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IMMOBILIZATION OF THE β-GALACTOSIDASE FROM *Aspergillus niger* ON CHITOSAN[1]

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Crude or purified *Aspergillus niger* β-galactosidase preparations were immobilized on chitosan (deacetylated chitin, activated with glutaraldehyde). The most active immobilized systems were obtained with *crude* enzyme preparations. The immobilized enzyme hydrolyzed lactose in pure lactose solutions, ultrafiltrate whey permeates, or acid wheys at similar rates. The pH activity profiles and K_m values of the chitosan-bound enzyme were not significantly altered on immobilization, and its stability on repetitive use up to 60°C was increased by reduction with $NaBH_4$. After 8 weeks on discontinuous operation (8 h use per day), β-galactosidase–chitosan columns were found to retain about 90, 50, or 60% of their initial activities with lactose, ultrafiltrate permeate, or acid whey solutions, respectively. The efficiency of the β-galactosidase–chitosan conjugate appears to be comparable or greater than those of other described systems, and its stability should allow its use on an industrial scale.

INTRODUCTION

Immobilization of the enzyme β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) is attractive, in view of its industrial applications (1,2). Bacterial (3,4), yeast (5,6), or fungal (7–11) β-galactosidases have been immobilized on various carriers, such as polyacrylamide gels, porous cellulose sheets, activated Sepharose derivatives, cellulose acetate fibers, collagen, modified ion-exchange resins, glass beads, ZrO_2-coated glass beads, TiO_2 particles, and chitin. We demonstrated previously that chitosan (deacetylated chitin from arthropod shells) was suitable for covalent enzyme fixation, e.g., protease immobilization (12). We now report the use of chitosan as an insoluble support for immobilization of either crude or purified *Aspergillus niger* β-galactosidase preparations. The aim of this work was to design an insoluble β-galactosidase system characterized by good storage and operational stability, as well as by favorable chemical and mechanical properties.

[1]A preliminary report of this work has already been presented at the 4th International Enzyme Engineering Conference (September 25–30, 1977, Bad Neuenahr, FRG).

MATERIALS AND METHODS

Chemicals

Chitosan was purchased from Fluka (Switzerland), and glutaraldehyde from Merck (25% aqueous solution). Acid whey powder (70% lactose, 11% protein, 2% ashes) was supplied by the Linor Laboratories (Orbe, Switzerland). Dry ultrafiltrate (UF) whey permeate (81% lactose, 4% protein, 8% ashes) was obtained from the Nestlé factory in La Mauffe (Normandy, France). The bacteriostatic agent Vantocil IB (a polymeric biguanide salt) was a product of Imperial Chemical Industries. All other chemicals were obtained in the purest form available from commercial sources.

Enzymes

A commercial β-galactosidase preparation (13% protein and 68% total sugars) was purchased from the Rapidase Company (Seclin, France). This commercial preparation was further purified and resolved into three isoenzymes, as described previously (13). The pure isoenzyme used in this work accounted for 83% of the total β-galactosidase activity of the commercial preparation. *A. niger* glucose oxidase and horseradish peroxidase were products of Sigma.

Protein Determination

The determination of protein in solution was performed using the method of Lowry et al. (14), with bovine serum albumin as the standard.

Enzyme Immobilization

A 2.5% (wt/vol) acidic chitosan solution was prepared using 0.3 M acetic acid. After 4 h vigorous stirring at room temperature, the viscous solution was filtered through three cheesecloth layers to eliminate any undissolved material, poured, and spread on nonadhesive (Teflon-treated) metallic plates (50 ml solution/100 cm^2). The chitosan layer was then dried at 70°C for 20 h, and the resulting rigid dessicated film (thickness ~0.1 mm) was broken and sifted (2-mm mesh). Figure 1 shows the structure of the particles, as revealed by scanning electron microscopy (Cambridge S4-10 Stereoscan, used at an accelerating voltage of 20 kV, with a tilt angle of 45°). The particles were swollen for 15 min in 0.5 M sodium acetate buffer (pH 6.0) at room temperature. Aqueous glutaralde-

