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PREPARATION AND APPLICATION OF IMMOBILIZED β -GALACTOSIDASE
OF SACCHAROMYCES LACTIS

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A variety of dairy products has been prepared in which the lactose has been partially or completely hydrolyzed by soluble β -galactosidases added to milk or whey (Kosikowski and Wierzbicki, 1971; Wendorff et al., 1971; Woychik and Wondolowski, 1973). These products were generally of good quality and acceptability and established the feasibility of using enzymes to hydrolyze lactose. Due to the high cost of soluble β -galactosidase it would appear that use of the enzyme in an immobilized form could offer significant economic advantages in the production of low-lactose dairy products.

Although studies of immobilized β -galactosidases of Escherichia coli (Sharp et al., 1969), Saccharomyces lactis (Dahlqvist et al., 1973), and Aspergillus niger (Olson and Stanley, 1973; Woychik and Wondolowski, 1972, 1973) have been reported in the literature, evaluation of the bound enzymes for lactose hydrolysis in dairy products has been limited primarily to the A. niger β -galactosidase. This enzyme has a pH optimum of 4.0 which limits its efficient use to acid wheys (pH 4.5). Skim milk and sweet whey (pH 6.3-6.8) require the use of a galactosidase with a higher pH optimum. The recent commercial availability of the β -galactosidase of the yeast Saccharomyces lactis (pH optimum, 7.0) prompted us to evaluate it in an immobilized form for lactose hydrolysis near neutral pH's.

This report is concerned with the study of the properties of S. lactis β -galactosidase immobilized on porous glass beads and on a new support, comminuted hide collagen.

MATERIALS AND METHODS

Gorning's Controlled-Pore porous glass beads (mean pore size 2050 A, 80-120 mesh) were purchased from Electro-Nucleonics, Inc. A partially purified β -galactosidase of *S. lactis* (Maxillact) was generously supplied by Enzyme Development Corporation. The fibrous collagen was prepared from comminuted split hides which were dry ground, in the presence of solid carbon dioxide, to pass a 2 mm screen and lyophilized. The comminuted hide collagen is a research product of the Eastern Regional Research Center, Philadelphia. Reconstituted skim milk was prepared from commercial skim milk powder; the sweet whey was obtained from the Dairy Products Laboratory, ERRC, Washington, D.C.

Preparation of Bound Enzyme

The bound enzyme was prepared by two methods. (1) The β -galactosidase was attached to aminoalkylated glass beads using the glutaraldehyde procedures reported previously (Robinson et al., 1971; Woychik and Wondolowski, 1973). The aminoalkyl glass was suspended in a cold 1% aqueous solution of glutaraldehyde for 30 min, rinsed with water, and then suspended in cold phosphate buffer (pH 7, 0.1 M phosphate containing 0.01 M magnesium chloride) containing the β -galactosidase. After 2 hr, the glass was washed with phosphate buffer containing 0.1 M sodium chloride until no soluble galactosidase activity could be eluted. (2) The enzyme was bound to collagen by glutaraldehyde cross-linking. The lyophilized, fibrous collagen was allowed to swell in phosphate-magnesium buffer containing galactosidase (10 ml/g collagen) for 3 hr at room temperature. Sufficient 25% glutaraldehyde was then added to give a final glutaraldehyde concentration of 0.5% and the cross-linking allowed to proceed for 15 min. The collagen-enzyme preparation was washed repeatedly as above until the supernatant was free of galactosidase activity. The amount of protein bound to the supports was estimated by dividing the number of units bound per g support by the specific activity of the soluble enzyme, assuming the bound enzyme retained 100% of its original activity. Using the above techniques, preparations were obtained containing 8-14 mg enzyme protein/g support. Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Enzyme Activity and Lactose Hydrolysis

Enzymatic activity was determined by measuring the amount of glucose released following incubation at room temperature (25°) of either free or bound β -galactosidase with lactose. Glucose was determined using the glucose oxidase procedure of Jasewicz and

Wasserman (1961). Aliquots of digests containing the free enzyme were inactivated by the pH drop accompanying the addition of the glucose oxidase reagent (2 M sodium acetate, pH 4.0) or by placing in a boiling water bath for 3 min. Activities of the immobilized enzymes were determined by pumping substrate in downward flow through columns of bound enzyme or by incubation with a stirred suspension. Appropriate aliquots were analyzed directly for glucose content by the glucose oxidase procedure. A unit of activity is defined as the amount of enzyme which liberates 1 μ mole of glucose/min at room temperature (25°) using 5% lactose as substrate in pH 7.0 phosphate buffer. Specific activity is expressed as units/mg protein.

