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Immobilization of Cellulase Using Polyurethane Foam

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ABSTRACT

Cellulase was covalently immobilized using a hydrophilic polyurethane foam (Hypol® FHP 2002). Compared to the free enzyme, immobilized cellulase showed a dramatic decrease (7.5-fold) in the Michaelis constant for carboxymethylcellulose. The immobilized enzyme also had a broader and more basic pH optimum (pH 5.5–6.0), a greater stability under heat-denaturing or liquid nitrogen-freezing conditions, and was relatively more efficient in utilizing insoluble cellulose substrates. High molecular weight compounds (Blue Dextran) could move throughout the foam matrix, indicating permeability to insoluble celluloses; activity could be further improved 2.4-fold after powdering foams under liquid nitrogen. The improved kinetic and stability features of the immobilized cellulase combined with advantageous properties of the polyurethane foam (resistance to enzymatic degradation, plasticity of shape and size) suggest that this mechanism of cellulase immobilization has high potential for application in the industrial degradation of celluloses.

Index Entries: Cellulase; enzyme immobilization; polyurethane foam; cellulose degradation.

INTRODUCTION

Cellulose is the single largest biomass and greatest renewable resource available on the earth yet to date industrial processes, such as the paper and textile industries, make use of only a small fraction of the cellulose

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produced annually (*1,2*). Key to the exploitation of this vast resource are the development of methods for the conversion of cellulose polymers into useful sugar products. The glucose derived from the breakdown of cellulose has numerous uses including feedstocks for single-cell protein processes, fermentation into useful chemical precursors, and conversion into fructose syrups (*1,2*).

Enzymatic degradation of cellulose by cellulases has already proven to be the preferred method, compared to chemical methods, for cellulose processing and numerous researchers are involved in optimizing the process. One drawback to enzymatic degradation is the need to use high concentrations of cellulase (because of low specific activities of these enzymes) and hence, the expense involved when soluble cellulases are lost in the further processing of product (*3*). Immobilization of cellulase on solid supports that can be recycled and reused offers a practical method for overcoming such waste. Immobilized enzymes can also possess other advantages over enzymes free in solution, including ease of separation of products from enzymes (*3*) and improved enzyme stability to extremes of pH and temperature (*2*).

Immobilization of enzymes also can create its own problems. External and internal diffusion barriers can develop for enzymes immobilized in polymer matrices. Conformational changes can be induced for covalently immobilized enzymes. These changes can result in enzyme inactivation, subunit dissociation, and/or the loss of allosteric properties (*2*).

Numerous methods, of varying utility, have been developed for immobilizing cellulase and other enzymes (*3,4*). We have recently begun to work with a hydrophilic polyurethane polymer (Hypol® FHP 2002 from W. R. Grace Co.). The foams produced from this polymer have major advantages as supports for covalently-bound enzymes including a wide porosity range, advantageous mechanical properties (flexibility, variable sizes, and shapes), a high additive loading capacity and high water absorbance (from 10–30 times their weight of water), and resistance to enzyme of microbial attack (*5,6*); W. R. Grace and Co. product information).

The present study investigates the use of this polyurethane foam for the covalent immobilization of cellulase. Kinetic properties and stability parameters (pH, salt, high temperature, and freezing effects) were compared for free and immobilized cellulase from *Trichoderma reesei* using both soluble and insoluble cellulose substrates. The results demonstrate an excellent potential for polyurethane foam immobilization of cellulases in cellulose biotechnology.

MATERIALS AND METHODS

Chemicals

All biochemicals and coupling enzymes were purchased from Sigma Chemical Co., J. T. Baker Chemical Co., or Boehringer Mannheim Corp.

Polymer

The foamable hydrophilic prepolymer (Hypol® FHP 2002) was graciously supplied by W. R. Grace and Co., Lexington, MA. In contrast to conventional hydrophilic foams, Hypol 2002 can utilize a variety of water to prepolymer ratios (*5*), ranging from 35–200 parts of water per 100 parts prepolymer (Hypol product information).