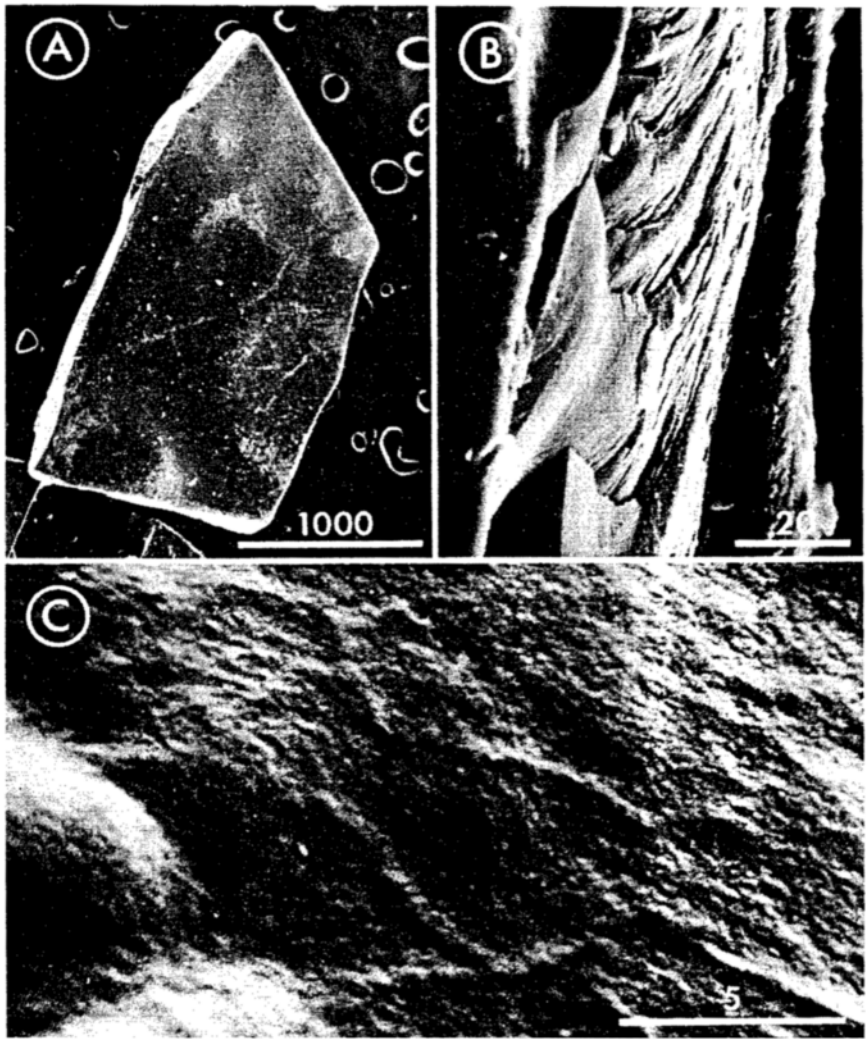

FIG. 1. Structure of the processed chitosan particles. The magnification was 22, 750, and 6000 for micrographs A, B, and C, respectively (the bars indicate lengths in micrometers). Native chitin and nonprocessed chitosan have porous structures (pore size 100–500 nm). On the contrary, processed chitosan as seen in this figure does not appear to have such a porous structure. This might minimize diffusional effects for the immobilized β-galactosidase.

