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Immobilization of a Lactase onto a Magnetic Support by Covalent Attachment to Polyethyleneimine-Glutaraldehyde-Activated Magnetite

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ABSTRACT

A magnetic immobilized lactase has been prepared using magnetite as the magnetic material. Magnetite was functionalized by treatment with polyethyleneimine and crosslinked with glutaraldehyde. Lactase was then covalently coupled to the activated magnetic matrix via the aldehyde groups. The conditions for optimal immobilization of enzyme are described. Eighty percent of the lactase activity was lost on immobilization and is thought to be owing to the orientation of enzyme binding to the matrix. The amount of protein coupled was 80% of that applied. The maximum lactase activity retained on the matrix following immobilization was 360 U/g matrix. The immobilized lactase showed optimal activity at pH 4.5 and 65°C. The immobilized lactase was more heat stable than the free enzyme, and retained 83% of its original activity after 14 d at 55°C. Galactose competitively inhibited the immobilized lactase preparation (Ki 20 m*M*). The presence of high initial concentrations of galactose (10% w/v) did not prevent total hydrolysis of lactose. Glucose and calcium ions were activators of the immobilized enzyme. The immobilized enzyme hydrolyzed high concentrations of lactose (up to 25% w/v) to completion within 4–6 h in a stirred batch reactor at 55°C. There was no evidence of substrate inhibition at high substrate concentrations. The efficiency of hydrolysis of lactose by the immobilized lactase was better than that of the free enzyme. The magnetic immobilized lactase was demonstrated to be suitable for use in the enzymatic hydrolysis of both pure, and cheese whey permeate, lactose.

Index Entries: Magnetically immobilized lactase; polyethyleneimine-glutaraldehyde activated magnetite; conditions of immobilization; enzymatic hydrolysis of lactose.

INTRODUCTION

The application of magnetism to biotechnology can be useful in the recovery of enzymes (and cells) through their immobilization onto magnetic supports. This makes use of their acquired magnetic properties and allows their selective recovery from solution and from suspended-solids by application of an electromagnetic field. The supports may be porous or nonporous. Nonporous magnetic supports (e.g., iron, iron and chromium oxides, nickel) have advantages over porous supports in that they appear to be more resistant to fouling, diffusional limitation, and attrition (*1*). Porous supports, however, offer a greater surface area for immobilization. Nonporous magnetic supports can also be prepared that are composed of highly crosslinked organic polymers with encapsulated magnetic particles (e.g., γ—Fe_2O_3, Fe_3O_4, CrO_2) (*2*).

Inorganic matrices such as glass, ceramics, metal oxides, and magnetic materials such as magnetite generally have few, or no, functionalized pendant groups on their surfaces that can be used to covalently couple enzymes. It is thus necessary to functionalize these support materials to provide binding sites to covalently link an enzyme to the matrix. There are numerous reports in the literature describing the immobilization of enzymes onto inorganic support matrices (*3*). Many techniques for improving the enzyme loading capacity of nonporous inorganic matrices have been developed. This can be accomplished using the commonly reported method of silanization (*4*), which produces an aminopropyl-activated matrix; or by coating the matrix with polyamines such as proteins (e.g., bovine serum albumin (*5*)), polylysine (*6*), polyglucosamines such as chitosan (*7*), and polyethyleneimine (*8,9*), and crosslinking them with a bifunctional reagent (e.g., glutaraldehyde); or by chelation using a transition metal (e.g., Ti) to form metal-salt bridge adducts (*10*).

Enzymes have been immobilized onto magnetic supports

1. Through direct physical adsorption onto the magnetic material and crosslinked with a bifunctional reagent (e.g., glutaraldehyde) (*11*);
2. By adsorption of nonspecific proteins (e.g., bovine serum albumin) onto the magnetic material followed by crosslinking with glutaraldehyde and coupling enzyme to the crosslinked protein by covalent attachment (*12*);
3. By silanization of the magnetic material followed by covalent attachment (*13*);
4. By formation of a metal–salt bridge conjugate that can immobilize (*10,14*) the enzyme by using the chelation properties of a transition metal (e.g., Ti, Zr); and

5. By encapsulating magnetic particles within a polymer matrix (organic (*15*) or biologic (*14,16*)), on which the enzyme can be covalently coupled.

Previous attempts to immobilize β-glucosidase onto magnetite by the silanization technique, and also by formation of transition metal complexes through chelation using $TiCl_4$, resulted in low immobilized enzyme activities (unpublished results). Precipitation of a layer of hydrous titanium (IV) oxide onto magnetite, as outlined in ref. (*10*), produced a magnetic β-glucosidase immobilized preparation of high activity, but resulted in enzyme leaching from the matrix. These methods thus proved to be unsatisfactory for use with magnetite, and prompted a search for other procedures that could produce functionalized pendant groups on the surface of magnetite that, in turn, could be employed to covalently couple enzymes. Activation of inorganic carrier supports (*8*), including a magnetic calx (prepared by calcining an inorganic oxide and Fe_3O_4) (*17*), by using polyethyleneimine (PEI) crosslinked with glutaraldehyde, has been reported to be suitable for enzyme immobilization. It was of interest, therefore, to determine whether the same activation approach could be used directly with magnetite (Fe_3O_4), thereby producing a magnetic matrix suitable for enzyme immobilization. This investigation thus formed the basis of the work reported herein and describes the preparation of a magnetic immobilized lactase (β-D-galactosidase, EC 3.2.1.23). Also reported in this paper are the physicochemical properties of the magnetically immobilized lactase and its use in the hydrolysis of cheese whey lactose.

MATERIALS AND METHODS

Materials

Biolactase, a powdered lactase preparation derived from *Aspergillus oryzae,* was kindly donated by Biocon (Australia) Pty. Ltd., Australia. Magnetite was obtained from Steetley Chemicals, Melbourne, and was pretreated with HCl and NaOH prior to use. Polyethyleneimine (50% aqueous solution), glutaraldehyde (Grade 1, 25% aqueous solution), *O*-nitrophenyl-β-D-galactopyranoside, glucose oxidase (Type V, *Aspergillus niger*), peroxidase (Type II, horseradish) and *O*-dianisidine dihydrochloride, were purchased from Sigma Chemical Co. Cheese whey ultrafiltered permeate lactose was a kind gift from United Milk Tasmania, Australia.

Analytical Procedures

Protein was determined by the Lowry method (*18*) as modified by Hartree (*19*). Bovine serum albumin (Sigma, Fraction V) was used as the standard.

Glucose resulting from the hydrolysis of lactose was determined by a modified glucose oxidase method, as described elsewhere (*20*).

Enzyme Assays

β-Galactosidase

β-Galactosidase activity was assayed by measuring the amount of *O*-nitrophenol (ONP) liberated from *O*-nitrophenyl-β-D-galactopyranoside (ONP Gal) by a procedure that was similar to that described for β-glucosidase (*20*).

Lactase

Lactase activity was determined with lactose as substrate. The amount of glucose produced as a consequence of hydrolysis was measured by the glucose oxidase method, as described above. Lactase activity was assayed by incubating lactose (0.8 mL of a 25% (w/v) lactose solution made up in 50 m*M* acetate buffer, pH 5.0) with enzyme (0.1 mL of a suitably diluted enzyme solution) and buffer (0.1 mL) at 50°C for 10 min. An aliquot (usually 0.1 mL) was withdrawn, suitably diluted with water, and a portion of this (e.g., 0.1 mL) assayed for glucose by the glucose oxidase method. In assaying immobilized lactase activity, free enzyme was replaced by the magnetic immobilized lactase preparation (25–50 mg dry wt). The suspension was then demagnetized (Bulk Tape Eraser, Realistic) and the digest incubated at 50°C for 10 min while shaking (100–150 rpm). All enzymic digests and assays were performed in duplicate.