Immobilization of Cellulase

Cellulase (Celluclast®), prepared from *Trichoderma reesei*, was kindly provided by Novo Industries (Bagsvaerd, Denmark). Cellulase was also purchased from Sigma Chemical Co. The standard amounts of cellulase immobilized were as follows: 74 U for Novo cellulase and 11 U for Sigma cellulase (where 1 U = 1 nmole glucose produced/mL/min at V_{max} conditions).

The standard buffer used for all experiments was 20 m*M* sodium acetate, pH 4.5. To prepare the foam-immobilized enzyme, cellulase was dissolved in 2 mL of buffer in a petri dish. A 1.0 g aliquot of prepolymer was then added. The mixture was agitated vigorously to achieve a homogeneous distribution of enzyme within the prepolymer. Agitation was stopped when extensive polymerization was detected visually and by increased viscosity of the mixture. The resulting foams were allowed to cure at room temperature for at least 20 min before use. After polymerization was complete, the foams were washed with 6 mL of buffer and squeezed repeatedly to ensure complete adsorption of the wash into the foam.

Cellulase Assay

Cellulase activity was determined by measuring the glucose liberated from soluble or insoluble celluloses suspended in 20 m*M* sodium acetate buffer (pH 4.5). Standard reactions were carried out at 23°C using 1% (w/v) solutions of cellulose unless otherwise indicated. To initiate a reaction, foams were squeezed to remove excess wash buffer and then placed in a petri dish containing 4 mL of the appropriate cellulose solution. The foam was again squeezed to absorb the substrate into the foam. To sample the reaction mixture, foams were gently squeezed and aliquots of reaction mixture (0.1 mL) were sampled and immediately mixed with 0.1 mL 100 m*M* Tris buffer (pH 9.5). The resulting pH change stopped any cellulase activity (if present). As needed, excess Tris buffer was added to dilute samples with high glucose concentrations before assay. Glucose concentrations in samples were assayed enzymatically using the hexokinase/glucose-6-phosphate dehydrogenase coupled enzyme assay described by Lowry and Passonneau (*7*).

Protein Assay

Protein was determined by the Coomassie Blue dye binding method using the BioRad Laboratories prepared reagent. Total protein (before ad-

dition to the foam) and protein recovered in the wash buffer were measured; protein bound to the foam was determined by subtraction. Direct measurements of foam-bound protein were not possible since the foam reacted with the dye. Foams polymerized without added protein provided controls to show that dye-reacting material from the foam was not released into the wash buffer.

Kinetic Parameters

The kinetic parameters for cellulase were determined using carboxymethylcellulose (CMC(7LF)) as the substrate. The CMC, a soluble cellulose derivative, was kindly provided by Hercules Inc. (Wilmington, DE). Glucose production after 30 min at room temperature was determined for reactions using 1.0, 2.5, 5.0, and 10.0 mg/mL of CMC for both the free and immobilized cellulases. Km and Vmax values were calculated from Lineweaver-Burke (double reciprocal) plots.

Insoluble Celluloses

Four insoluble substrates were tested: cellulose acetate, microcrystalline cellulose, cellulose (boiled in acid), and Solka Floc (BW 40). Because of the lower reactivity of cellulase with the insoluble substrates, the standard amounts of cellulase and cellulose substrates used were doubled for these studies (to 148 U and 2%, respectively). Foams containing immobilized cellulase were also chopped up to yield small fragments (approximate volume of 60 mm^3) of higher surface area. These fragments were mixed with substrate by continuous inversion in a mechanical rotator during incubation.

Optimum Temperature and Thermal Inactivation

Free and immobilized cellulases were tested using two substrates, one soluble (CMC) and one insoluble (microcrystalline cellulose). Glucose production after 1 h was measured at a variety of temperatures in order to establish the optimum temperature of hydrolysis for both forms of cellulose. To examine thermal inactivation, free and immobilized cellulases were incubated at 60°C for 2 h with sampling at 30 min intervals to determine glucose production.

Optimum pH

Foams containing immobilized cellulase were pre-equilibrated by washing with 6 mL of 20 m*M* acetate buffer of the appropriate test pH. Free cellulase samples were prepared in 20 m*M* acetate buffer of the desired pH. Substrate solutions of 1% (w/v) CMC in 20 m*M* acetate buffer at the appropriate pH were prepared and added to the foams; aliquots were sampled for glucose production after 1 h at 23°C.

