

## Characterization and Immobilization of *E. coli* (ATCC-26) $\beta$ -Galactosidase

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### Summary

The  $\beta$ -galactosidase from *Escherichia coli* ATCC-26 was partially purified and characterized. It was found to be comparable to galactosidases from other *E. coli* strains in stability, pH and temperature maxima, and activity requirements, but it had a more favorable ratio of activity toward lactose versus synthetic substrates. The galactosidase was immobilized on porous glass beads by three covalent bonding methods. Kinetic data for the free and bound enzymes were determined using natural and synthetic substrates. Activity characteristics of the free and immobilized enzymes were comparable, however, the bound forms were less stable to heat.

### INTRODUCTION

A variety of  $\beta$ -galactosidases (lactases) have been investigated for their potential application in the utilization of cheese whey lactose.<sup>1-6</sup> These enzymes were primarily of fungal<sup>1-3</sup> or yeast<sup>4,5</sup> sources, although some effort has been made with bacterial galactosidases.<sup>6</sup> The galactosidases of selected *E. coli* strains have been investigated<sup>7</sup> but, in general, have not been demonstrated to possess as great an activity toward lactose as toward the synthetic nitrophenyl-galactoside substrates. In the course of a recent survey of various bacterial lactases, we found that the *E. coli* ATCC-26 galactosidase had an activity toward lactose equal to 40% of that toward the synthetic substrates. We therefore characterized this enzyme in both soluble and immobilized forms and studied the effects of three methods of covalent enzyme bonding to insoluble supports. The results of these investigations are the subject of this report.

## MATERIALS AND METHODS

### *Chemicals*

*o*-Nitrophenyl- $\beta$ -D-galactopyranoside (ONPG) and *p*-nitrophenyl- $\beta$ -D-galactopyranoside (PNPG) were purchased from Calbiochem.\*

1-Cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluene sulfonate was purchased from Aldrich Chemical Company, Inc.

Controlled-pore glass (CPG-10), 120/200 mesh, pore diameter 700 Å, was obtained from Corning Glass Works.

DE-32, a microcrystalline diethylaminoethyl cellulose, was purchased from Whatman.

### *Growth Conditions*

The bacteria were cultivated in 1500 ml Fernbach flasks in an incubator-shaker (175 rpm) at 34°C. The culture medium (1000 ml) was a 2% Bacto lactose broth, pH 6.0. The cultures were harvested by centrifugation ( $7000 \times g$ , at 4°C) after 18 hr growth.

### *Enzyme Purification*

The harvested cells (3–4 g/liter medium) were ground with powdered glass in a cold mortar and extracted by stirring for 30 min in 10 volumes of cold 0.05M potassium phosphate buffer, pH 6.8, containing 0.01M 2-mercaptoethanol and 0.01M magnesium chloride (buffer A). The extract was centrifuged at 4°C for 10 min at  $10,000 \times g$  and the extraction repeated. The combined extracts from 100 g of cells yielded 600–800 mg protein with a specific activity toward ONPG of 1–2.

The combined extracts (~200 ml) were applied to a  $2 \times 30$  cm column of DE-32 equilibrated at pH 7 with Buffer A. The column was developed at 50 ml/hr using a 1 liter linear sodium chloride gradient from 0 to 1.0M. Protein elution was monitored with a continuous-flow UV analyzer. Activity, located using ONPG, eluted after 250 ml of gradient, as seen in Figure 1. The active fraction was further purified by rechromatography. Since ultrafiltration or lyophilization resulted in activity losses, eluted fractions (100 ml) were concentrated after overnight dialysis against buffer A by adsorption on a  $1 \times 10$  cm DE-32 column equilibrated with buffer A. The enzyme was desorbed (10 ml volume, 50–75 units/ml) with 1.0M sodium chloride.