The kinetic studies were done with the soluble and immobilized enzymes using comparable amounts of activity (0.5-1.0 lactose units) as determined under the standard conditions. One lactose unit was equivalent to 66 μ g of enzyme and was used as the standard weight for the kinetic comparisons. The data were treated by the least squares analysis using the weighting methods of Wilkinson (1961).

RESULTS AND DISCUSSION

Enzyme Immobilization

A wide variety of insoluble supports and methods of enzyme immobilization have been reported; however, no single method or support has been universally accepted. Porous glass beads have probably been the most widely used support and have indeed proven extremely useful in laboratory studies. Collagen offers several advantages over other supports (Venkatasubramanian et al., 1972) and has been utilized in membrane form for the immobilization of a variety of enzymes (Wang and Vieth, 1973). The excellent mechanical strength and hydrophilicity of collagen led us to investigate its potential as an enzyme support in a particulate form.

The lyophilized collagen fibers swell rapidly and take up approximately 100% their weight of buffered enzyme solution. Although maximum buffer uptake required approximately 1 hr, the fibers were normally swelled in buffered enzyme solution for 3 hr. Protein-protein interactions can lead to non-covalent bonding of enzyme to the collagen fibers; however, after 16 hr, less than 0.5 mg enzyme/g collagen was bound in this manner. Therefore, glutaraldehyde was used to form cross-links between the enzyme and collagen. Figure 1 shows that the amount of enzyme bound in the presence of glutaraldehyde depended on the protein concentration, with a maximum binding of 8 mg/g collagen. Collagen preparations containing 8 mg enzyme/g were used in the subsequent experiments. Comparative experiments were done with the glass-bound galactosidase having similar levels of enzymatic activity.

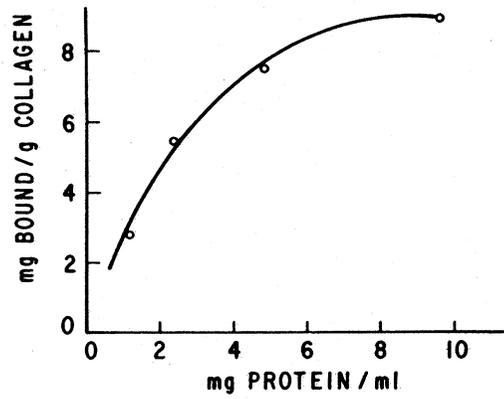


Figure 1. Influence of β -galactosidase concentration on the amount of enzyme bound to collagen.

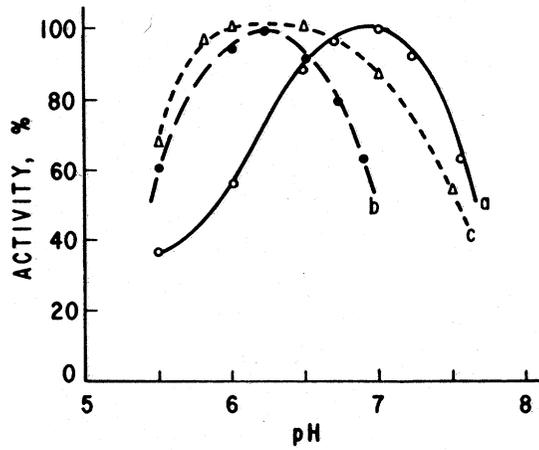


Figure 2. Influence of pH on the activity of *S. lactis* β -galactosidase in free solution (-o-o-), bound to glass (--Δ--Δ--) and bound to collagen (-●-●-).