The unit (U) of lactase activity is defined as the amount of enzyme required to liberate 1μ mole glucose from lactose per min under the conditions of assay. The unit of lactase activity is expressed as μ moles glucose/min/mL for the free enzyme, and as μ moles glucose/min/g immobilized matrix for the immobilized enzyme.

Enzyme Immobilization Onto Polyethyleneimine-Glutaraldehyde-Activated Magnetite (General Procedure)

Magnetite (1–10 g) was treated with an aqueous solution of 10% (w/v) polyethyleneimine (PEI) at a ratio of 4 mL PEI solution per g magnetite. The suspension was stirred at 100 rpm for 1 h at room temperature (RT). Excess PEI solution was removed by decanting and the PEI-coated magnetite recovered by filtration on a sintered-glass filter and air-dried.

The PEI-coated magnetite was then treated with 25% (w/v) aqueous glutaraldehyde (1.5 mL per g PEI-magnetite) and left at RT for 1h. Excess glutaraldehyde was decanted and the PEI-glutaraldehyde-activated magnetite (referred to hereafter as PGM) thoroughly washed with water until there was no odor of glutaraldehyde remaining. The matrix was recovered by filtration and air-dried.

The immobilization step consisted of adding enzyme (Biolactase, 20 mg powder) and 50 m*M* acetate buffer (pH 5.0) in a final volume of 10 mL to PEI-glutaraldehyde-activated magnetite (1 g) followed by shaking the

suspension in a reciprocal shaker (100 rpm) at 5°C for 16–20 h. The immobolized enzyme preparation (IME) was recovered by decanting off the supernatant while applying a magnetic field followed by thoroughly washing the IME with 50 m*M* acetate buffer (pH 5.0), then twice with 20–30 mL volumes of 1 *M* NaCl in 50 m*M* acetate buffer (pH 5.0) and the washings collected, followed by further washing with buffer. The IME was collected on a sintered-glass filter, air-dried, and stored at 5°C. The above washing protocol was used on all immobilized enzyme preparations after the enzyme immobilization step.

Magnetite, and enzyme-immobilized magnetite, was demagnetized prior to the activation and immobilization steps, and also before use in enzymic digests. This practice avoided formation of aggregates of magnetite, which occurs during magnetization, and which would otherwise reduce the available surface area of the magnetic matrix during use.

Experimental Conditions in Optimizing Enzyme Immobilization

Factors Affecting PEI Coating of Magnetite

Aqueous PEI solutions of concentrations ranging from 2–18% (w/v) were prepared from a 50% PEI stock solution and 4 mL volumes of these added to 1 g magnetite and then left at RT for 1h whilst shaking (100 rpm). Each of the various PEI-coated magnetite preparations were then crosslinked using 25% (w/v) aqueous glutaraldehyde (1.5 mL/g matrix, 1 h at RT) and enzyme immobilized, as described above. In further experiments, PEI at a concentration of 10% (4 mL/g magnetite) was used to examine the following—the effect of temperature (RT, 30, 40, and 50°C for 1 h) on PEI coating of magnetite, the influence of time of contact (1, 2, 4, and 6 h at RT) of PEI with magnetite, the effect of drying (100°C/18 h) the PEI-coated magnetite prior to crosslinking with glutaraldehyde, and the influence of the pH of the PEI solution (>10.5, 8.5, 6.5) during the coating step.

The Effect of Glutaraldehyde Concentration

PEI-coated magnetite (1 g, coated with a 10% PEI solution) was reacted with aqueous glutaraldehyde solutions of concentrations ranging from 1 to 25% (w/v) at RT for 1 h while shaking (100 rpm). The volume of glutaraldehyde per g PEI-coated magnetite was examined using 25% aqueous glutaraldehyde.

The Effect of the Amount of PEI-Glutaraldehyde-Activated Magnetite

PGM was prepared using 10% PEI and crosslinked using 25% glutaraldehyde, as described above. Lactase was immobilized using a constant amount of enzyme (2765 U lactase) and variable amounts of PGM (rang-

ing from 0.3 to 2.5g) in a final volume of 10 mL. The suspension was incubated at 5°C overnight with shaking.

The Effect of Protein Loading

A standard solution of lactase (2175 U lactase and 8.89 mg protein per mL) was used to prepare enzyme solutions of protein concentrations ranging from 2.22 to 22.3 mg/10 mL. To 10 mL aliquots of enzyme solution were added 1 g PGM (prepared using 4 mL 10% PEI/g and 1.5 mL 25% glutaraldehyde) and the suspensions incubated at 5°C for 16–20 h.

Enzyme Coupling Efficiency

Enzyme coupling efficiencies were calculated from the expression

$$E_{IME} / E_A - E_R \times 100\%$$

where E_{IME} is the enzyme activity of the IME preparation (U/g); E_A, total enzyme applied for immobilization (U); and E_R, total enzyme recovered following immobilization (U).

Extent of Protein Coupling

The amount of protein disappearing (presumed to have been covalently coupled to the matrix) during immobilization was determined by difference, i.e., total protein applied minus protein remaining after immobilization.

Physicochemical and Kinetic Characterization of the Immobilized Lactase Preparation

Details of the methods used to characterize the physicochemical and kinetic properties of the immobilized enzyme were essentially those that were described elsewhere (*21*).

The Effect of Galactose on Immobilized Lactase Activity

IME-lactase (~25 mg) was incubated with 20% lactose in 50 m*M* acetate buffer (pH 5.0, 1 mL) and varying amounts of D-galactose (0 to 10%, w/v) at 55°C for 10 min. Enzyme activity was assayed by measuring the amount of glucose produced. In another experiment, IME-lactase (~25 mg) was incubated with 20% (w/v) lactose solution containing 10% (w/v) galactose (1 mL) in the presence (final concentration in the digest was 150 m*M* $CaCl_2$) and the absence of Ca^{++} ions at 55°C for 10 min. Aliquots were removed, suitably diluted, and assayed for glucose.

The Effect of Glucose on Immobilized Lactase Activity

IME-lactase (~25 mg) was incubated with 20% lactose containing 0–50 mg glucose (1 mL) at 55°C for 10 min. Aliquots were removed, appropriately diluted to compensate for the large amount of glucose initially present, and then assayed for glucose. Appropriate blanks were included that contained buffer, lactose, and glucose but no enzyme, and were diluted to the same extent as those of the lactose hydrolyzates prior to assay for glucose. The glucose values of the blanks were deducted from the glucose values obtained from the lactose hydrolyzates. The corrected glucose values were then used to calculate the lactase activity.

Enzymic Hydrolysis of Lactose

Enzymic digests contained lactose (1 mL, 20% (w/v) pure lactose, or whey permeate lactose, in 50 m*M* acetate buffer, pH 5), and IME-lactase and were incubated at 55°C in a stirred batch reactor. At each time interval, aliquots were withdrawn, suitably diluted with water, and portions removed for glucose assay.

Following hydrolysis, the IME was recovered with the aid of a magnet, washed well with water and buffer, and reused for a further hydrolysis period. The hydrolysis procedure was repeated several times using the same IME; recycling procedure.

RESULTS AND DISCUSSION

Enzyme Immobilization

Functionalization of magnetite with PEI and glutaraldehyde produced a magnetic matrix that was found to be suitable for immobilizing lactase. The immobilized enzyme preparation was stable and showed no evidence of enzyme leaching. At the pH of PEI (pH>10), PEI does not bind covalently, or ionically, to the magnetite. At alkaline pH, magnetite (pI ~6.0) is negatively charged, whereas the NH_2 groups of PEI remain largely undissociated. The PEI thus forms a surface layer (or coat) on the magnetite that is easily removed upon suspension in an aqueous solution or on washing. This can be prevented by crosslinking with glutaraldehyde, which entraps the magnetic particles within a polymer network, and also provides aldehydic groups that can be employed to covalently couple enzyme.