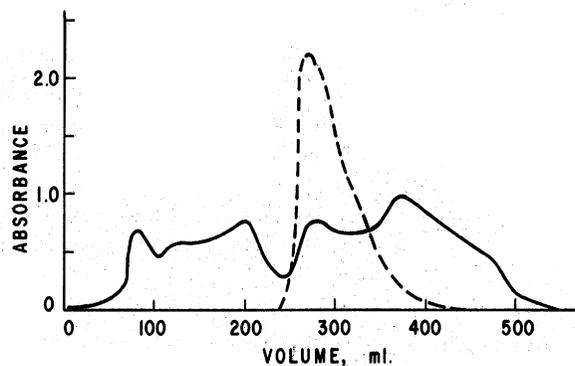


Fig. 1. DE-32 chromatogram of an extract of *E. coli* ATCC-26 cells, using buffer A and a linear salt gradient. Protein was monitored at 280 nm (—). Activity was located by measuring *o*-nitrophenol production by hydrolysis of ONPG at 405 nm (---).

The enzyme was dialyzed overnight in the cold against buffer A and stored at 4°C.

#### *Analytical Methods*

Enzyme activities toward ONPG, PNPG, and lactose were determined at 37°C using the specific buffer found to yield maximum activity with each substrate. Hydrolysis of PNPG was measured in pH 6.8 potassium phosphate buffer (0.05*M*) containing 0.01*M* magnesium chloride and 0.01*M* 2-mercaptoethanol. Sodium chloride (0.1*M*) increased hydrolysis of ONPG and was therefore added to the preceding buffer. Lactose activities were determined in the absence of 2-mercaptoethanol and 0.1*M* sodium chloride because both compounds interfered with the glucose determination. Aliquots of soluble enzyme (10–100  $\mu$ l) were added to 1 ml of 5*mM* ONPG or PNPG, or 146 *mM* lactose, and incubated at 37°C for 3 to 5 min. Digestion of the synthetic substrates was stopped by the addition of 2 ml of 0.5*M* sodium carbonate and the amount of liberated *o*-nitrophenol or *p*-nitrophenol was measured directly at 410 nm or 405 nm, respectively. Lactose digests were inactivated by the drop in pH to 4.0 resulting from the addition of the glucose oxidase reagent (in 2*M* sodium acetate, pH 4.0).<sup>8</sup> The amount of glucose liberated was quantitated using appropriate standard curves.

Activities of the immobilized enzymes were determined by suspending homogeneous samples of the glass beads with overhead stir-

ring in 5 ml of substrate. One milliliter aliquots of the digest supernatants were removed at appropriate time intervals (5–15 min) and analyzed as above. In all assays, less than 1% of the available substrate was utilized. One unit of activity was defined as the amount of enzyme which liberated 1  $\mu$ mole of product/min at 37°C. Specific activities were expressed as the number of units/mg protein.

Protein concentrations were determined by the Lowry procedure<sup>9</sup> using bovine serum albumin as the standard.

#### *Enzyme Immobilization*

Controlled-pore glass beads were aminoalkylated with a 2% solution of 3-aminopropyl triethoxysilane in acetone according to the method of Robinson et al.<sup>10</sup> A single preparation of aminoalkyl glass was used in all experiments. Four to five milligrams of the  $\beta$ -galactosidase preparation ( $SA = 22$  ONPG units/mg) were bound to 1 g aminoalkyl glass beads by glutaraldehyde coupling,<sup>10</sup> by diazo linkage,<sup>11</sup> and by means of carboxyls activated with a carbodiimide at pH 6.5.<sup>12</sup>

#### *Influence of Temperature and pH*

The influence of temperature on the galactosidase activity was determined using ONPG as the substrate over the range of 25 to 60°C. Enzyme stabilities were determined after 30 min exposure to temperatures in the above range followed by analysis at 37°C.

The pH activity curve in the range 5.8–7.6 was determined for the free enzyme at 37°C using each of the three substrates. The activity curves of the bound enzymes were determined using a single sample of enzyme for pH's from 7.0 to 5.8, and another from 7.0 to 7.6. The pH stability in the range 3–11 was determined at 37°C in either acetate, phosphate, or borate buffers (0.05M) containing 0.01M magnesium chloride, 0.01M 2-mercaptoethanol, and 0.1M sodium chloride. Activities relative to original values were determined at 37°C in pH 6.8 buffer following 45 min exposure at selected pH values.