Characterization of Bound β -Galactosidase

The pH activity curves were obtained for the free and bound enzymes at room temperature, using 5% lactose (0.146 M) in 0.1 M phosphate-0.01 M magnesium chloride buffers. Activities of the bound enzymes were determined in column operations. These results are presented in Figure 2. The maximum activity for the free enzyme (solid curve) occurred at pH 7.0, whereas the optima for the glass and collagen bound galactosidases occurred at pH 6.3-6.5. As noted in Figure 2, the curve for the enzyme bound to glass is quite broad in comparison to the other curves. The shifts in the pH optima can be attributed to either microenvironmental effects or to changes in the enzyme caused by the reaction with glutaraldehyde.

The effect of substrate concentration on the rate of lactose hydrolysis by the free and immobilized galactosidases is shown in Figure 3. The plots reflect the similarity of behavior of the free and bound enzymes. The values for K_M and V calculated from this data are presented in Table I. The slightly higher K_M values determined for the immobilized enzyme can be attributed

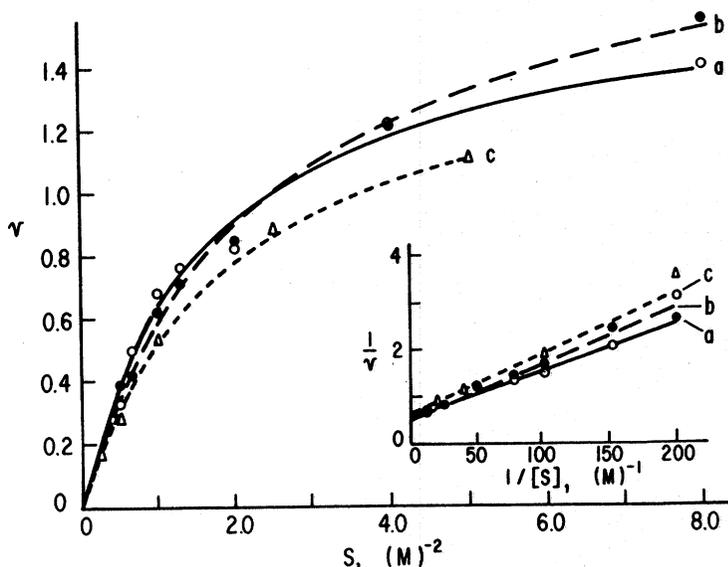


Figure 3. V versus S and Lineweaver-Burk plot showing the effect of substrate concentration on the hydrolysis of lactose by soluble β -galactosidase (a) and by β -galactosidase bound to collagen, (b) and bound to glass (c). The bound enzymes were analyzed in stirred reactors. Velocity is expressed as μ moles glucose released/min/standard amount of enzyme.

to steric factors resulting from covalent bonding to the supports which may cause decreased binding of lactose to the active site. The maximum velocities were comparable for the free and bound enzymes.

TABLE I
Values of K_M and V for the Hydrolysis
of Lactose by Soluble and Immobilized
 β -Galactosidase

	K_M (M)	V^a
Soluble	0.016	1.66
Glass-bound	0.019	1.60
Collagen-bound	0.022	1.92

^a μ moles/min/standard amount of enzyme.

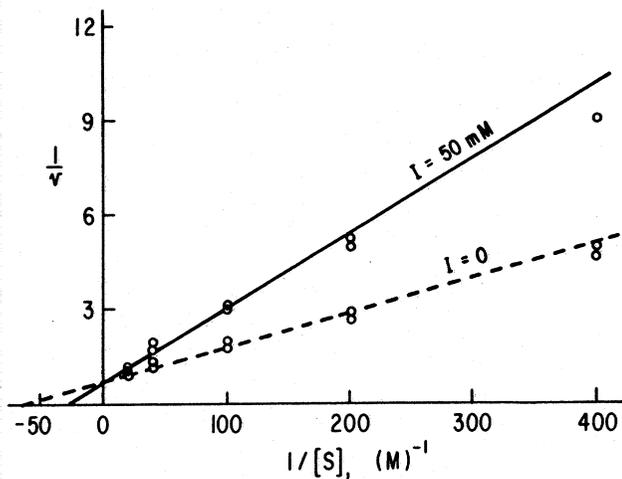


Figure 4. Effect of galactose on the hydrolysis of lactose by soluble β -galactosidase in the presence of varying substrate concentrations. Velocity is expressed as μ moles glucose released/min/standard amount of enzyme.