The influence of PEI concentration on the amount of lactase immobilized onto magnetite is shown in Fig. 1. A PEI concentration of about 10% appeared optimal for producing an immobilized enzyme preparation that contained maximal lactase activity. The temperature at which PEI

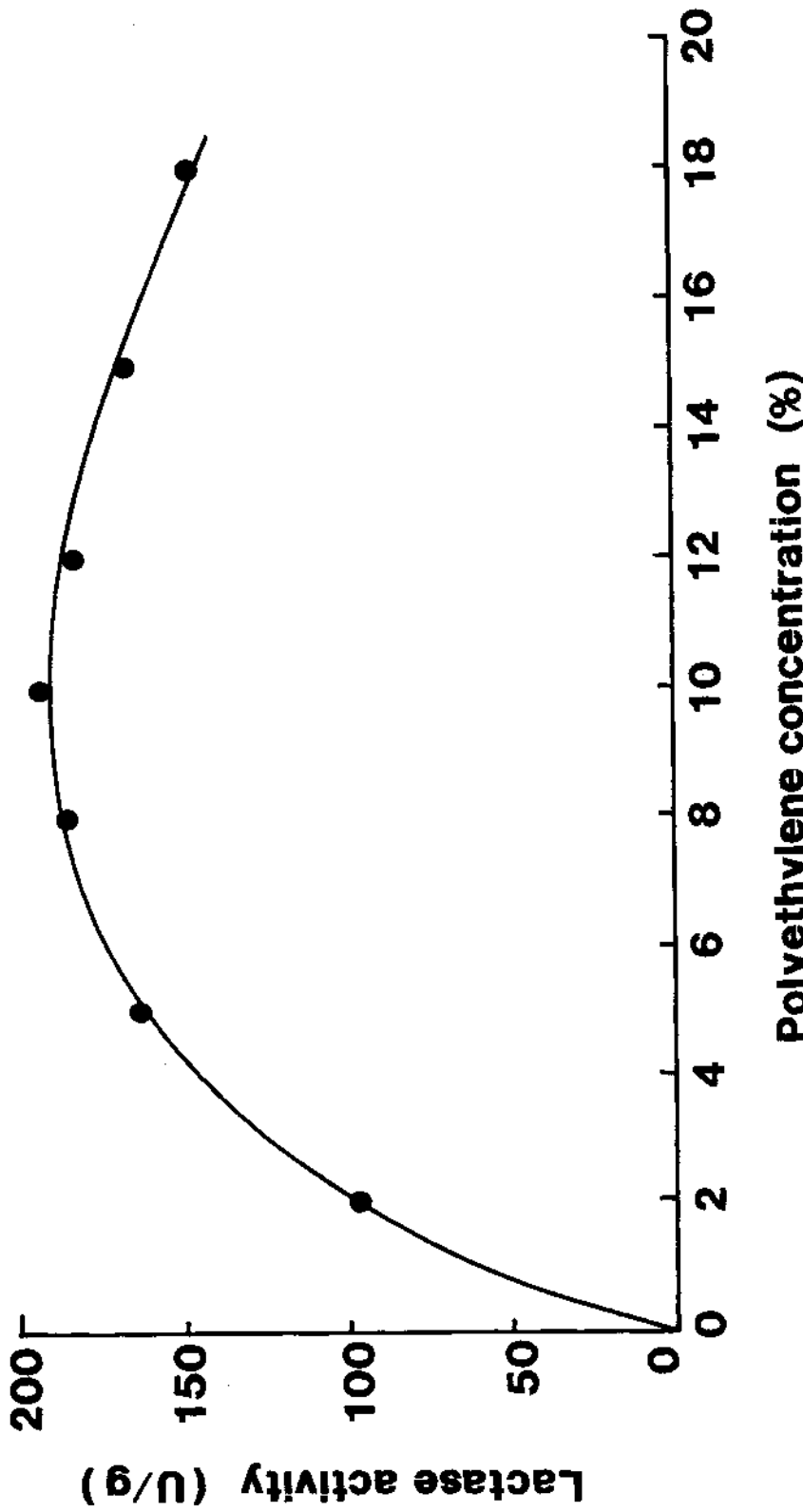

Fig. 1. The influence of polyethyleneimine concentration on immobilization of lactase. The glutaraldehyde concentration used was 25% (w/v).

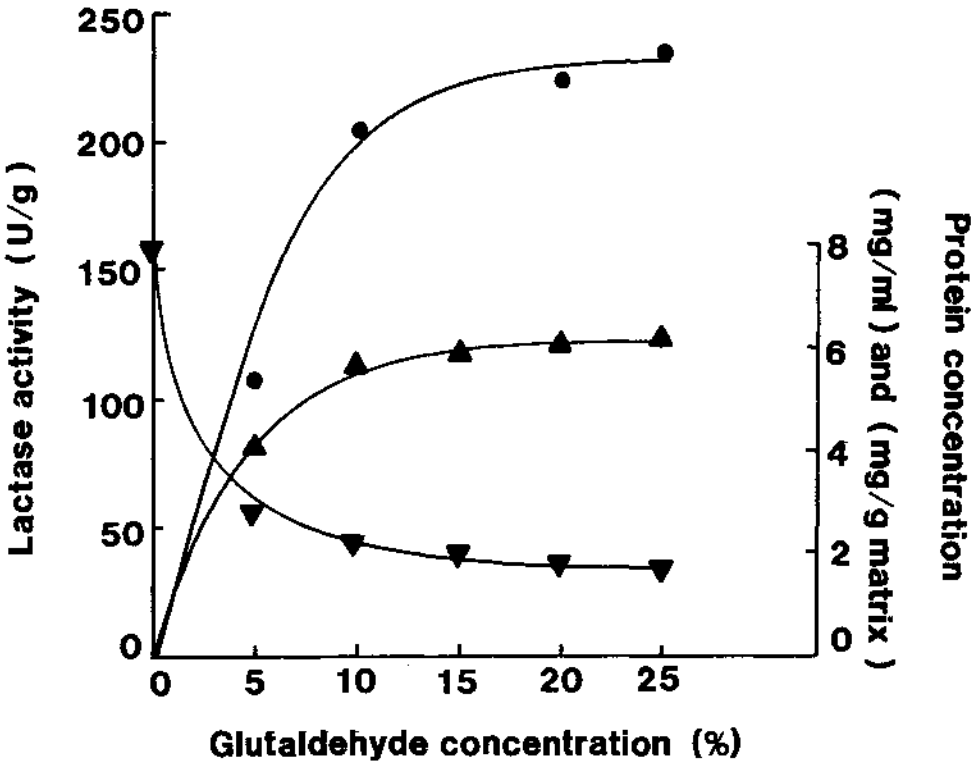

Fig. 2. The effect of glutaraldehyde concentration on immobilization of lactase. The polyethyleneimine concentration used was 10% (w/v). ●, immobilized lactase activity; ▲, protein coupled; ▼, protein remaining in solution.

was coated onto magnetite did not appear to influence the extent of coating of PEI, as judged by the amount of lactase activity on the IME preparation. Temperature did, however, affect enzyme immobilization when the PEI-coated magnetite was dried by heating at 100°C (18 h) prior to the crosslinking step. This resulted in a marked decrease in the immobilized lactase activity (162 U/g matrix c.f. 320 for no heating). The pH of the PEI solution during coating of the magnetite also markedly affected the final immobilized lactase activity, being less as the pH was progressively lowered. The effect of time at which magnetite was in contact with PEI did not appear to influence the amount of enzyme that was coupled when the magnetite was exposed to PEI for periods of up to 6 h at ambient temperature.

