

BBA 66778

COVALENT BONDING OF FUNGAL  $\beta$ -GALACTOSIDASE TO GLASS

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(Received June 26th, 1972)

## SUMMARY

*Aspergillus niger*  $\beta$ -galactosidase ( $\beta$ -D-galactoside galactohydrolase, EC 3.2.1.23) was purified by affinity chromatography on porous glass columns containing the inhibitor, *p*-aminophenyl- $\beta$ -D-thiogalactopyranoside. The enzyme was coupled to glass by diazo-linkage and retained 75% of its theoretical activity. Enzyme properties, such as temperature and pH optima, and enzyme stability, were not affected by insolubilization. Calculated  $K_m$  values for the free and bound enzymes with lactose as the substrate were comparable; however, the  $V$  of the bound enzyme was decreased by 60%. Galactose was a competitive inhibitor of the *A. niger*  $\beta$ -galactosidase.

## INTRODUCTION

In recent years numerous studies have established a pattern of milk intolerance among non-Caucasian children and adults<sup>1</sup>. This problem is attributed to decreased levels of intestinal  $\beta$ -galactosidase ( $\beta$ -D-galactoside galactohydrolase, EC 3.2.1.23), which results not only in lactose malabsorption, but in a general impairment of normal digestive processes. The etiology of  $\beta$ -galactosidase deficiency is unknown, although genetic, dietary and other factors have been implicated. Lactose intolerance and its related aspects has recently been the subject of a detailed review<sup>2</sup>.

Recent progress in enzyme immobilization<sup>3</sup> suggests that these techniques may serve as the basis for the development of economical, continuous methods for lactose hydrolysis. Although  $\beta$ -galactosidases have been isolated from a wide variety of microbial sources, only that of *Escherichia coli* has been evaluated after immobilization<sup>4,5</sup>. The commercial availability of crude extracts and of partially purified  $\beta$ -galactosidase preparations isolated from *Aspergillus niger* prompted our investigation of the effects of immobilization on this enzyme. Purified preparations of the enzyme were coupled to porous glass beads by diazo-linkage and the properties of the product evaluated.

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## MATERIALS AND METHODS

Corning's Controlled-Pore\* glass beads (pore size  $700 \pm 70 \text{ \AA}$ , 120–200 mesh) were purchased from Analabs;  $\gamma$ -aminopropyltriethoxysilane from Aldrich Chemical; *p*-nitrophenyl- $\beta$ -D-galactopyranoside and *p*-aminophenyl- $\beta$ -D-thiogalactopyranoside from Calbiochem. A partially purified preparation of *A. niger*  $\beta$ -galactosidase (Lactase LP) was a gift of Wallerstein Laboratories.

*Diazo coupling to glass*

$\beta$ -Galactosidase and its inhibitor were covalently attached to porous glass beads by the diazotization procedure reported by Weetal<sup>6</sup>. Glass beads were refluxed for 16 h in toluene containing 10%  $\gamma$ -aminopropyltriethoxysilane. The silanized beads were reacted with *p*-nitrobenzoyl chloride in chloroform containing 5% triethylamine. Reduction of the nitrated derivative was obtained by refluxing with 4%  $\text{Na}_2\text{S}_2\text{O}_4$  for 1 h. The diazonium chloride was prepared at 4 °C by suspending the glass in 0.66 M HCl and adding 10 2-ml aliquots of ice cold 20%  $\text{NaNO}_2$  over a 15-min period. The beads were filtered, washed with cold 1 mM hydrochloric acid, and immediately transferred to cold 0.05 M sodium pyrophosphate (pH 8.0) containing either the galactosidase or its inhibitor. After allowing the coupling to proceed for 4 h with stirring, the material was allowed to stand overnight in a cold room. The glass beads were washed with 0.05 M phosphate buffer containing 0.5 M NaCl and stored at 4 °C in 0.05 M phosphate buffer (pH 6.5). The amount of protein bound to glass was determined from amino acid analyses after 24 h hydrolysis at 110 °C in 6 M HCl in sealed, evacuated tubes.

*Affinity chromatography*

The  $\beta$ -galactosidase was purified by affinity chromatography using the substrate analogue inhibitor, *p*-aminophenyl- $\beta$ -D-thiogalactopyranoside covalently coupled to porous glass by the diazotization procedure described below. The column was equilibrated with 0.2 M sodium acetate (pH 4.0) and operated at room temperature. A solution of the crude enzyme preparation (0.2–0.4% protein) in acetate buffer was applied to the column at a rate of approximately 50 ml/h. Protein elution was monitored by measuring the absorbance at 280 nm with a continuous-flow ultraviolet analyzer. Following sample application, the column was developed with additional buffer until all unadsorbed protein was eluted. The adsorbed  $\beta$ -galactosidase was eluted at pH 8.0 with 0.10 M sodium pyrophosphate. Enzyme activity was determined using *o*-nitrophenyl- $\beta$ -D-galactopyranoside.