hyde was added to a final concentration of 2.5 or 5%. After 1 h reaction with the free amino groups of chitosan, excess glutaraldehyde was removed by centrifugation (supernatant). The active particles (pellet) were washed three times with distilled water and suspended in 50 mM sodium phosphate

buffer (pH 7.5). The particles having a sedimentation velocity inferior to 1.5 mm/s were discarded. All subsequent operations were performed at 4°C. Various amounts (17–2400 mg) of commercial (crude) or purified *A. niger* β-galactosidase were dispersed in 20 ml of 50 mM sodium phosphate buffer (pH 7.5), and these enzyme solutions were added to the activated chitosan preparations (total amount of dry chitosan 1.0–2.4 g). After 12 h slow stirring, the obtained β-galactosidase–chitosan conjugates (GCC) were thoroughly washed with 0.15 M NaCl, 0.5 M sodium phosphate buffer (pH 7.5), and water, down to zero 280 nm absorbance.[2] The insoluble preparations were used without additional treatment, or after reduction by $NaBH_4$ (see later). All preparations were stored at 4°C in 50 mM sodium acetate buffer (pH 4.0), containing 0.5% (vol/vol) Vantocil IB.

Reduction by $NaBH_4$

The active complex (10 g moist weight) suspended in 50 ml of 50 mM sodium phosphate buffer (pH 7.5) was reacted for 3 h at 4°C with $NaBH_4$ (3 mg reductant/ml buffer). The treated particles were then thoroughly washed with distilled water and the storage buffer. Treatment by $NaBH_4$ may transform imine derivatives into more stable secondary amines, thereby enhancing the overall stability of the immobilized β-galactosidase complex. $NaBH_4$ is a mild reducing agent, and did not appear to affect soluble or immobilized β-galactosidase activity.

Monitoring of β-Galactosidase Activity

The activity of the immobilized enzyme preparations was determined at 30°C using 5 mM *o*-nitrophenyl-β-D-galactopyranoside (ONPG) or 45 mM lactose as the substrate (in 50 mM sodium acetate buffer, pH 4.0), unless otherwise indicated. Hydrolysis of ONPG was monitored at 405 nm, which corresponds to the absorbance peak of *o*-nitrophenol at acidic pH values. Glucose evolution with lactose as the substrate was analyzed using the glucose oxidase/peroxidase system (16). Other details are given in the Results section.

Determination of K_m and V_{max} values

The kinetic constants of immobilized β-galactosidase were determined with stirred batch procedures. With ONPG as the substrate, we used

[2]The various theories developed to explain the reactions of glutaraldehyde with proteins have recently been reviewed (15).

the apparatus described previously (17) (20 mg drained GCC in 25 ml substrate solutions). With lactose as the substrate, 100 mg drained GCC was reacted in 50 ml substrate solutions (propeller stirring, to minimize abrasion). The time course of lactose hydrolysis was monitored by performing the glucose oxidase/peroxidase assay (16) on reaction medium aliquots withdrawn at various time intervals (total volume withdrawn $\leq$ 500 μl).

Operational Stability

Chitosan-bound β-galactosidase was packed into K 9/15 Pharmacia columns (3.5 g drained GCC per column), which were fed with a pure lactose solution, reconstituted UF permeate, or reconstituted acid whey, respectively. The three solutions were prepared in 50 mM sodium acetate buffer (pH 4.0), with a lactose concentration adjusted to 4% (i.e., 111 mM) in each case. The reconstituted permeate and whey solutions were clarified by filtration through a Celite 535 filter-aid bed, on G-3 sintered glass funnels. After pH adjustment to 4.0 with glacial acetic acid when necessary, all three solutions were pumped through GSWP 14250 Millipore filters. The columns were operated 8 h/day at 40°C. The flow rate was adjusted to 125 ml/cm^2/h (1 ml/min a column), which ensured an initial lactose hydrolysis of approximately 50%. The columns were stored overnight at 20°C, after having been filled with 0.5% (vol/vol) Vantocil IB in 50 mM sodium acetate buffer (pH 4.0) to prevent bacterial growth.[3]

Miscellaneous

The specific flow resistance of the immobilization support was determined using a 150 × 16 mm horizontal column packed with activated chitosan. The pressure drop at 20°C as a function of flow rate was indicated by three manometers connected to the column at various intervals. The solution and swelling medium was distilled water. Flow rates from 5 to 20 ml/cm^2/min were used. Measurements were done each time 20 min after initiation of the flow.