The relationship between glutaraldehyde concentration and the amount of lactase immobilized is shown in Fig. 2. Maximum coupling of enzyme occurred at high glutaraldehyde concentrations (20–25%, w/v). At low glutaraldehyde concentrations, most of the aldehydic groups appeared to be involved in crosslinking PEI and were thus not available for enzyme immobilization. The effect of the volume-to-weight ratio of glu-

taraldehyde to PEI-magnetite did not differ significantly beyond 1.0 mL/g when using 25% (w/v) glutaraldehyde, and so 1.5 mL was chosen as a convenient working volume.

The relationship between protein loading of the crude commercial lactase preparation per g activated matrix (i.e., PGM) and immobilized lactase activity is shown in Fig. 3. Maximum immobilized lactase activity occurred at a protein loading of about 10 mg/g PGM. No further increases in immobilized lactase activity occurred beyond 10 mg protein/g matrix, although further coupling of protein continued to about 20 mg protein/g matrix. The further coupling of protein to the matrix may have been owing to that of nonenzyme protein (the enzyme preparation used was crude), or that enzyme protein was further bound but in an inactivated form through binding to the active center groups.

Figure 4 shows the relationship between the amount of activated magnetite (PGM) and the amount of lactase immobilized when offered a constant amount of lactase during immobilization. The amount of lactase immobilized was directly proportional to the amount of PGM. The amount of lactase remaining in solution after immobilization decreased with increasing amounts of PGM and was presumed to have coupled, albeit in an inactive form, since its disappearance could not be accounted for by higher lactase activity on the matrix.

The highest immobilized lactase activity obtained in this study was around 360 lactase U/g matrix. This represented an enzyme coupling efficiency of about 20%. Thus, 80% of the enzyme activity was lost upon coupling, or inactivated during the immobilization step. The amount of protein coupled represented about 80% of that applied. When the results (*see* Fig. 3) were expressed in terms of specific activity (i.e., units of lactase activity per mg protein coupled per g matrix) and plotted against protein loading, then the optimal protein loading appeared to be around 5 mg/g matrix (data not shown), and resulted in a specific activity of 60 lactase U/mg protein coupled/g matrix.

The reason(s) for the low enzyme coupling efficiencies (i.e., low immobilized lactase activities) is not known. Factors such as surface charge (the zeta potential of the immobilized lactase preparation was +3.72 mV, which indicates a net positive surface charge), surface chemistry phenomena, binding of enzyme at the active center sites, and conformational changes in enzyme induced upon binding, may influence retention of enzyme activity on the IME preparation. Cowan et al. (22) found similar low lactase activities upon immobilization and ascribed this to the orientation of enzyme binding that sterically hindered the active site.

Physicochemical and Kinetic Properties

The physicochemical properties of the free and immobilized lactase preparations are summarized in Table 1. Optimal lactase activity occurred at 65°C for both forms of the enzyme. The pH optimum of 5.0 for the free

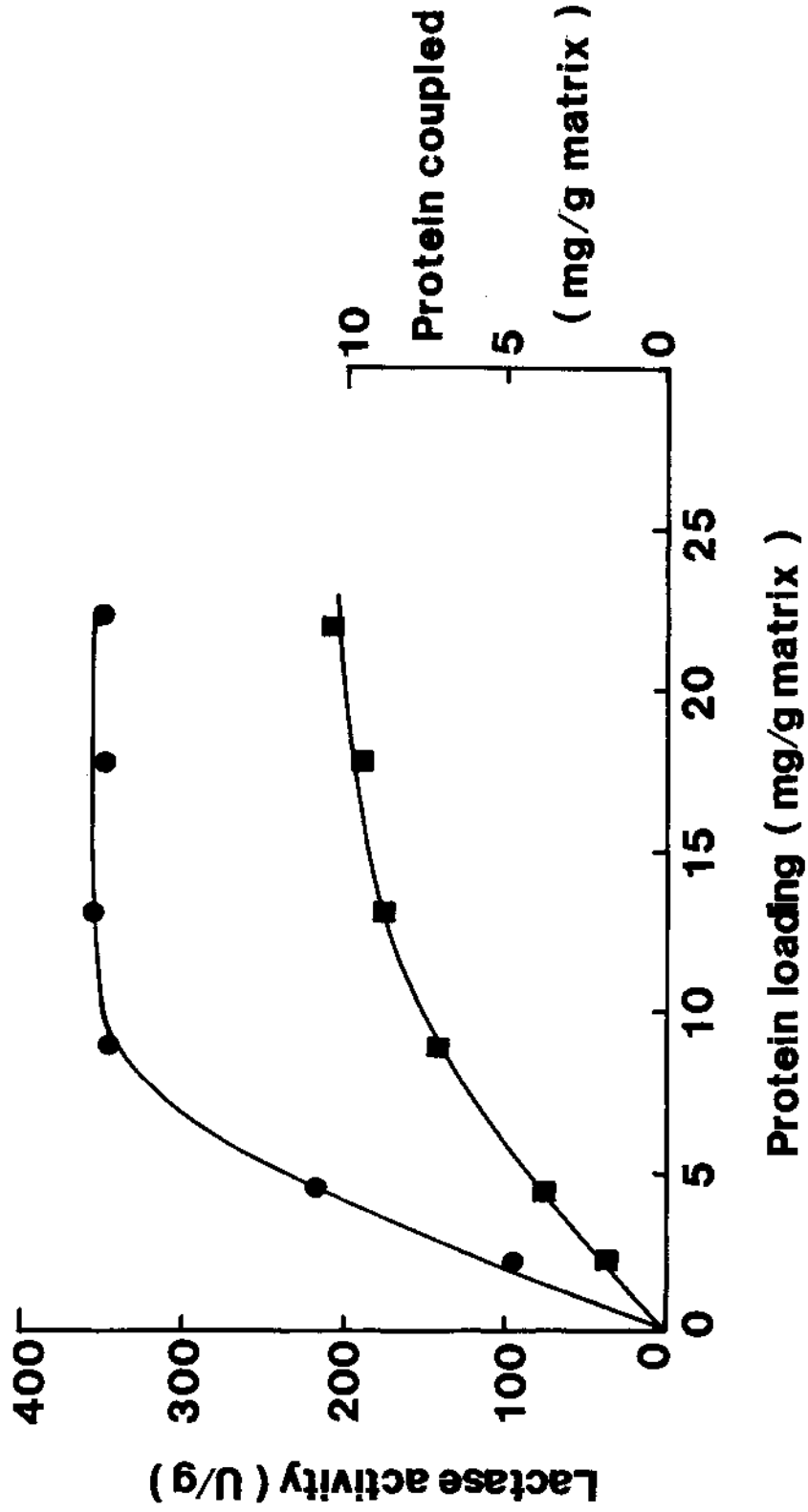

Fig. 3. The relationship between protein loading and lactase immobilized. ●, immobilized lactase activity; ■, protein coupled.