#### *Kinetics*

The kinetic studies of soluble and immobilized enzymes used comparable amounts of activity (.05 to .10 ONPG units/ml substrate) as determined under standard conditions. One ONPG unit was equivalent to 40  $\mu$ g of enzyme, and this was the standard weight used for the kinetic comparisons. The data were treated by the least squares analysis using the weighting methods of Wilkinson.<sup>13</sup> Kinetic parameters for the free enzyme were determined using the three sub-

strates. Product inhibition was examined at constant concentrations of glucose or galactose while varying the substrate concentrations. The effects of added buffer components on kinetics were determined with ONPG and PNPG. Kinetic parameters for the immobilized enzymes were determined using lactose and ONPG in stirred reactors. A single aliquot of bound enzyme was used for a complete set of experiments varying substrate concentrations.

## RESULTS

### *Purification and Immobilization*

Purification of the galactosidase was carried out as described above and the results of a typical isolation are presented in Table I. The enzyme was coupled to glass beads by diazo, glutaraldehyde, and carbodiimide procedures by reacting approximately 4 mg protein with 1 g of aminoalkylated glass.

TABLE I  
Enzyme Purification

Fraction	Total units	Protein (mg)	Specific activity (units/mg)	Recovery (%)
Crude extract	1428	819	2	100
DE-32 chromatography	1051	73	16	74
DE-32 rechromatography	718	20	33	50

No enzymatic activity was found in the supernatants of the coupling reactions, even at extended digest times. No protein was detected in the supernatants after dialysis against distilled water. A uniform sample of the washed enzyme-beads was analyzed for activity toward ONPG. The beads were then dried and weighed, and the activity bound to 1 g of glass was calculated. At these low concentrations of protein, all the enzyme appeared to be bound to the glass beads with no loss in activity. These results are shown in Table II.

### *Enzyme Stabilities*

No loss in activity of the free enzyme was observed after 90 days storage in buffer at 4°C, nor for the bound enzymes stored for more than 30 days. The free enzyme retained full activity after 30 min incubation at temperatures up to 50°C. A 10% loss was observed

TABLE II  
Binding Efficiency of Immobilization Methods

Immobilization method	Activity added (ONPG units)	Activity recovered	
		ONPG units/weight glass (mg)	Total (ONPG units/g glass)
Carbodiimide	114	1.83/15.6	117
Diazo	100	0.98/10.2	96
Glutaraldehyde	83	1.03/12.0	86

at 55°C, while complete inactivation resulted from incubation at 60°C. Figure 2 shows that, after 4 days incubation at 37°C, no loss of activity was observed for the free enzyme. In contrast the bound enzymes lost approximately 50% of their activity. Losses of activity were also observed with the bound enzymes incubated at 25°C but the rate of inactivation was less rapid.

The free enzyme retained at least 90% of its activity after 45 min incubation at room temperature at pH's between 5.5 and 9.5 (Fig. 3). Complete loss of enzymatic activity was observed at pH 4.0; losses above pH 9.5 were more gradual. The bound enzymes retained 90% of their activity only between pH 5.5 to 8.0, as seen in the composite curve in Figure 3.

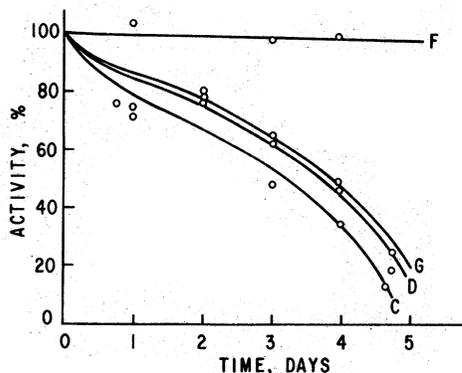


Fig. 2. Thermal stability of free  $\beta$ -galactosidase (F) and of the glass-enzymes linked by glutaraldehyde (G), by diazo (D), and by carbodiimide (C) methods, at 37°C storage in buffer A containing also 0.1M NaCl.