Increasing Surface Area

Foams containing immobilized cellulase were frozen using liquid nitrogen and ground into a fine powder. Control foams (approx 15,400 mm^3) were compared to chopped foams (approx volume of each piece was 60 mm^3) and to powdered foams for their ability to hydrolyze CMC over 30 min.

Polyethylene Glycol (PEG) Effects

Free cellulase was tested for its ability to hydrolyze CMC in the presence and absence of 10% (w/v) PEG 8000. The PEG was mixed with the substrate prior to addition of the enzyme.

Salt Effects

Free and immobilized cellulase were tested for the ability to break down CMC in the presence and absence of 2 *M* KCl. The salt solution was mixed with the substrate prior to addition to the enzyme.

Long-Term Cellulase Activity

Foams containing immobilized cellulase were tested for their ability to hydrolyze CMC over a 6-wk period. Glucose production after 1 h at 23°C was measured. Foams were thoroughly washed before each use and then fresh substrate was added. Between uses foams were stored at 4°C in substrate solutions containing 0.04% sodium azide.

Diffusion Limitation

Two methods were used to determine the role of "diffusion limitation" in the system under study. If enzyme:substrate diffusion is limiting in an immobilized enzyme system, one or both of the following should be observed: deviations in the Arrhenius relationship (relating temperature and reaction velocity), especially at high temperatures; and alterations in enzyme kinetic parameters (slope and shape of the Lineweaver-Burke plot) (*15*).

RESULTS

Immobilization of Cellulase

Compared to the activity of an equal amount of free cellulase (74 U Novo cellulase, 11 U Sigma cellulase, set at 100%), the recovery of activity after immobilization was $33 \pm 0.5\%$ bound to the foam for NOVO cellulase and $12 \pm 2\%$ bound for the Sigma enzyme. Protein immobilized into the foam was approximately 80% of the total added in both instances. Non-

immobilized enzyme was recovered in the first 6 mL wash; second and subsequent washes contained no significant enzyme activity or protein.

Kinetic Properties

The K_m and V_{max} values for free and immobilized cellulases are presented in Table 1. Both enzymes showed dramatic decreases in the K_m value for CMC after immobilization. K_m decreased by 7.5-fold for NOVO cellulase and by 3-fold for Sigma cellulase. The calculated V_{max} value for Sigma cellulase was that expected based on the percentage of added enzyme (12%) that was immobilized. For the NOVO enzyme, the decrease in V_{max} was greater than that accounted for by the percentage of added activity that was immobilized into the foam.

Insoluble Substrates

Table 2 shows activity of free cellulase using different types of insoluble cellulose as substrates. Glucose production was only a few percent (3.6% at most) of that achieved using CMC as a substrate. Based on these results, microcrystalline cellulose and Solka Floc were chosen as substrates for further studies.

Table 3 shows the activities of free and immobilized Novo and Sigma cellulases utilizing soluble (CMC) and insoluble (Solka Floc and micro-

Table 1
Kinetic Parameters for Cellulase[a]

Kinetic parameter	Novo cellulase	Sigma cellulase
K_m, mg/mL		
Free	15.26±0.55	4.85±0.22
Immobilized	2.02±0.21	1.58±0.26
V_{max}, (nmol glucose min^{-1} mL^{-1})		
Free	74.2 ±2.0	10.9 ±0.3
Immobilized	8.8 ±0.3	1.2 ±0.1

[a]Data are means ± SEM, $n=3$. The substrate used was carboxymethylcellulose.

Table 2
Substrate Preferences
for Insoluble Substrates by Free Cellulases[a]

Substrate	Novo cellulase	Sigma cellulase
Cellulose acetate	1.2%	0%
Microcrystalline cellulose	2.8%	1.0%
Cellulose, boiled in acid	2.4%	1.0%
Solka floc, BW 40	3.6%	0.5%

[a]Activities are given as a percentage of V_{max} using CMC as a substrate.