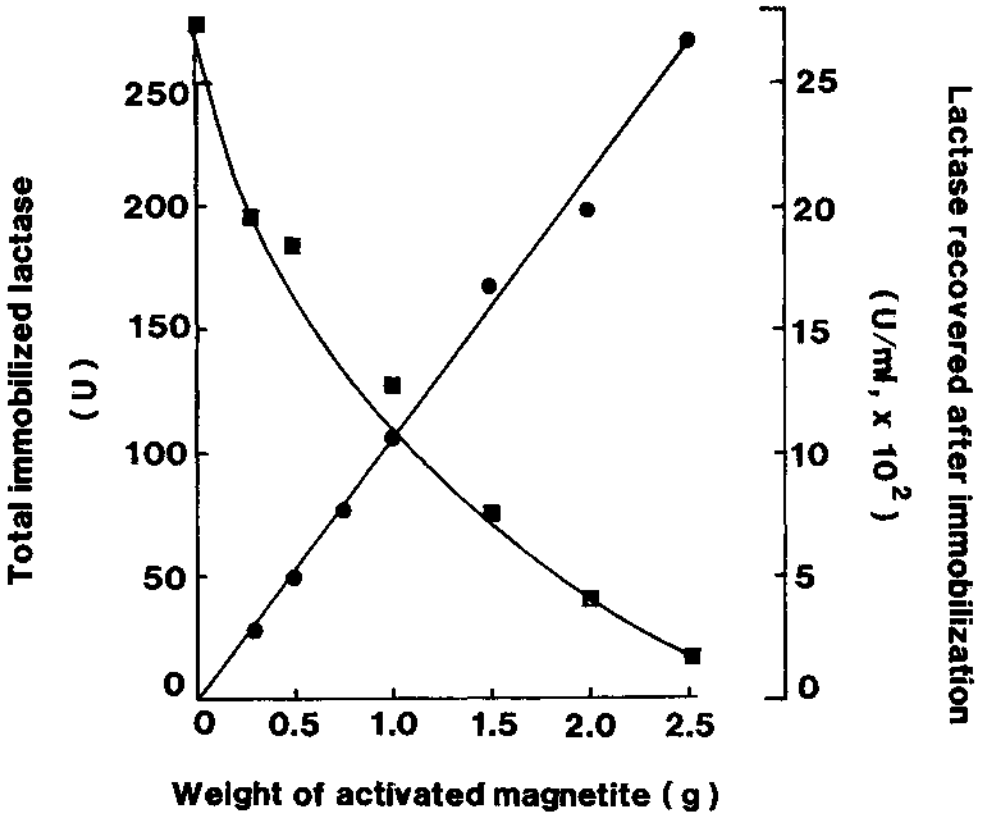

Fig. 4. Relationship between activated matrix (PGM) loading and lactase immobilized per constant amount of enzyme applied for immobilization. ●, immobilized lactase activity; ■, lactase remaining in solution following immobilization.

enzyme was lowered to 4.5 upon immobilization of the enzyme. Similar pH shifts have also been reported for other lactases when immobilized (23). The immobilization of lactase also narrowed the pH range over which the IME was stable. The activation energy (E_A) for both the free and immobilized lactase was similar, and compares to that of an IME-lactase from *A. niger* (24). However, the deactivation energy (E_{DA}) differed significantly (Table 1), being lower for the immobilized enzyme and indicated that the immobilized lactase was less susceptible to heat inactivation than the free enzyme. The marked difference between E_A and E_{DA} of the free and immobilized lactase preparations indicated that conformational changes of the enzyme occurred upon immobilization, which conferred thermal stability on the enzyme. Evidence to support this was furthermore corroborated from thermostability profiles (data not shown) of both enzyme forms. The free enzyme was less stable to heat and showed a half-life of 4.5 h at 50°C, and 0.5 h at 60°C. The immobilized lactase preparation, by contrast, was remarkably heat stable, and when kept at 55°C

Table 1
Comparison of the Physicochemical and Kinetic Properties of the Free and Immobilized Lactase Preparations

Property	Free lactase	Immobilized lactase
pH optimum	5.0	4.5
pH stability range	3.5–7.5	4.5–6.5[a]
Temperature optimum	65°C	65°C
Temperature stability range	0–50°C	ND
Stability half-life	4.5 h at 50°C/0.5 h at 60°C	>14 d at 55°C
Activation energy, kJ/mole/°K	29.9[b]	31.4
Deactivation energy, kJ/mole/°K	−158.3[b]	−273.6
K¹m, ONP Gal,	1.54 m*M*	ND
lactose	84.1 m*M*	218.4 m*M*
Vm, ONP Gal,	200[c]	ND
lactose	234[c]	45.2[d] (236.6)[e]
Inhibitor	galactose	galactose
Type of inhibition	ND	competitive
Ki (galactose)	ND	20 m*M*
Activator	ND	Glucose
Relationship between enzyme activity and time of hydrolysis	linear up to 15 min	linear up to 45 min

[a]1 h at 55°C.
[b]Using ONPGal as substrate.
[c]μmoles/min/mg protein.
[d]μmoles/min/mg coupled-protein/g matrix.
[e]μmoles/min/g IME.
[f]Partial activator.

(in buffer) for 14 d retained 83% of its original enzyme activity. There was a linear relationship between lactase activity and time of hydrolysis for both forms of enzyme (Table 1).

Salts such as Ca, Na, and K are present in milk and cheese whey in substantial amounts and have been reported (*25*) to affect lactase activity. The extent to which the cations affect lactase activity was dependent upon the source of the enzyme (*25*). Concentrations as high as 1000 m*M* NaCl did not appear to affect IME-lactase activity, whereas Ca^{++} as high as 300 m*M* activated the enzyme. Optimal IME-lactase activity occurred at 150 m*M* and resulted in an increase of 22% in immobilized lactase activity.

Lactose hydrolysis by the IME-lactase showed typical Michaelis-Menten kinetics behavior. Both the free, and immobilized, lactase preparations were not inhibited by substrate at concentrations as high as 25% (w/v) lactose, or 10 m*M* ONPGal. The apparent Km and Vm values for both forms of lactase are presented in Table 1. These values were deter-

mined from several different forms of plot (e.g., Lineweaver-Burk, Hanes, and Woolf-Hofstee plots), and were computed from regression analysis of lines of best fit. The IME-lactase showed a lower affinity (high Km) for lactose than the free enzyme, whereas the Vm of the free enzyme was correspondingly higher (5.2-fold) than that of the immobilized enzyme. The change in Km and Vm of lactase upon immobilization indicates evidence of steric hindrance (*26*). Thus, the enzyme freed from the constraints of immobilization has a kinetic advantage over the enzyme-bound form.

Galactose is well-known as an inhibitor of β-galactosidase (and lactase) activity, usually inhibiting the enzyme competitively (*25*). The effect of initial galactose concentration on immobilized lactase activity is shown in Fig. 5 and demonstrated that up to 85% inhibition occurred at 10% (w/v) galactose concentration. A similar observation has also been reported by others (*22,27*). It should be borne in mind that initial high concentrations of galactose do not normally exist in the early stages of lactose hydrolysis. As hydrolysis proceeds, more and more galactose is produced that will, during the final stages of hydrolysis, exert a pronounced affect on lactase activity; hence the asymtotic curves seen in Fig. 5.

The inhibition constant (Ki) for the immobilized lactase was determined from a Dixon plot (data not shown) and was found to be 20 m*M* galactose. This value is rather high compared to other IME-lactases (*23,27*). The Lineweaver-Burk plot (data not shown) at different galactose concentrations revealed that galactose inhibited the immobilized enzyme in a competitive manner. The inhibitory effect by galactose was furthermore found to be reversible, and full lactase activity of the immobilized enzyme preparation was restored following the removal of galactose. An examination of the effect of glucose (the other hydrolysis product) on IME-lactase activity revealed that glucose activated the immobilized enzyme preparation (Fig. 6a). This observation was in contrast to that reported (*28*) for a lactase from *Escherichia coli*, which was uncompetitively inhibited by glucose (Ki 630 m*M*), and an IME-lactase from a *Thermus* sp. (*22*), which was strongly inhibited by glucose. A replot of the slopes and intercepts from a Lineweaver-Burk plot (Fig. 6a) against glucose concentration (Fig. 6b) furthermore indicated that glucose was a partial activator of IME-lactase activity. Thus, the lactase preparation was inhibited by galactose but activated by glucose. This unusual behavior probably explains why this lactase preparation (from *Aspergillus oryzae*) was capable of hydrolyzing high concentrations of lactose (20–25%, w/v) to completion (*see below*).