With regard to the hydrolysis products, only galactose produced competitive inhibition; this is shown in the Lineweaver-Burk plot in Figure 4. The calculated K_i for galactose was 0.042 M.

The β -galactosidase of *S. lactis* is labile to temperatures above 30°; the stability was not materially improved by immobilization. Extended column operation of the bound enzymes was therefore limited to room temperatures. Neither the glass-bound nor collagen-bound galactosidases showed any loss of activity after 5 days of continual hydrolysis of 5% lactose at pH 7.0. After 3-4 days operation at room temperature, bacterial growth began to impair flow-rates. Although large amounts of bacteria could be removed from the supports by batch washing procedures, sufficient bacteria remained adsorbed and caused renewed plugging and channeling after 10-20 hr operation. A number of bactericides were studied to determine their potential use as column sanitizing agents. Hydrogen peroxide, benzoic acid, phenol, and iodofors all caused either partial or total inactivation of the galactosidase after 30 min exposure to working concentrations of these reagents. Although column operations at low temperatures (5-10°) reduced bacterial growth, column plugging still resulted after several days operation.

Application of Immobilized β -Galactosidase

The activity of the β -galactosidase toward lactose was influenced by the non-lactose solids in sweet whey and skim milk. At comparable pH's and lactose concentrations, the activity was decreased approximately 10% in sweet whey and 13% in skim milk when compared with the activity in buffered lactose solutions. These reductions in activity were also observed with the *A. niger* β -galactosidase (Woychik et al., 1973).

TABLE II
Hydrolysis of 5% Lactose^a by
Collagen-Bound β -Galactosidase

Flow-Rate, l./hr	% Hydrolysis
2.3	93
3.6	84
4.4	80
5.2	76

^ain 0.1 M phosphate, pH 6.7 containing 0.01 M magnesium chloride.

Some results obtained with a 70 g collagen-galactosidase column (4.5 x 35 cm) are presented in Table II. The amount of lactose hydrolyzed at pH 6.7 decreased with increased flow-rates and indicates that 75% or more hydrolysis is obtained at flow-rates up to 5 l./hr. A large column containing 375 g of collagen-bound galactosidase was operated at room temperature for the hydrolysis of lactose in sweet whey. Better than 85% hydrolysis was obtained at flow-rates up to 18 l./hr.

Although the collagen columns could be operated in downward flow for several days at slow flow-rates, rapid column packing resulted from flow-rates greater than 1 l./hr. Upward flow is therefore recommended for all column operations with collagen.

SUMMARY

The β -galactosidase of *S. lactis* has been immobilized to porous glass beads and to a new support, comminuted collagen. Collagen has been demonstrated to be comparable to other supports currently in use, and because of its hydrophilicity, it may facilitate substrate diffusibility in aqueous systems. The new properties conferred on the collagen by the reaction with glutaraldehyde make the tanned fibers quite suitable for batch or column operations.

The *S. lactis* β -galactosidase appears to have the necessary characteristics for successful applications to the hydrolysis of lactose in a variety of dairy products. The enzymic properties remain essentially unaltered after coupling with glutaraldehyde to either glass or collagen. This is reflected in the comparable kinetic data for the soluble and the immobilized forms.

The problem of bacterial growth associated with rich nutrient fluids, such as milk and whey, remains the major obstacle to commercial adaptation of an immobilized β -galactosidase system. The bacteria adsorbed to the insoluble supports, and their associated growth, result in column-plugging and in a generally unsanitary situation. There remain to be developed adequate bactericides or bacteriostats which will permit routine sanitization of immobilized enzyme reactors.

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