The amount of chitosan-bound protein was estimated from a mass balance (difference between the amount of protein in solution before and after immobilization, and in the combined washings), using Lowry's

[3]Vantocil IB has recently been developed by ICI as a biocide suited for the food industry (18). Preliminary experiments showed that this compound is an uncompetitive inhibitor of the *A. niger* β-galactosidase. The inhibition appeared to be fully reversible, since GCC stored for 10 days at 20°C in 50 mM sodium acetate buffer (pH 4.0) containing 0.5% (vol/vol) Vantocil IB showed no significant loss of enzymatic activity after adequate washing.

method. When pure β-galactosidase was used (see Table 1, preparation 4), the amount of immobilized enzyme was also determined by acid hydrolysis of the active matrix, with subsequent amino acid analysis (Beckman Multichrom B amino acid analyzer). The value given in Table 1 is the average of the results obtained by both methods.

RESULTS

Reactivity of Chitosan-Bound β-Galactosidase

Tables 1 and 2 present the results obtained with four different typical GCC preparations. The efficiency of the chitosan conjugates is seen to depend on various parameters involved in the immobilization process, e.g., glutaraldehyde concentration, enzyme/support weight ratio, and the purity of the enzyme solutions. Lowering the glutaraldehyde concentration from 5 to 2.5% increased the specific activity of the immobilized enzyme (compare preparations 1 and 2 in Table 2). A lowering of the enzyme/support weight ratio from 1.0 to 0.5 had the same effect (compare preparations 2 and 3), with almost complete binding of the available protein. Immobilization of pure enzyme (preparation 4) adversely affected the specific activity for lactose, which declined by more than 30%. Therefore, enzyme purification does not appear to be a prerequisite for an adequate immobilization yield. This substantiates previous findings indicating that inert protein reduces the loss of specific activity when glutaraldehyde is used in the immobilization process (19). Consequently, the experiments described in this report were performed with immobilized *crude* enzyme, except for the determination of K_m and V_{max} values.

Table 3 compares our results with those of previous studies.

Chemical and Mechanical Data

Chemical basis: partially deacetylated chitin. The structures of chitin and chitosan are given in Fig. 2. The degree of deacetylation, which corresponds to the percentage of free amino groups, was found to be 64%, using a potentiometric method (25). This percentage corresponds to 3.7 μmol $-NH_2$/mg dry matrix. These nucleophilic groups are involved in the chitosan–glutaraldehyde coupling reaction.

Functional groups after activation: aldehyde groups –CHO. These reactive groups are theoretically involved in the immobilization process, but various theories explaining the reactions of glutaraldeyde with proteins are still discussed (see footnote 2).

TABLE 1. **Immobilization of *Aspergillus niger* β-Galactosidase on Chitosan**

Preparation	Enzyme (mg)	Chitosan[a] (g)	Total initial activity		Immobilized protein		Total bound activity			
			ONPG units[b]	Lactose units[b]	mg	%	ONPG units[c]	%	Lactose units[d]	%
1[e]	2400	2.4	9360	3870	201	64	4960	53	1660	43
2[e]	2400	2.4	9360	3870	190	61	5520	59	1970	51
3[e]	500	1.0	1950	810	61	94	1850	95	680	84
4[f]	17	1.0	1410	590	16.6	98	1380	98	342	58

[a] Dry weight.
[b] One unit is defined as the amount of enzyme that hydrolyzes 1 μmol substrate/min at 30°C (ONPG concentration 5 mM in 50 mM sodium acetate buffer, pH 4.0; lactose concentration 45 mM in the same buffer).
[c] Theoretical values (calculated differences between the total units initially used for immobilization and the total units detected in the supernatants and washings). It was not possible to determine lactose units in supernatants and washings for all four preparations. Because pH adjustment to 7.0 (for the glucose-oxidase/peroxidase assay) would have caused inappropriate dilution in the case of preparations 3 and 4.
[d] The values reported here were ascertained using a stirred batch procedure comparable to that described for the determination of K_m.
[e] The crude enzyme was used (13% protein). Glutaraldehyde concentration, 2.5%, except for preparation 1(5%).
[f] The purified enzyme was used. Glutaraldehyde concentration, 2.5%.