Enzymic Hydrolysis of Lactose

Lactose was hydrolyzed batchwise in a stirred reactor at 55°C and at a concentration of 20%, w/v. The rate and extent of hydrolysis of lactose by the immobilized lactase preparation was dependent upon the amount of

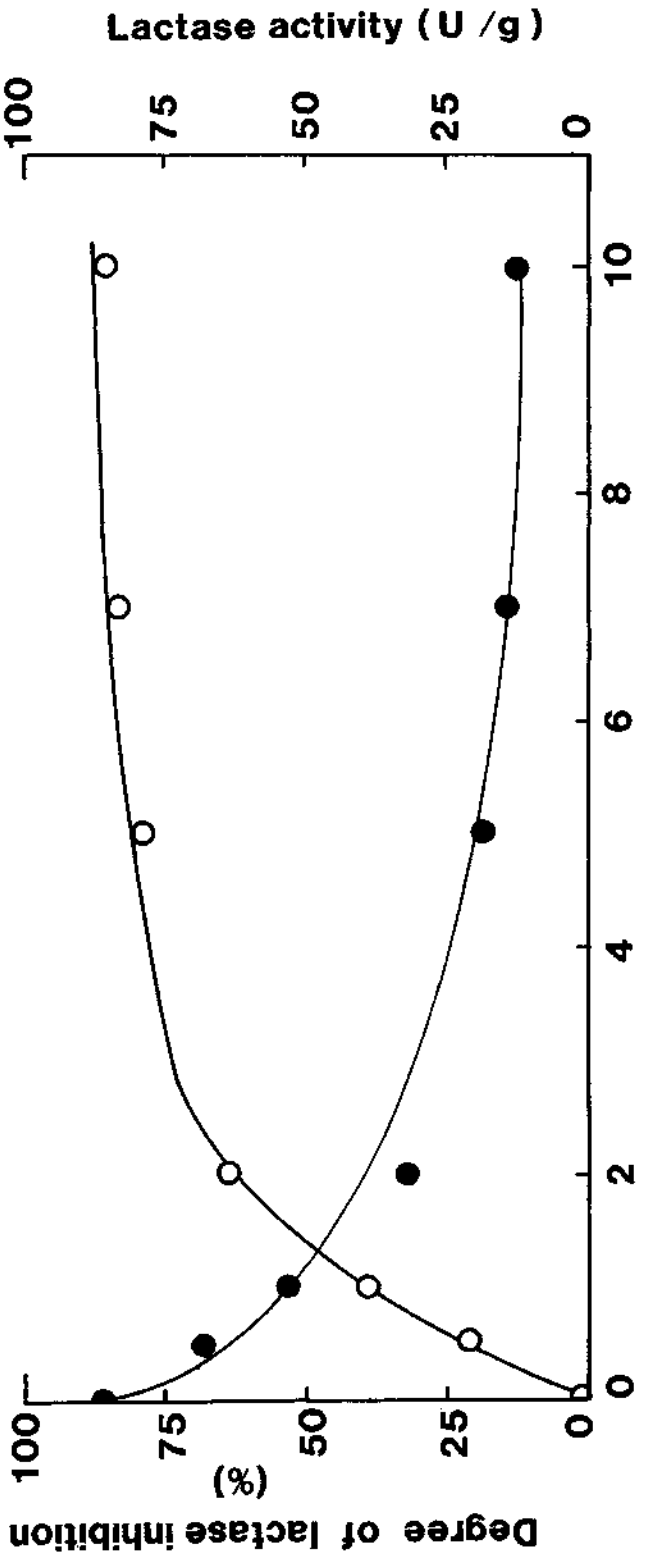

Fig. 5. Influence of galactose on immobilized lactase. ○, degree of inhibition; ●, immobilized lactase activity.

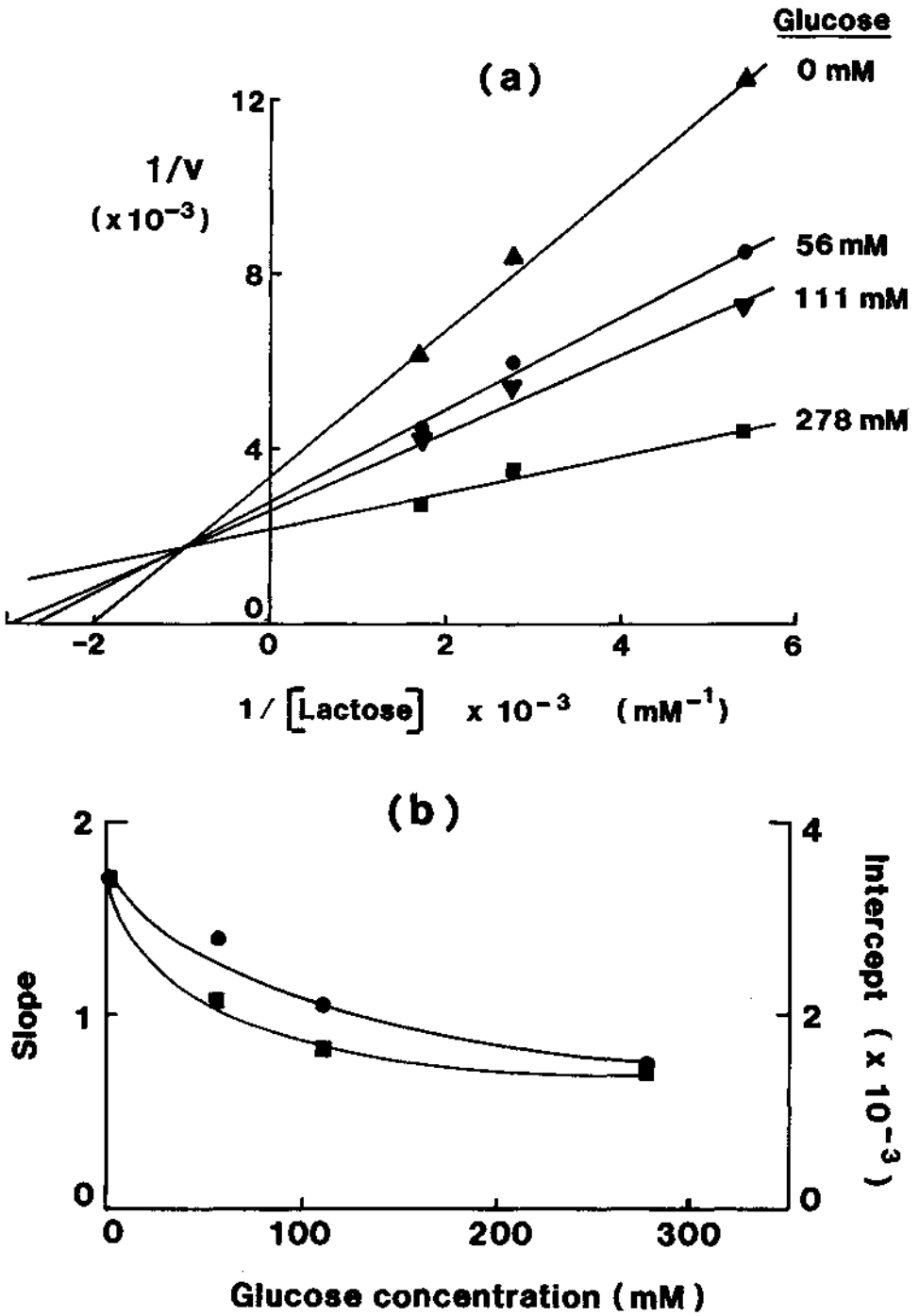

Fig. 6. Influence of glucose on immobilized lactase activity. (a) Lineweaver-Burk plot; (b) Replot of slopes and intercepts of (a) against glucose; ■, slope; ●, intercept.