Table 3
Use of Soluble and Insoluble Cellulose Substrates by Free and Immobilized Cellulases[a]

Enzyme substrate	Novo cellulase		Sigma cellulase	
Carboxymethylcellulose				
Free	21.0±0.1		7.0±0.07	
Immobilized	7.0±0.3		1.1±0.1	
Solka floc				
Free	2.4±0.2	(11.4%)	0.9±0.2	(12.9%)
Immobilized	1.1±0.4	(15.7%)	0.1±0.2	(7.3%)
Microcrystalline cellulose				
Free	3.3±0.7	(15.7%)	1.6±0.2	(22.9%)
Immobilized	1.4±0.4	(20.0%)	0.1±0.0	(7.3%)

[a]Enzyme activity is given as nmol glucose produced min^{-1} mL^{-1}, means ± SEM, $n=3$. For insoluble cellulose substrates, the relative activities compared to the corresponding free or immobilized enzyme using CMC as substrate are given in parentheses. Reactions were carried out with foams that were cut up into 60 mm^3 pieces and a mechanical rotator was employed for continuous mixing of both free and immobilized enzymes.

crystalline cellulose) celluloses. The immobilized Novo cellulase appeared to be relatively more efficient than the free form in utilizing the insoluble substrates; for example, compared to their respective activities with CMC, relative activities with microcrystalline cellulose as the substrate were 20% for the immobilized enzyme and 15.7% of the free enzyme. Results with Sigma cellulase were the opposite, free cellulase having a greater relative activity with the insoluble substrates.

Time Courses for Thermal Inactivation

Figure 1A shows the time course for the thermal inactivation of Novo cellulase at 60°C using CMC as the substrate. Activity of the free enzyme declined sharply after 30 min, whereas activity of the immobilized enzyme was little affected up to 90 min. Hence, it appeared that immobilization provided some protection against thermal inactivation of the enzyme.

Figure 1B shows the same experiment with microcrystalline cellulose as the substrate. Both enzymes showed increasing activity over the initial 90 min at 60°C. This continued to 2 h for the immobilized cellulase, whereas inactivation of the free enzyme had begun to occur at this time. Insoluble substrates appeared, therefore, to stabilize the enzymes of the cellulase complex, whether free or immobilized, against thermal denaturation.

Optimum Temperature

The optimum temperature for cellulose hydrolysis is shown in Table 4 for Novo and Table 5 for Sigma cellulases. Both immobilized and free Novo cellulase exhibited maximal activity at 58°C using either soluble