TABLE 2. **Comparison between Specific Activities**[a]

Preparation	Enzyme/support weight ratio	Initial specific activity[b]		Specific bound activity[b]		Percentage of initial specific activity	
		ONPG	Lactose	ONPG	Lactose	ONPG	Lactose
1	1.0	30.0	12.4	24.7	8.3	82	67
2	1.0	30.0	12.4	29.1	10.4	97	84
3	0.5	30.0	12.4	30.0	11.1	100	90
4	—	83.1	34.6	83.1	20.6	100	60

[a]Data calculated from Table 1.
[b]Units per milligram of protein. See footnotes *c* and *d* in Table 1.

Chemical stability of the activated matrix. Native chitosan is insoluble in water, organic solvents, and alkali, but it dissolves easily in mineral or organic diluted acids (except sulfuric acid) (24). By contrast, no dissolution or alteration was observed when glutaraldehyde-activated chitosan was kept for a week under stong acidic or alkaline conditions (up to 1.0 N H^+ or OH^- at room temperature).

Water regain: At pH 4 (50 mM sodium acetate buffer), 2.4 ml/g; at pH 6 (50 mM citrate phosphate buffer), 2.4 ml/g; at pH 8 (50 mM Tris-HCl), 2.9 ml/g.

The following mechanical properties were determined:

Particle size before swelling: ~0.1-mm thickness, 0.1–1.8 mm length (bell-shaped distribution curve).

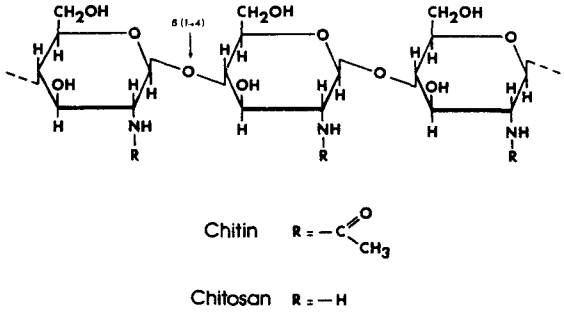

FIG. 2. Chemical structure of chitin (*N*-acetyl-2-amino-2-deoxy-D-glucose) and chitosan (partially deacetylated chitin). Commercial chitosan preparations have molecular weights ranging from 45,000 to 120,000 (23,24).

TABLE 3. **Comparison between the Activities of *Aspergillus niger* β-Galactosidase Immobilized on Various Supports**

Reference	Support	Conditions	Bound activity (μmol lactose hydrolyzed/min)
This work (preparation 3 in Table 1)	Chitosan (glutaraldehyde coupling)	45 mM lactose; pH 4.0; 30°C	195/g drained chitosan
		Same at 40°C	260–290/g drained chitosan
		Same at 60°C	330–480/g drained chitosan
7	Collagen	139 mM lactose; pH 4.0; 37°C	43/g complex
8	Duolite S-30 (glutaraldehyde coupling)	400 mM lactose; pH 4.0; 45°C	200/g drained resin
9	Glass beads (diazo coupling)	140 mM lactose; pH 4.0; 37°C	88/g complex
10, 20	Glass beads (glutaraldehyde coupling)	555 mM lactose; pH 3.5; 40°C	579/g complex
	ZrO_2-coated glass beads (glutaraldehyde coupling)	555 mM lactose; pH 3.5; 60°C	800/g complex
	TiO_2 particles (glutaraldehyde coupling)	555 mM lactose; pH 3.5; 60°C	500/g complex
11	Chitin (glutaraldehyde coupling)	400 mM lactose; pH 3.0; 40°C	60–110/g drained chitin
21	Glass beads (diazo coupling)	Acid whey (~125 mM lactose) pH 4.5; 55°C	89/g complex
22	Polyvinylpyrrolidone (polymerization by γ-rays)	139 mM lactose; pH 4.5; 40°C	14/g gel

Particle size after swelling at pH 4: ~0.15-mm thickness, 0.15–2.5 mm length (bell-shaped distribution curve).