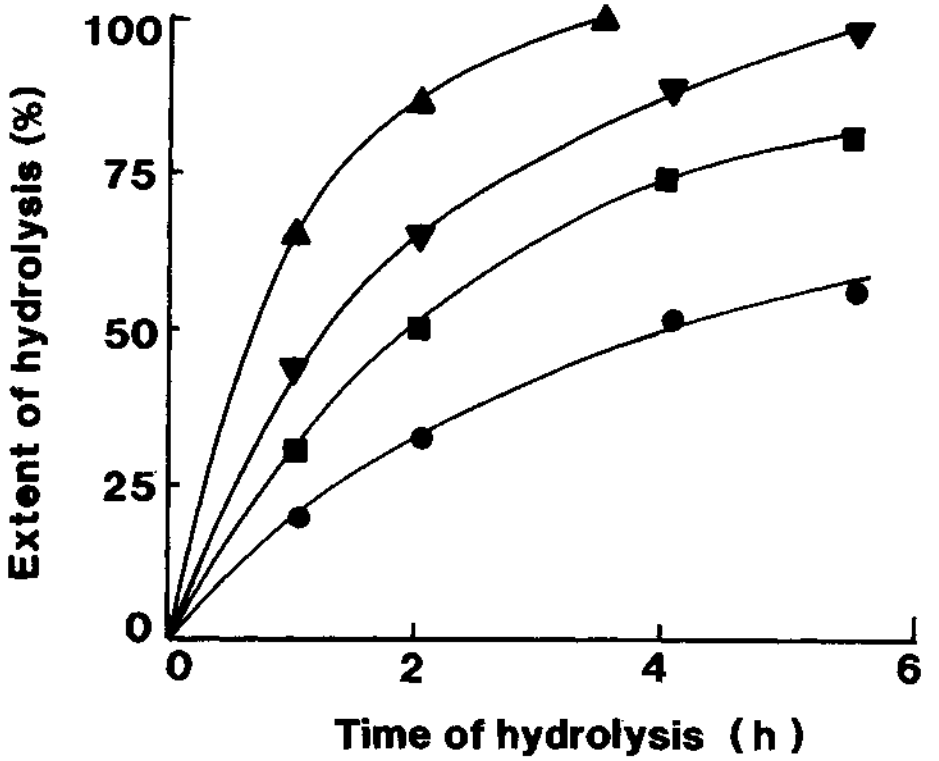

Fig. 7. Influence of the concentration of immobilized lactase on the rate and extent of lactose hydrolysis. ▲, 40 immobilized lactase U; ▼, 20 immobilized lactase U; ■, 10 immobilized lactase U; ●, 5 immobilized lactase U.

lactase used (Fig. 7). At high enzyme concentrations (e.g., 40 U per digest) hydrolysis was virtually complete within 4 h, whereas at lower concentrations the extent of hydrolysis was proportionately lower. A comparison of the hydrolytic activity of the free and immobilized lactase toward lactose at equivalent lactase activities is shown in Fig. 8. The efficiency of hydrolysis (i.e., the amount of glucose produced per unit of immobilized lactase), and the rate and extent of hydrolysis, was higher for the IME preparation, and resulted in complete hydrolysis within 6 h. The difference in hydrolytic activity is probably attributable to the greater thermostability of the IME-lactase preparation at 55°C. For example, at short hydrolysis times (10 min), the efficiencies were similar but diverged thereafter (*see* Fig. 8.).

Since galactose and calcium ions, respectively, inhibited and activated IME-lactase activity, experiments were performed to examine the effects of the presence of initial galactose on the hydrolysis performance of the immobilized lactase preparation, and whether calcium could reverse the effects of inhibition by galactose. The results of that investigation are presented in Fig. 9 and show that, although galactose suppressed lactase activity and lactose hydrolysis, the IME-enzyme still managed to hydrolyze

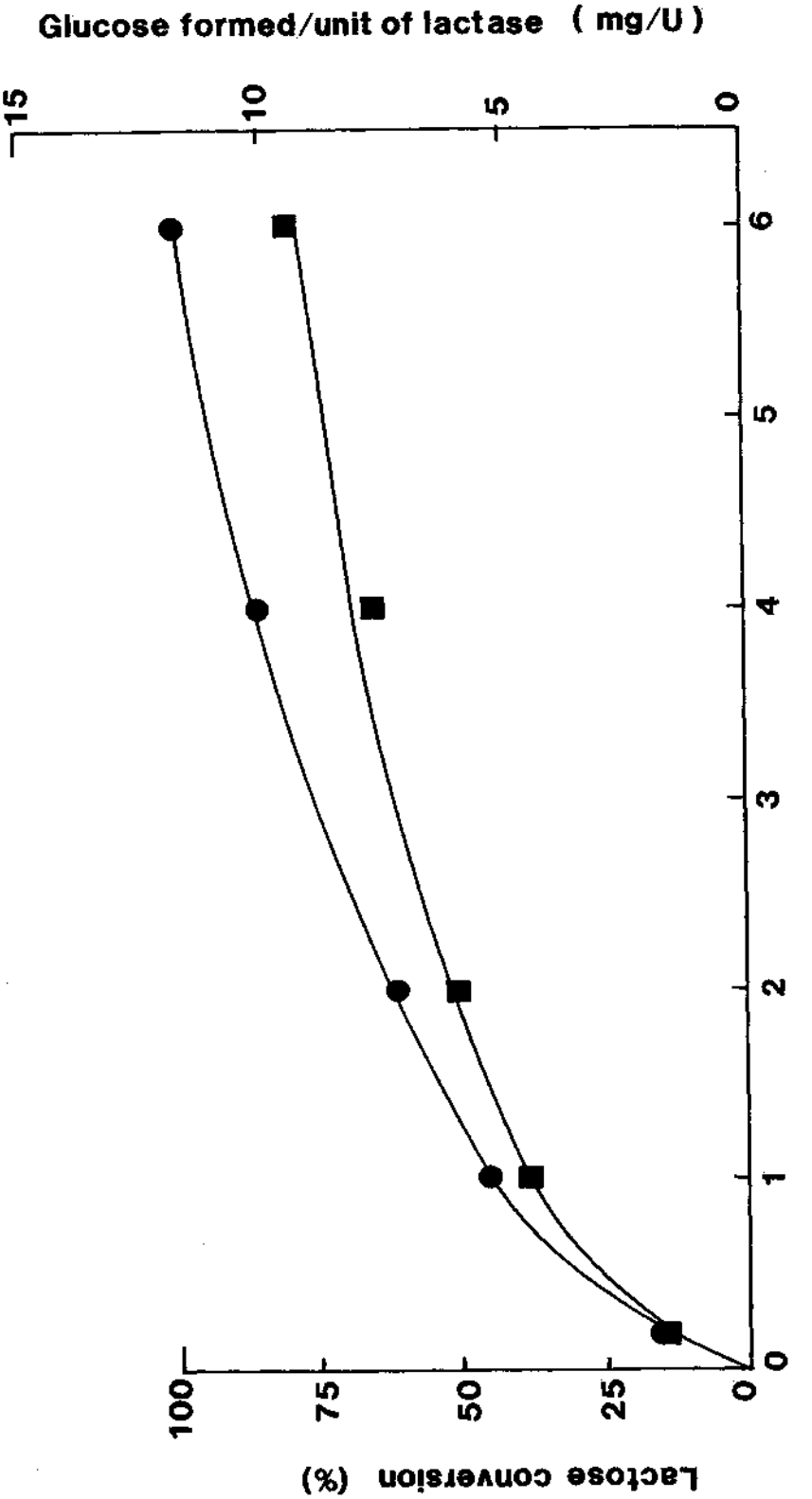

Fig. 8. Comparison of the hydrolytic activity of the free and immobilized, lactase preparations. ●, immobilized lactase; ■, free lactase. Digests contained 10 U lactase and were incubated at 55°C.

