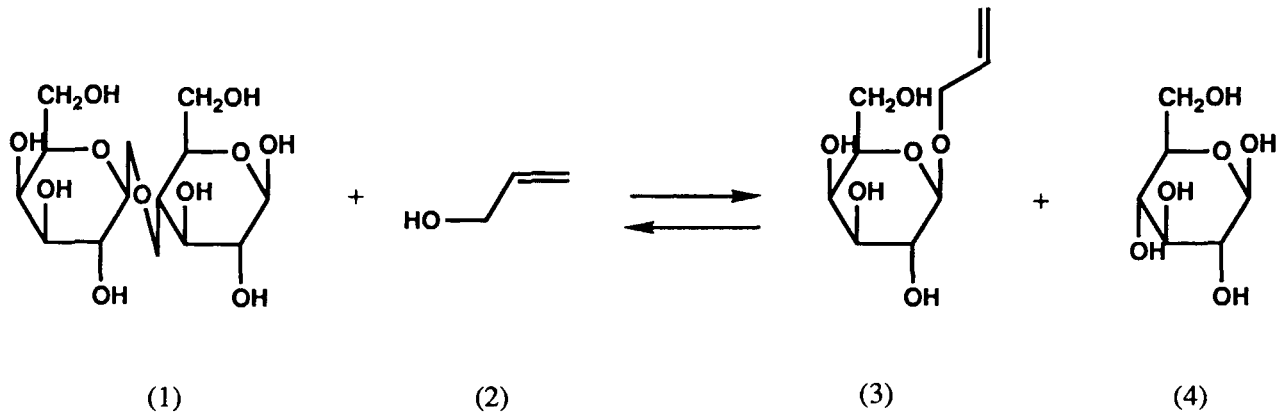


Figure 3. Reaction scheme for the synthesis of allyl-β-D-galactopyranoside (3) from lactose (1) and allyl alcohol (2). As a by-product, glucose (4) is formed.

Table I. Enzyme activity of commercial enzyme, soluble fusion protein, immobilized enzyme and immobilized enzyme after the first, second, and third subsequent batches.

Sample	Protein (mg/mL)	Specific activity (U/mg protein)	Activity remaining (%)
Commercial β-gal	0.6	390	—
Soluble $(His)_6$-β-gal	0.2	420	100
Immobilized $(His)_6$-β-gal	0.2	269	64
Immobilized $(His)_6$-β-gal after batch I	0.2	85	20
Immobilized $(His)_6$-β-gal after batch II	0.2	85	20
Immobilized $(His)_6$-β-gal after batch III	0.2	85	20

The enzyme activity was determined following the formation of *o*-nitrophenol at 410 nm cleaved off from the synthetic *o*-nitrophenol-β-D-galactopyranoside. The protein concentration of the soluble enzymes was determined by the Bio-Rad protein microassay. The protein concentration of the immobilized enzyme was also estimated by the Bio-Rad protein microassay using 20–40-μL aliquots of suspended resin.

immobilized form of β-galactosidase, our system appears advantageous since the enzyme activity is stabilized and can be reused in subsequent preparative cycles. In addition, the immobilized system renders a continuous production process possible.

Recently the use of enzymes as catalysts for the synthesis of carbohydrates has been reviewed as a promising alternative to the classical synthesis of carbohydrates.[8,20] By taking advantage of the specific binding of genetically engineered fusion proteins to an affinity adsorbent, immobilized enzymes can be utilized as biocatalysts in organic chemistry.

We thank Mary Graves for her help in constructing the expression plasmid. We are also grateful to Yu-Ching Pan for protein sequencing.

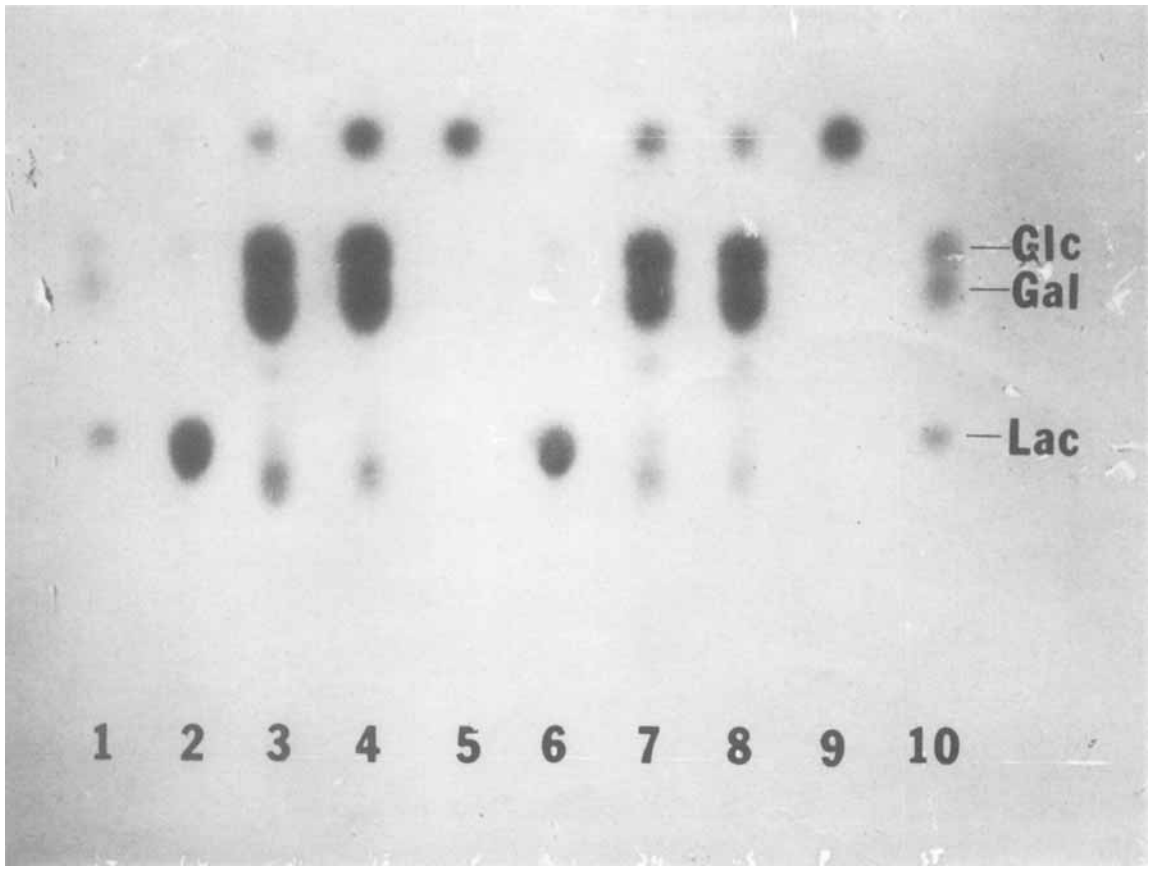


Figure 4. Thin-layer chromatography comparing the results of the enzymatic synthesis of allyl-β-D-galactosidase of batch I to batch III. (1, 10) Sugar standards: lactose (Lac), galactose (Gal), and glucose (Glc). (2) Batch I, after starting the reaction. (3) Batch I, after 24 h. (4) Batch I, after 48 h. (5) Batch I, isolated product. (6) Batch III, after starting the reaction. (7) Batch III, after 24 h. (8) Batch III, after 48 h. (9) Batch III, isolated product.

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