Sedimentation velocity: ≥ 1.5 mm/s.

Bed volume in columns (*pH* 4): 1.1 ml/g drained matrix.

Flow resistance: ~ 0.3 bar $\times$ s^2/cm $\times$ g. This low coefficient was found to be constant within 5% up to the high flow rate of 20 ml/cm^2/min, indicating negligible compressibility of the chitosan bed.

Abrasion by magnetic stirring devices was not negligible, which indicated that chitosan conjugates are more suited to column operations than to stirred tank procedures.

Stability of Chitosan-Bound β-Galactosidase

pH–Activity Profile. The possible alteration of the pH–activity profile on immobilization was investigated. Figure 3 shows that the profile was not dramatically altered. A positive net charge of glutaraldehyde-treated chitosan might be caused by protonated residual amino groups, and could explain the moderate shift of the pH profile toward lower values (26). Curve B closely resembles the pH activity profile obtained with the same enzyme immobilized on ZrO_2-coated glass particles (10). The shoulder on the right of curve B has also been found for the DEAE-Sephadex-sorbed β-galactosidase from *Curvularia inaequalis* (27).

Immobilized β-galactosidases would not be used in practice at pH values as low as 2.5 (i.e., the pH optimum seen on Fig. 3). This explains why most of the experiments described here were performed at pH 4.0, unless otherwise indicated. This pH value is close to that of acid whey, which is an abundant prospective feed for β-galactosidases.

Temperature Stability. The three purified *A. niger* β-galactosidase isoenzymes were found to be heat stable up to about 60°C (13). This stability was only moderately increased on immobilization (Fig. 4).

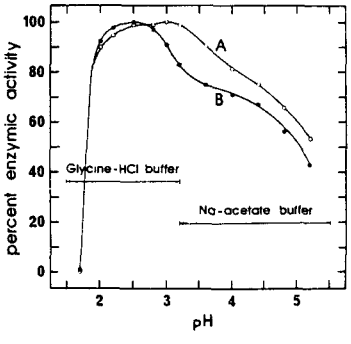

FIG. 3. pH–activity profiles of soluble and immobilized *A. niger* β-galactosidase, with 45 mM lactose as the substrate, in 50 mM buffers. Curve A, soluble crude enzyme; curve B, immobilized enzyme (50 mg drained GCC was packed in a 50 × 5 mm column; flow rate, 0.5 ml/min, ensuring a maximum lactose hydrolysis at pH 2.5 of ~15%). Both buffers were used at pH 3.2, with slightly different results. Therefore, the activities reported at this abscissa are average results. For each curve, the maximum rate was arbitrarily set at 100%.

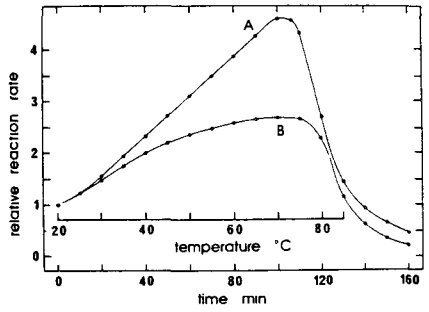

FIG. 4. Thermal stability of normal and $NaBH_4$-reduced immobilized *A. niger* β-galactosidase. Normal and reduced GCC (20 mg, curves A and B, respectively) were packed in 20×5 mm columns immersed in a programmable thermostated bath. The flow rate was set at 0.7 ml/min per column, and a linear gradient of temperature was established from 20 to 85°C (temperature increase per hour, 30°C; the temperature reached 85°C after 130 min and was then kept constant). The enzymatic activity was monitored using 5 mM ONPG as the substrate. The extent of ONPG hydrolysis was continuously determined by recording the 405-nm absorbance of the undiluted eluates. ONPG hydrolysis never exceeded approximately 10% (i.e., a 405-nm absorbance of 1.5).