*Activity measurements*

Activity of the free enzyme was determined by the addition of a suitable aliquot to 1 ml of substrate (5 mM *o*-nitrophenyl- $\beta$ -D-galactopyranoside or 140 mM lactose) in pH 4.0 sodium acetate buffer (0.1 M) and incubating for 5–15 min at 37 °C. The *o*-nitrophenyl- $\beta$ -D-galactopyranoside digests were stopped by the addition of 2 ml 0.5 M  $\text{Na}_2\text{CO}_3$  and the lactose digests by placing the tubes in a boiling water bath for 3 min. The absorbance of the liberated *o*-nitrophenol was measured directly at 405

\* Mention of companies or products is for the convenience of the reader and does not constitute an endorsement by the U.S. Department of Agriculture.

nm; the glucose content of the lactose digest aliquot was determined by a glucose oxidase procedure<sup>7</sup>. Quantitation of absorbance values was obtained from standard curves. Activity of the immobilized enzyme was determined by pumping substrate through jacketed columns of bound enzyme or by incubation of substrate with the bound enzyme suspended by means of overhead stirring; aliquots of the digest were withdrawn at appropriate intervals and analyzed as above. One unit of activity is defined as the amount of enzyme which liberates 1  $\mu$ mole of product per min at 37 °C. The specific activity was expressed as the number of units per mg of protein. Protein was determined by the Lowry procedure<sup>8</sup> using bovine serum albumin as the standard.

## RESULTS AND DISCUSSION

$\beta$ -Galactosidase was purified by affinity chromatography on columns of porous glass beads containing the covalently bound inhibitor, *p*-aminophenyl- $\beta$ -D-thiogalactopyranoside<sup>9</sup>. Chromatography of the partially purified commercial preparation (Fig. 1) resulted in recovery of over half the applied protein in the unadsorbed fraction; all of the enzymatic activity was adsorbed to the inhibitor column. The adsorbed enzyme retained activity toward *o*-nitrophenyl- $\beta$ -D-galactopyranoside and lactose but could not be eluted in a sharp zone with buffers containing high concen-

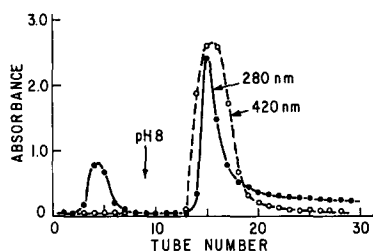


Fig. 1. Chromatographic purification of *A. niger*  $\beta$ -galactosidase on a 1 cm  $\times$  15 cm column of porous glass beads containing covalently bound *p*-aminophenyl- $\beta$ -D-thiogalactopyranoside. 50 mg of a partially purified preparation (9 ml) was applied in 0.2 M acetate buffer (pH 4) and yielded 21 mg of purified enzyme. Fraction volume, 3 ml.

trations of substrate. Elution of the enzyme was accomplished at pH 8.0 with 0.1 M sodium pyrophosphate; no additional activity could be eluted at pH 10 with 0.1 M borate buffer. Protein recoveries were based on Lowry determinations due to the presence of colored breakdown products of the derivatized glass in the eluting buffers. The affinity chromatography increased the specific activity of the enzyme toward *o*-nitrophenyl- $\beta$ -D-galactopyranoside from 30 to 60, which was comparable to activities obtained when purified by usual chromatographic techniques<sup>10,11</sup>.

The affinity column used had a maximum binding capacity of 6 mg enzyme/g beads. Although some breakdown of the glass beads occurs with enzyme desorption, no significant decrease in binding capacity occurred after several chromatographic cycles. Steers *et al.*<sup>9</sup> reported that when the inhibitor was coupled directly to Sepharose 4-B, little binding capacity for *E. coli*  $\beta$ -galactosidase was evident. However, good enzyme adsorption was obtained when a 21-Å hydrocarbon arm was placed between the Sepharose backbone and inhibitor. The satisfactory enzyme adsorption obtained

with diazo-coupled inhibitor indicated the ligand was positioned far enough away from the matrix to permit binding of enzyme to the inhibitor.