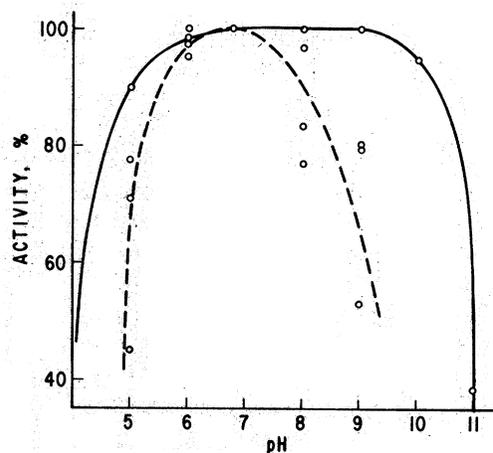


Fig. 3. Activity remaining after 45 min exposure to pH's from 4 to 11 was greater for the free enzyme (—) than for the bound enzymes (---). The curve for the glass-bound enzymes is a composite of data for the three preparations.

#### Activity Profiles

The pH activity curve obtained was comparable to curves for galactosidases isolated from other *E. coli* strains. Immobilization did not result in a shift of the pH maximum from 7.0, but did narrow the range of full activity (Fig. 4).

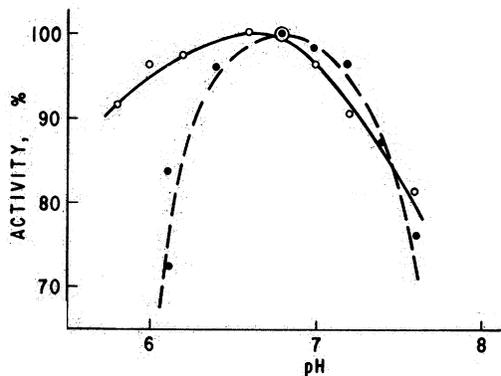


Fig. 4. Relative activity of soluble (—) and immobilized (---)  $\beta$ -galactosidases is similar on the basic side of the pH optimum (6.8). On the acid side, the free enzyme has higher relative activity. The curve for the glutaraldehyde-bound enzyme is representative of the three bound forms.

The temperature maximum for the free enzyme was 55°C in salt-free buffers, but was shifted to 60°C by the presence of 0.1M sodium chloride.

### Enzyme Kinetics

The enzyme obeyed simple Michaelis-Menten kinetics toward both natural and synthetic substrates as demonstrated by straight lines obtained for double reciprocal plots. The effect of mercaptoethanol and sodium chloride on the kinetic parameters was investigated and is reported in Table III, together with  $K_M$  and  $V$  for the three substrates using soluble enzyme. Lineweaver-Burk plots for the free and immobilized enzymes, using lactose as a substrate, are presented in Figure 5 and the values for  $K_M$  and  $V$  calculated from this graph are shown in Table IV. Comparable data was obtained for both free and bound enzymes using either lactose or ONPG as substrates.

TABLE III  
Michaelis Constants and Maximum Velocities

Substrate <sup>a</sup>	Buffer additive	$K_M(10^{-4}M)$	$V(\mu\text{moles}/\text{min}/\text{ONPG unit})$
Lactose	—	45.0	0.32
PNPG	—	3.9	0.47
PNPG	0.01M mercaptoethanol	6.3	0.67
ONPG	0.01M mercaptoethanol	4.8	0.64
ONPG	0.01M mercaptoethanol + 0.10M NaCl	4.1	1.40

<sup>a</sup> In 0.05M potassium phosphate, 0.01M MgCl<sub>2</sub>, pH 6.8.