Immobilization could even be considered as detrimental when Arrhenius plots are examined (Fig. 5). The plot for the soluble enzyme is linear in the range 25–65°C, with an activation energy E_a equal to 7.1 kcal/mol. Chitosan-bound β-galactosidase has the same E_a up to about 50°C (curve B), but for higher temperatures diffusional limitations may become significant, with a concomitant decrease of E_a (29). In the range 25–40°C, E_a for reduced GCC is equal to 5.8 kcal/mol (curve C on Fig. 5), and falls off significantly for higher temperatures. On the other hand, it was found that reduction by $NaBH_4$ permanently stabilized the immobilized enzyme

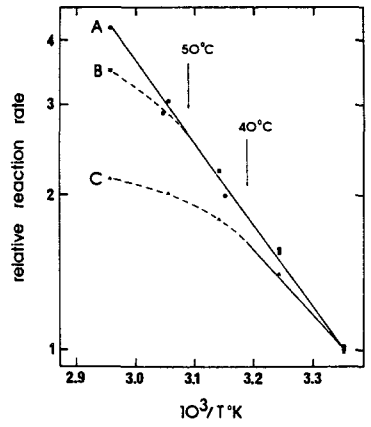

FIG. 5. Arrhenius plots for soluble and immobilized *A. niger* β-galactosidase. Curve A, soluble purified enzyme; curve B, normal GCC (replot from Fig. 4); curve C; $NaBH_4$-reduced GCC (replot from Fig. 4). The different values of activation energy E_a reported in the text were calculated from this graph as described by Jencks (28).

TABLE 4. **Kinetic Constants of *Aspergillus niger* β-Galactosidase**[a]

	$K_m(mM)$		V_{max} (μmol substrate hydrolyzed min/mg protein)	
	ONPG	Lactose	ONPG	Lactose
Soluble enzyme	2.4	85	122	100
Immobilized enzyme	3.9	95	38	65

[a]The purified enzyme was used.

toward heat. This was shown by experiments in which a normal and a reduced GCC were subjected to three temperature cycles (i.e., alternate temperature increases and decreases between 20 and 60°C). The normal GCC was characterized by significant and irreversible stepwise losses of enzymatic activity. By contrast, the reduced GCC recovered its entire initial activity each time. Therefore, the operational stability experiments described herein were performed with $NaBH_4$-reduced conjugates.

Kinetic Parameters

The kinetic parameters of the three soluble β-galactosidase isoenzymes have already been determined at pH 3.5, i.e., the average pH optimum with ONPG as the substrate (13, and manuscript in preparation). For comparison, the K_m and V_{max} values of chitosan-bound β-galactosidase were determined at the same pH value (Table 4). The results in Table 4 were calculated from double-reciprocal plots (least-squares fits). With both substrates the plots were adequately linear, indicating minimal diffusional effects (Fig. 6 shows the plots for soluble and immobilized β-galactosidase, using ONPG as the substrate). The increase in the K_m values on immobilization is accompanied by a notable reduction of the specific activities. Since both ONPG and lactose are uncharged low mole-

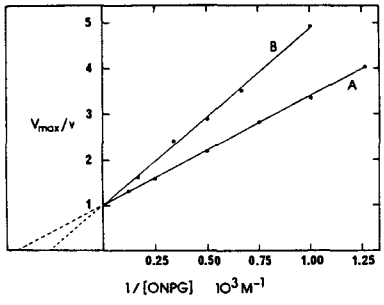

FIG. 6. Determination of K_m values for soluble and immobilized *A. niger* β-galactosidase, using ONPG as the substrate (normalized ordinate). Curve A, soluble enzyme; curve B; immobilized enzyme.