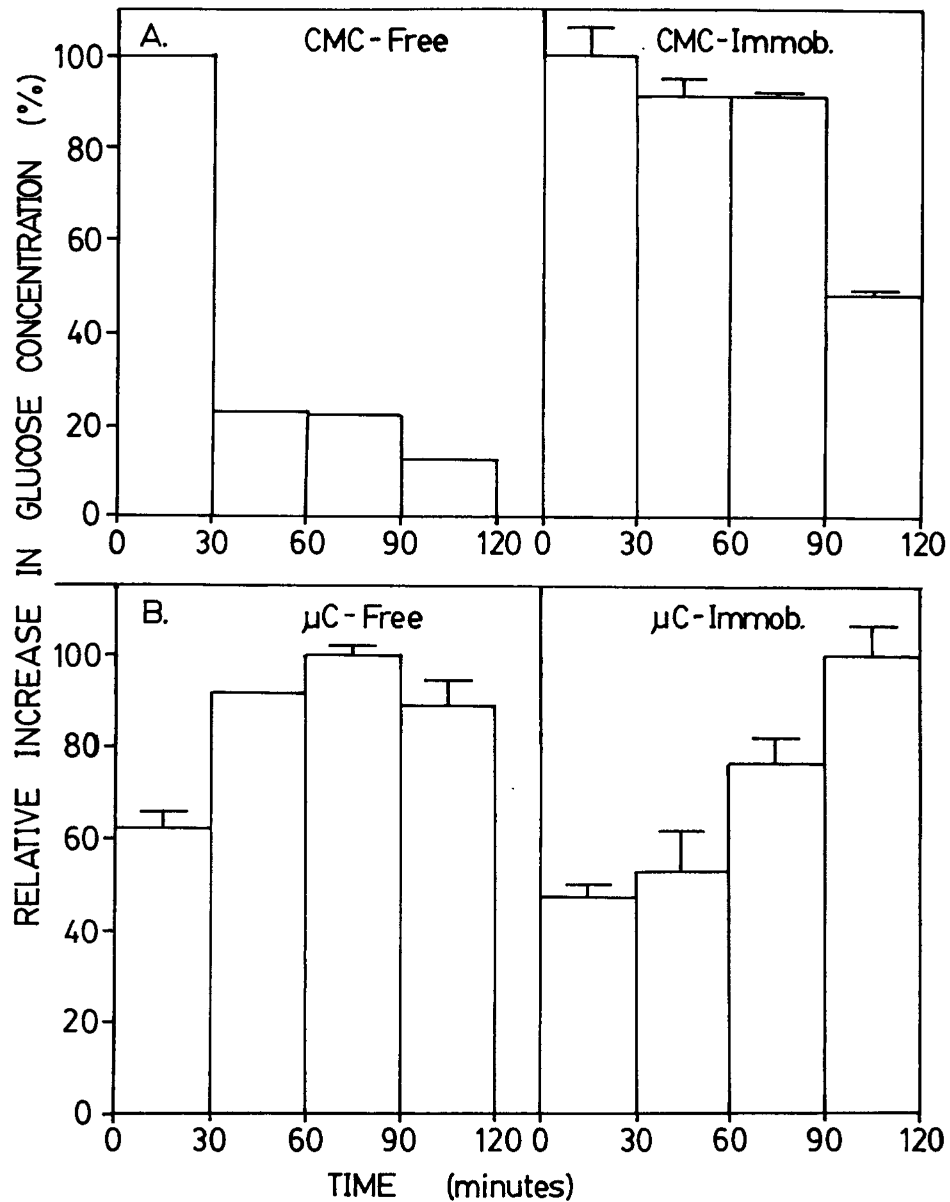


Fig. 1. Time course of NOVO cellulase inactivation at 60°C using CM cellulose (A) or microcrystalline cellulose (μC cellulose) (B) as substrate. The increases in glucose concentrations over 30-min intervals are expressed as percentages of the maximum increase observed. Data are means ± SEM, $n=3$. Where error bars are not shown, SEM values were within the dimensions of the symbol used.

Table 4
Effect of Temperature
on the Activity of Novo Cellulase[a]

Temperature	Free cellulase		Immobilized cellulase	
	CMC	μC[b]	CMC	μC[b]
5°C	660±10	60±20	260±20	40±4
22°C	880±10	60±10	340±10	40±2
58°C	1440±10	380±30	1020±90	140±2
65°C	1360± 4	190±10	960±60	130±4

[a]Activity is given as nmol glucose produced h^{-1} mL^{-1}, means ± SEM, $n = 3$.
[b]Microcrystalline cellulose.

Table 5
Effect of Temperature
on the Activity of Sigma Cellulase[a]

Temperature	Free cellulase		Immobilized cellulase	
	CMC	μC[b]	CMC	μC[b]
5°C	280±10	0	70±10	0
22°C	440±10	0	70±10	0
58°C	870±20	190±60	410±60	40±3
65°C	1380±60	210±30	60±50	0

[a]Activity is given as nmol glucose produced h^{-1} mL^{-1}, means ± SEM, $n = 3$.
[b]Microcrystalline cellulose.

(CMC) or insoluble (microcrystalline cellulose) substrates. Free Sigma cellulase had a temperature optimum of 65°C, whereas the immobilized Sigma cellulase showed a temperature optimum of 58°C.

Optimum pH

Figure 2 shows the pH activity profile of free and immobilized Novo cellulase. The pH optimum of the free enzyme was found to be approximately 5.0–5.5, identical to that reported by Novo Industries (Novo Industri A/S, 1984). The pH optimum of the immobilized cellulase underwent a shift toward a slightly more basic pH value in the range of 5.5–6.0. The immobilized cellulase also exhibited high activity over a wider pH range, when compared to the free enzyme.

Time Course of Cellulose Degradation

The time course of glucose production by free and immobilized Novo cellulase using CMC as the substrate is given in Fig. 3. The free cellulase showed greater initial activity compared to the immobilized form, but the rate of glucose production by the immobilized enzyme remained near linear for a longer time.