The amount of protein bound to glass is related to the amount of alkyl amino groups incorporated during refluxing of the glass with  $\gamma$ -aminopropyltriethoxysilane. Two preparations of bound enzyme using the same alkyl amine glass yielded 3.0 and 3.1 mg protein/g glass. The above preparations were used in the studies described below. Following the completion of much of this work, an improved method for preparing alkyl amine glass was reported<sup>5</sup> which permitted a several-fold increase in the amount of enzyme bound. Activity measurements indicated that the immobilized enzyme retained 75% of its free activity. No loss in activity was observed following storage for 2 months at 4 °C in pH 4 acetate buffer; however, freeze-drying resulted in complete loss of enzymatic activity. The free and bound  $\beta$ -galactosidases retained full activity when held in glycine-acetate-phosphate buffers in the pH range 2.5–8.0 for 1 h at 37 °C. Similarly, a comparison of the thermal stability of the bound and unbound enzyme exposed to 55 °C in acetate buffer (pH 4) results in no loss of activity; however, 65% of the activity was destroyed in each case at 65 °C. Thus, stability of the enzyme was not affected by the coupling procedure. The pH optimum for the free and bound enzymes was 4.0 using either *o*-nitrophenyl- $\beta$ -D-galactopyranoside or lactose as the substrate; maximum enzymatic activity was obtained at 55 °C.

The effect of substrate concentration on the hydrolysis of lactose by  $\beta$ -galactosidase in free solution and bound to glass was investigated. The results of these experiments are presented in Fig. 2 by the Lineweaver-Burk plots. The values for  $K_m$  and  $V$  were calculated from the results in Fig. 2 and are presented in Table I. The  $K_m$  values obtained for the free (20) and bound enzyme (18) were essentially identical and indi-

TABLE I

VALUES OF  $K_m$  AND  $V$  FOR THE HYDROLYSIS OF LACTOSE BY  $\beta$ -GALACTOSIDASE IN FREE SOLUTION OR COUPLED TO GLASS BEADS

	$K_m$ (mM)	$V^*$
Free solution	20	0.071
Bound enzyme	18	0.029

\* Expressed as  $\mu$ moles of lactose hydrolyzed/min per  $\mu$ g enzyme.

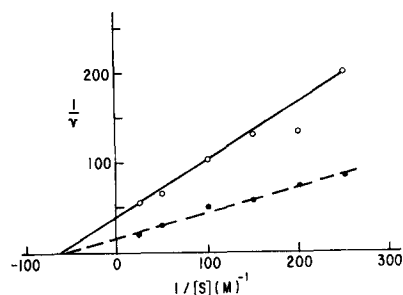


Fig. 2. Effect of substrate concentration on the hydrolysis of lactose by  $\beta$ -galactosidase in free solution (●---●) and chemically bound to glass beads (○---○). Velocity is expressed in  $\mu$ moles lactose hydrolyzed/min per  $\mu$ g enzyme.

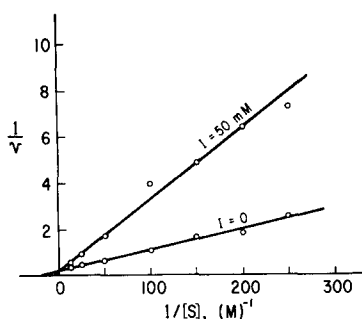


Fig. 3. Effect of galactose on the hydrolysis of lactose by a 0.1-cm<sup>3</sup> column of bound  $\beta$ -galactosidase in the presence of varying substrate concentrations. Velocity is expressed as  $\mu$ moles lactose hydrolyzed per ml at a flow rate of 100 ml/h.

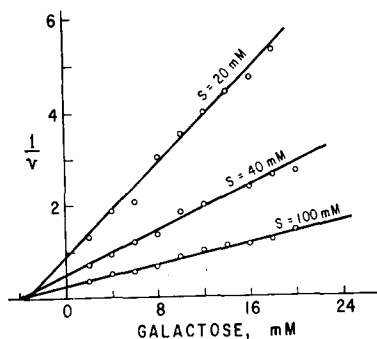


Fig. 4. Dixon plots of  $1/v$  versus galactose at 20, 40 and 100 mM lactose concentrations using a 0.1 cm<sup>3</sup> column of bound  $\beta$ -galactosidase. Velocity is expressed as  $\mu$ moles lactose hydrolyzed per ml at a flow rate of 100 ml/h.

cated that no limitations on substrate diffusion to the bound enzyme were operative in the stirred reactor. The value for  $V$  was decreased by 60% after immobilization of  $\beta$ -galactosidase.

Since the rates of lactose hydrolysis obtained with columns of immobilized  $\beta$ -galactosidase were not inversely proportional to the flow rates, product inhibition appeared to be operative. With regard to the lactose hydrolysis products, only galactose inhibited *A. niger*  $\beta$ -galactosidase<sup>10</sup>. The effects of galactose on the kinetics of lactose hydrolysis by  $\beta$ -galactosidase were studied with columns of immobilized enzyme (0.1 cm<sup>3</sup>) operating at 37 °C and flow rates of 100 ml/h. These results are presented in Figs 3 and 4. The Lineweaver-Burk plots (Fig. 3) showed straight line relationships and indicated that galactose acts as a competitive inhibitor. Dixon plots of  $1/v$  versus galactose concentration at 3 lactose levels are shown in Fig. 4 and confirm the competitive inhibition by galactose. Glucose did not inhibit  $\beta$ -galactosidase activity.

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