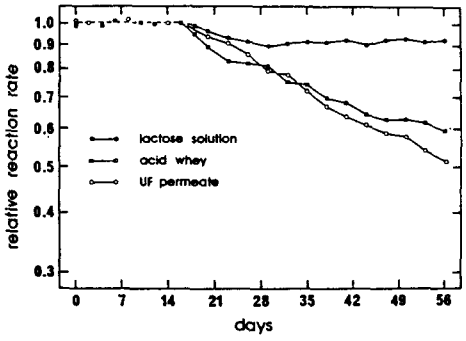

FIG. 7. Operational stability of GCC columns fed with a pure lactose solution, UF permeate, or acid whey. The initial activities were arbitrarily set at 1.0.

cular weight substrates, these effects must be mainly due to strains on the tertiary structure of the immobilized enzyme (29).

Operational Stability

Reduced GCC was stable for months when stored in the cold or at room temperature. Figure 7 shows that the operational stability was also notable. The initial hydrolysis rates were similar in all three cases, indicating little or negligible sensitivity of the immobilized enzyme to the ions contained in UF permeate and acid whey. Since the initial activities corresponded to about 50% hydrolysis only, the initial plateaus of stability for 2 weeks cannot be discounted as artifacts. Therefore, the decreases of activity do not appear to be exponential. This finding was not investigated further. The relatively high temperature and low pH of operation, as well as the Millipore filtration and intermittent soaking in Vantocil IB, prevented uncontrollable bacterial growth. The loss of activity with acid whey and UF permeate (~60 and 50% residual activity, respectively, compared to 90% with the pure lactose solution) might therefore be mainly due to sticking of inert peptides or proteins to the chitosan particles, with progressive embedding of the active enzyme molecules. Prolonged exposure to the ions contained in the permeate and whey solutions might also have eventually resulted in significant inactivation.

CONCLUSION

The enzyme β-galactosidase from *A. niger* was successfully immobilized on chitosan, using glutaraldehyde as the cross-linking agent. This novel

support had already been used, under various conditions, to immobilize proteases, urease, glucose isomerase, glucoamylase, glucose oxidase, and acid phosphatase (12,30,31). The efficiency of the immobilized β-galactosidase system described in this report can be shown to be comparable or greater than those of already existing systems.[4] The stability of the chitosan conjugates should allow their use on an industrial scale. Continuous-flow operations with packed-bed reactors would be the best procedures, ensuring minimization of mechanical abrasion and of inhibition by galactose (32,33). Chitosan is readily prepared from an abundant natural polymer (chitin), and its cost should therefore not be prohibitive (34). Furthermore, immobilization via glutaraldehyde is easy. Immobilized β-galactosidase might be useful in processing milk by-products into valuable sweeteners, or into nutritional media for fermentation processes. Its possible application to solving many problems related to the use of lactose (e.g., digestive intolerance, crystallization, and low sweetness) should also be considered.

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[4]For appropriate comparison, the results of Table 3 should be adjusted to a unique lactose concentration. Such calculations are not practicable for Table 3, because the K_m values are generally unknown. However, since the K_m value for the chitosan-bound enzyme with lactose as the substrate has been determined (see Table 4), as well as the pH-activity profile (Fig. 3), it is possible to extrapolate our results from 45 mM lactose to 555 mM lactose, i.e., the highest concentration given in Table 3 (pH 3.5, 60°C). The activity of 330–480 μmol lactose hydrolyzed min^{-1} g^{-1} drained chitosan (45 mM lactose, pH 4.0, 60°C) can thus be calculated to correspond to 920–1340 μmol lactose hydrolyzed min^{-1} g^{-1} drained chitosan (555 mM lactose, pH 3.5, 60°C).

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