Galactose inhibition was investigated with all substrates under the buffer conditions listed in Table III. Lineweaver-Burk plots at constant galactose concentrations demonstrated competitive inhibition (Fig. 6). Inhibitor constants calculated from this data were between 0.08 and 0.10M for all systems except for the hydrolysis of ONPG in the salt-containing buffer, where a  $K_i$  of 0.16M was obtained.

Glucose at 0.05 and 0.10M concentration was an uncompetitive inhibitor of the synthetic substrates, using buffer conditions in Table III. An experiment using PNPg is shown in Figure 7. Higher levels of glucose were required to demonstrate inhibition in the presence of 0.10M sodium chloride.

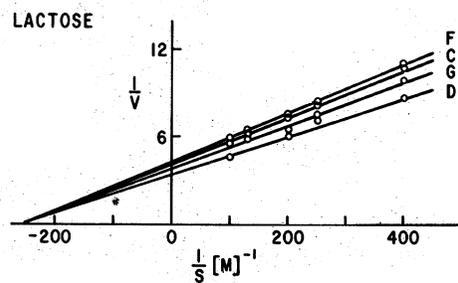


Fig. 5. Lineweaver-Burk plot for lactose hydrolysis by free (F)  $\beta$ -galactosidase and by  $\beta$ -galactosidase bound to glass by diazo (D), glutaraldehyde (G), and carbodiimide (C) procedures.

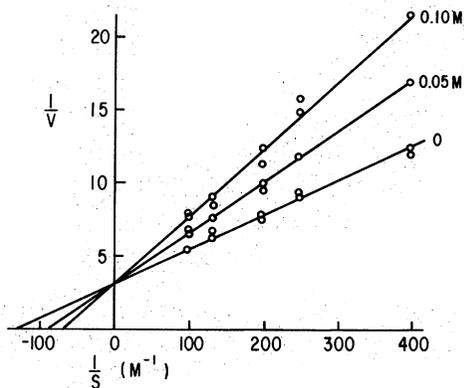


Fig. 6. Lineweaver-Burk plot demonstrating the effect of varying galactose concentrations on the hydrolysis of lactose by free  $\beta$ -galactosidase.

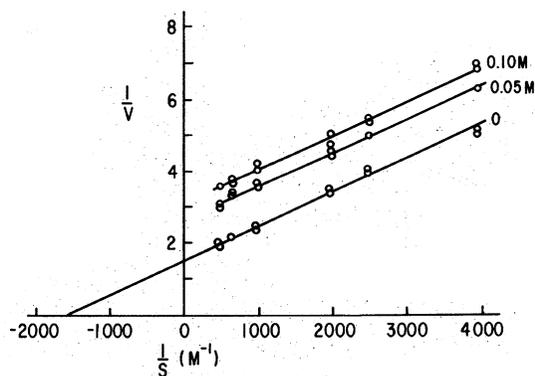


Fig. 7. Lineweaver-Burk plot demonstrating the effect of glucose on the hydrolysis of PNPG by free  $\beta$ -galactosidase.

### Salt Effects

The effects of several salts on the hydrolysis of each substrate were determined after 30 min incubation of the enzyme with each compound. The relative activities found are listed in Table V. The divalent cations magnesium, manganese, cobalt, iron, and barium increased activity of the enzyme toward at least two of the three substrates. Ammonium ion enhanced enzymatic activity, but the buffer salts, trishydroxymethylaminomethane and potassium citrate, were inhibitory. Cupric and mercuric ions reduced activity to zero; EDTA reduced activity by more than 75%. Sodium ion enhanced hydrolysis of ONPG by the enzyme but had no effect on the hydrolysis of either PNPG or lactose. Prior to this experiment, it was determined that neither potassium nor chloride ion, up to 0.10M concentration, affected activity toward any substrate.