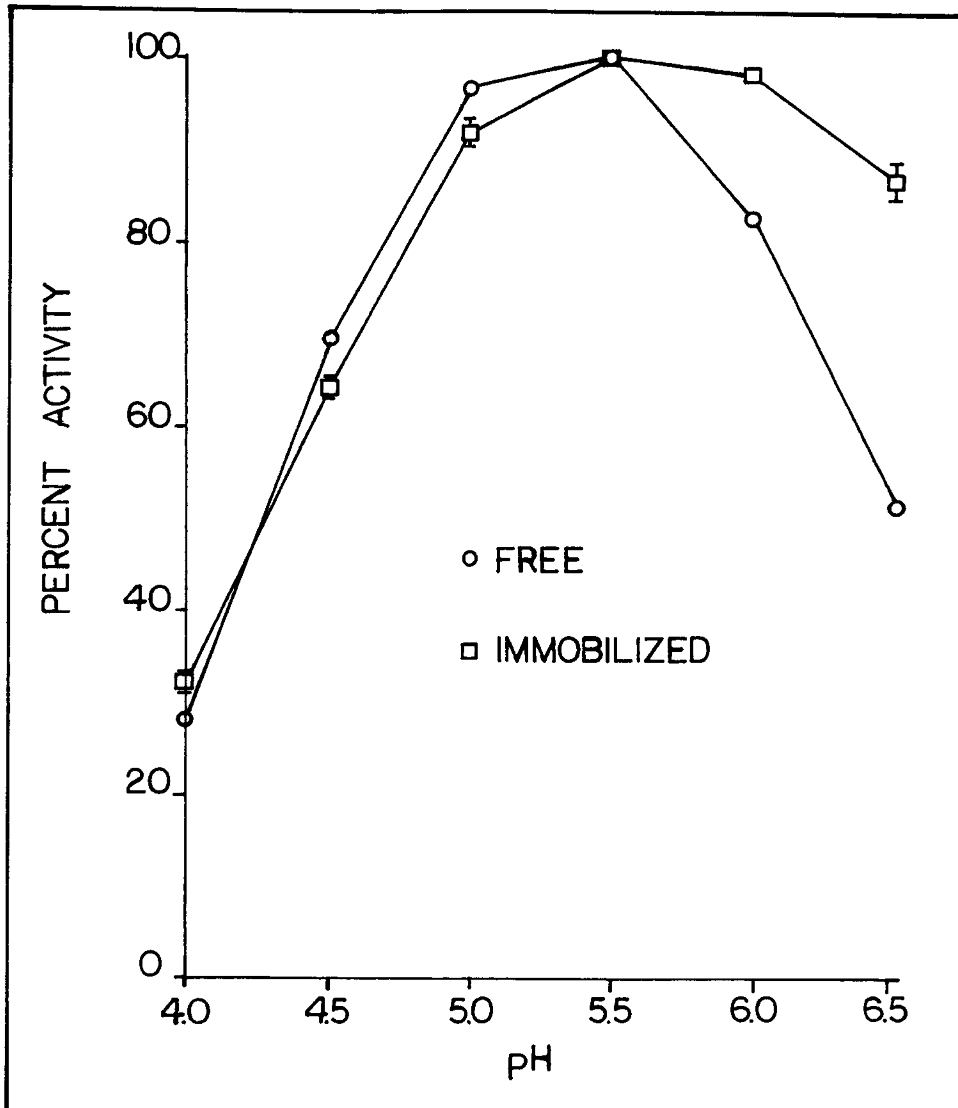


Fig. 2. Effect of pH on the relative activity of free and immobilized Novo cellulase at 23°C using 1% (w/v) CMC as substrate. Data are means ± SEM, $n=3$. Where error bars are not shown, SEM values were within the dimensions of the symbol used.

The time course of glucose production for Novo cellulase, using microcrystalline cellulose as substrate, is shown in Fig. 4. The immobilized cellulase showed a high rate of glucose production that continued throughout the incubation. Glucose production by free cellulase, however, declined sharply after the initial 10 min interval. Apparently, the immobilized cellulase could utilize the insoluble substrate more efficiently than could the free cellulase.