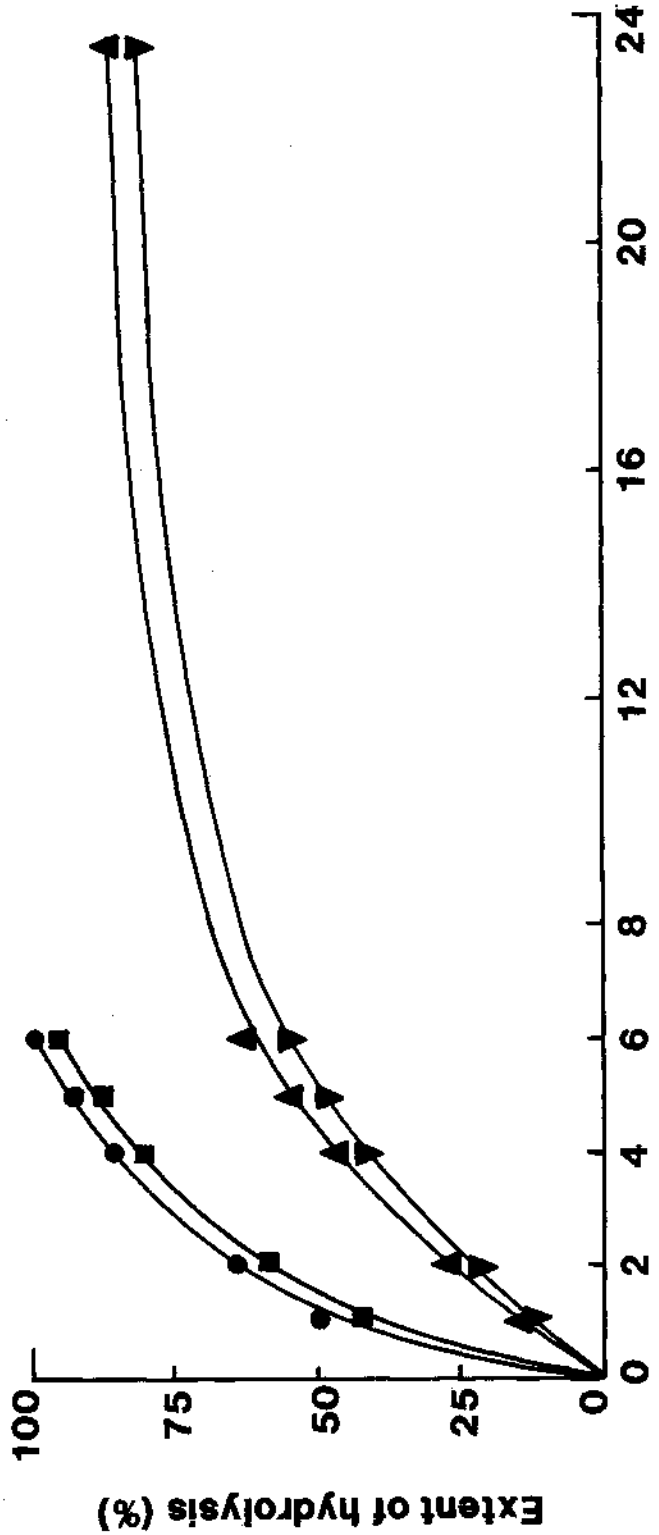

Fig. 9. Hydrolysis of lactose by immobilized lactase in the presence and absence of calcium and galactose. ■, control (only lactose); ●, calcium (150 m*M*); ▲, calcium and galactose (100 g/L); ▼ galactose (100 g/L). Digests contained 20 U immobilized lactase.

Table 2
The Effect of Recycling Immobilized Lactase
on the Extent of Hydrolysis of Whey Permeate Lactose

Time of hydrolysis, h	IME cycle number	Conversion of lactose into glucose,[a] Immobilized lactase concentration, U		
		5	10	20
1	1	19.5 (19.3)[b]	25.7 (30.6)	30.9 (43.2)
	2	16.6	23.5	36.3
	3	13.9	22.5	32.5
2	1	28.1 (32.8)	44.1 (49.4)	57.9 (64.1)
	2	26.4	33.4	49.3
	3	23.3	29.8	43.6
4	1	45.9 (51.8)	63.7 (74.7)	70.7 (88.2)
	2	42.6	49.9	67.4
	3	37.8	50.4	60.7
5.5	1	63.5 (ND)	74.3 (77.3)	87.2 (97.1)
	2	52.2	61.4	80.0
	3	47.1	58.1	70.1
	4	42.9	55.4	69.5

[a] Conversion factor for lactose into glucose was 0.5265.
[b] Figures in parentheses represent hydrolysis of pure lactose.

lactose (up to 85% within 24 h), albeit at a lower rate than those digests containing no initial galactose. The presence of calcium (150 m*M*) in the digests containing lactose and initial galactose did not alleviate the inhibitory effects of galactose on lactase activity. Calcium was effective, however, in promoting hydrolysis by reducing the hydrolysis time from 6–7 h (control, no calcium) to 5.5–6.0 h (Fig. 9).

The effect of recycling IME-lactase (at 3 different enzyme concentrations) on the extent of hydrolysis of whey permeate lactose is presented in Table 2. Recycling of the immobilized enzyme resulted in decreased conversions of lactose at the 3 enzyme levels examined. After recycling (and reuse) the IME-lactase four times, the extent of hydrolysis in a stirred batch reactor after 5.5 h decreased from 87% (first use of the enzyme) to ca. 70% using 20 U of IME-lactase/digest). The efficiency of hydrolysis was thus reduced by some 17% over a 4-recycle period. The decrease in hydrolysis performance upon reuse of the IME-lactase preparation was attributable to physical loss of IME encountered during the recovery and regeneration (washing) steps. IME was recovered by magnetization using a bar magnet. During the washing stages and recovery of IME, very fine magnetic particles were produced as a consequence of demagnetization/magnetization. These particles appeared to have a lower density than the bulk of the IME preparation and tended to "float" on the surface of the

hydrolyzates and washes. They were not attracted by the power of the magnet used and, hence, were lost during the decanting and recovery steps. Such losses would be responsible for the reduced hydrolysis yields upon multiple reuse of the IME preparation, particularly if they contained IME. I have also observed that the hydrolysis yields of whey permeate lactose were consistently lower than those arising from the use of pure lactose (*see* Table 2). The lower conversion yields may be attributable to fouling of the IME-preparation by impurities present in the whey lactose preparation: A solution of whey permeate lactose (prepared from the solids) was brightly colored yellow and contained a fine flocculent material.

CONCLUSION

A magnetic immobilized lactase was prepared by a simple procedure involving 3 steps. Magnetite was coated with PEI, then crosslinked with glutaraldehyde, and finally enzyme covalently coupled via the aldehyde groups. Although 80% of the lactase activity was lost upon immobilization, the active enzyme retained on the matix was capable of completely hydrolyzing lactose in relatively short hydrolysis times (e.g., 4–6 h at 55°C in a stirred batch reactor). The IME-lactase was highly stable at 55°C for prolonged times. Although galactose inhibited the IME-lactase, this did not prevent the complete hydrolysis of lactose. Glucose, by contrast, activated the enzyme. The IME preparation was demonstrated to be more efficient than the free enzyme in hydrolyzing lactose at 55°C. Stirred batch reactor studies showed that the magnetic immobilized enzyme when used in the recycled mode was suitable for hydrolyzing whey permeate lactose.

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