TABLE IV  
Michaelis Constants and Maximum Velocities for Free and Bound  $\beta$ -Galactosidase

	ONPG		Lactose	
	$K_M(10^{-4}M)$	$V^a$	$K_M(10^{-3}M)$	$V^a$
Free	3.9	1.37	4.5	0.26
Carbodiimide	3.7	0.79	4.1	0.24
Diazo	3.5	0.85	4.2	0.30
Glutaraldehyde	3.7	0.87	4.5	0.27

<sup>a</sup> $V = \mu\text{moles}/\text{min}/\text{ONPG unit.}$

### DISCUSSION

The  $\beta$ -galactosidase of *E. coli* ATCC-26 was comparable to  $\beta$ -galactosidases isolated from other *E. coli* strains<sup>7</sup> with regard to temperature and pH optima, and in its requirement of 2-mercaptoethanol and divalent cation for maximum activity and stability. Its ability to hydrolyze lactose at 40% the rate obtained with ONPG was particularly significant; other *E. coli* galactosidases hydrolyzed lactose at rates of 3% or less than for ONPG.<sup>7</sup>

The properties of the immobilized enzyme were independent of the binding method used, although each procedure involved a different

TABLE V  
Effect of Various Salts on  $\beta$ -Galactosidase Activity

Salt	Relative activities <sup>a</sup>		
	ONPG	PNPG	Lactose
0	1.00	1.00	1.00
$5 \times 10^{-4}M$ MgCl <sub>2</sub>	1.12	1.33	1.19
$5 \times 10^{-4}M$ CaCl <sub>2</sub>	.92	1.04	.66
$5 \times 10^{-4}M$ HgCl <sub>2</sub>	0	0	0
$5 \times 10^{-5}M$ CuSO <sub>4</sub>	0	0	0
$5 \times 10^{-5}M$ BaCl <sub>2</sub>	.79	1.23	1.24
$5 \times 10^{-5}M$ MnCl <sub>2</sub>	1.49	2.20	1.72
$5 \times 10^{-5}M$ FeSO <sub>4</sub>	.89	1.46	1.34
$5 \times 10^{-5}M$ CoCl <sub>2</sub>	1.05	1.83	1.83
$1 \times 10^{-1}M$ NH <sub>4</sub> C <sub>2</sub> H <sub>3</sub> O <sub>2</sub>	.92	1.55	1.06
$1 \times 10^{-1}M$ NaCl	1.19	.99	.95 <sup>b</sup>
$5 \times 10^{-2}M$ K <sub>3</sub> C <sub>6</sub> H <sub>5</sub> O <sub>7</sub>	.37	.79	.90
$5 \times 10^{-2}M$ THAM <sup>c</sup>	.41	.80	.57
$2.5 \times 10^{-2}M$ Na <sub>2</sub> EDTA	.07	.22	.22

<sup>a</sup> Compared to activity in 0.02M potassium phosphate, pH 6.8–7.0, 25°C.

<sup>b</sup> Corrected for interference of Na<sup>+</sup> with glucose oxidase reagent.

<sup>c</sup> Trishydroxymethylaminomethane.

amino acid. This indicated that no functional group essential to the enzymic activity was involved in the covalent bond formation. The apparent 100% efficiency of the coupling methods was most likely attributable to the very low protein concentrations, and would not be expected at higher protein to glass ratios. From the similarity of the Michaelis constants obtained with the three different covalently bound preparations, one can conclude that the active site remains accessible to substrate approach. The decreases in velocity for bound enzymes can be attributed either to steric hindrances or to charge effects which influence product release. Charge effects may be the more likely explanation since this decrease in velocity occurs only for ONPG hydrolysis in the presence of sodium ion. The marked increase in the velocity of the free enzyme and the higher  $K_i$  for galactose observed in this system may be due to similar charge effects. However, this was not a general salt effect since 0.10M KCl had no effect on velocity. The decreased stability observed for the bound enzyme was not expected, and reflects an atypical lability in this enzyme.

From the data presented it is evident that, although immobilization does alter some of the properties and the kinetics of *E. coli* ATCC-26  $\beta$ -galactosidase, the individual methods of bonding used in this study did not differ significantly in their effects.

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