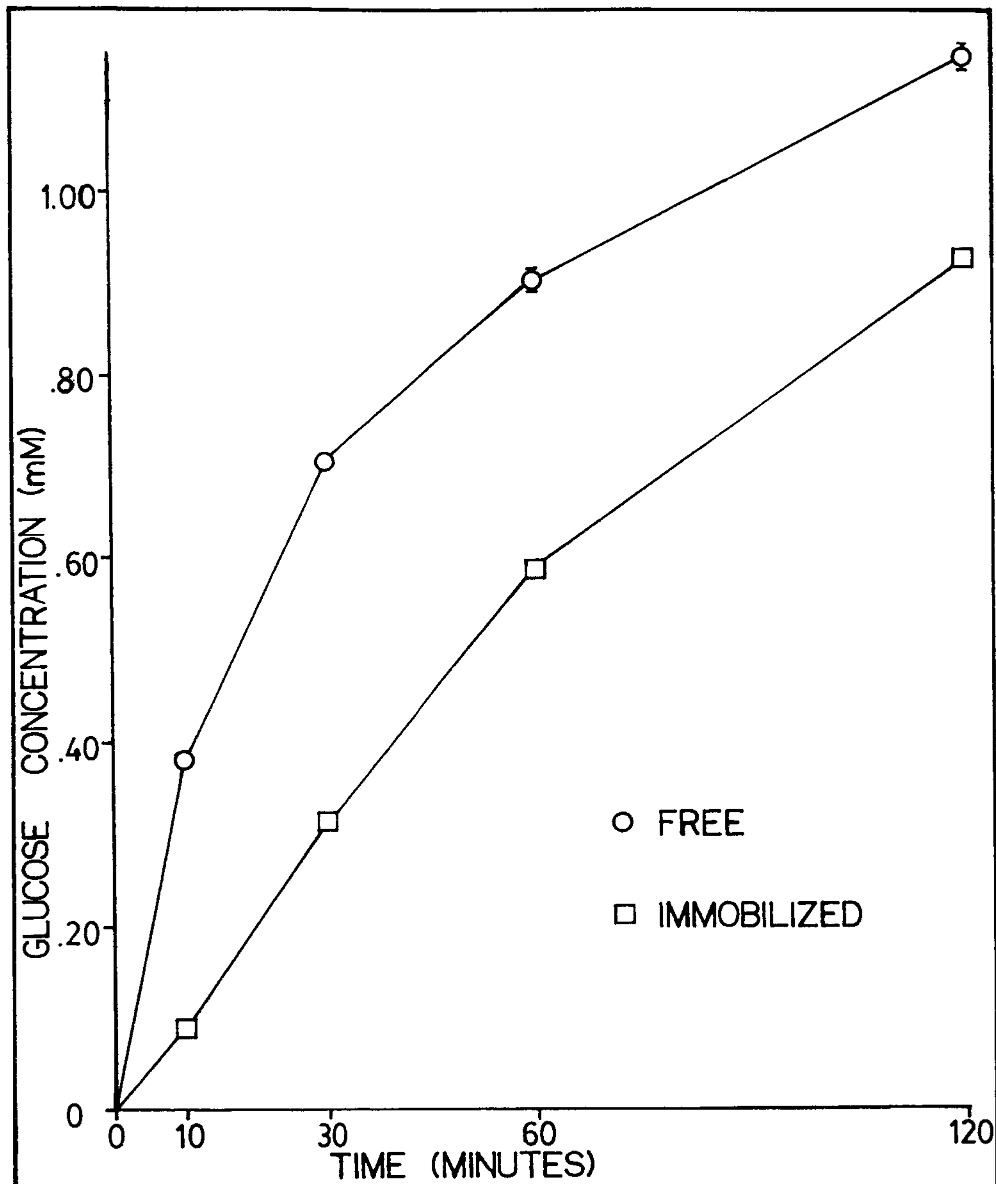


Fig. 3. Time course of glucose production for free and immobilized Novo cellulase at 23°C using CMC as substrate. Incubations contained 4 mL 1% (w/v) CMC.

Increasing Surface Area and Distribution of Binding

Table 6 shows the effects of two techniques that increase the surface area of the foam on the subsequent production of glucose from CMC. Chopping the foam into small pieces (approx 60 mm^3) slightly increased enzyme activity relative to the activity in the intact foam (approx 15,400 mm^3). Powdering the foam (frozen in liquid nitrogen and then ground

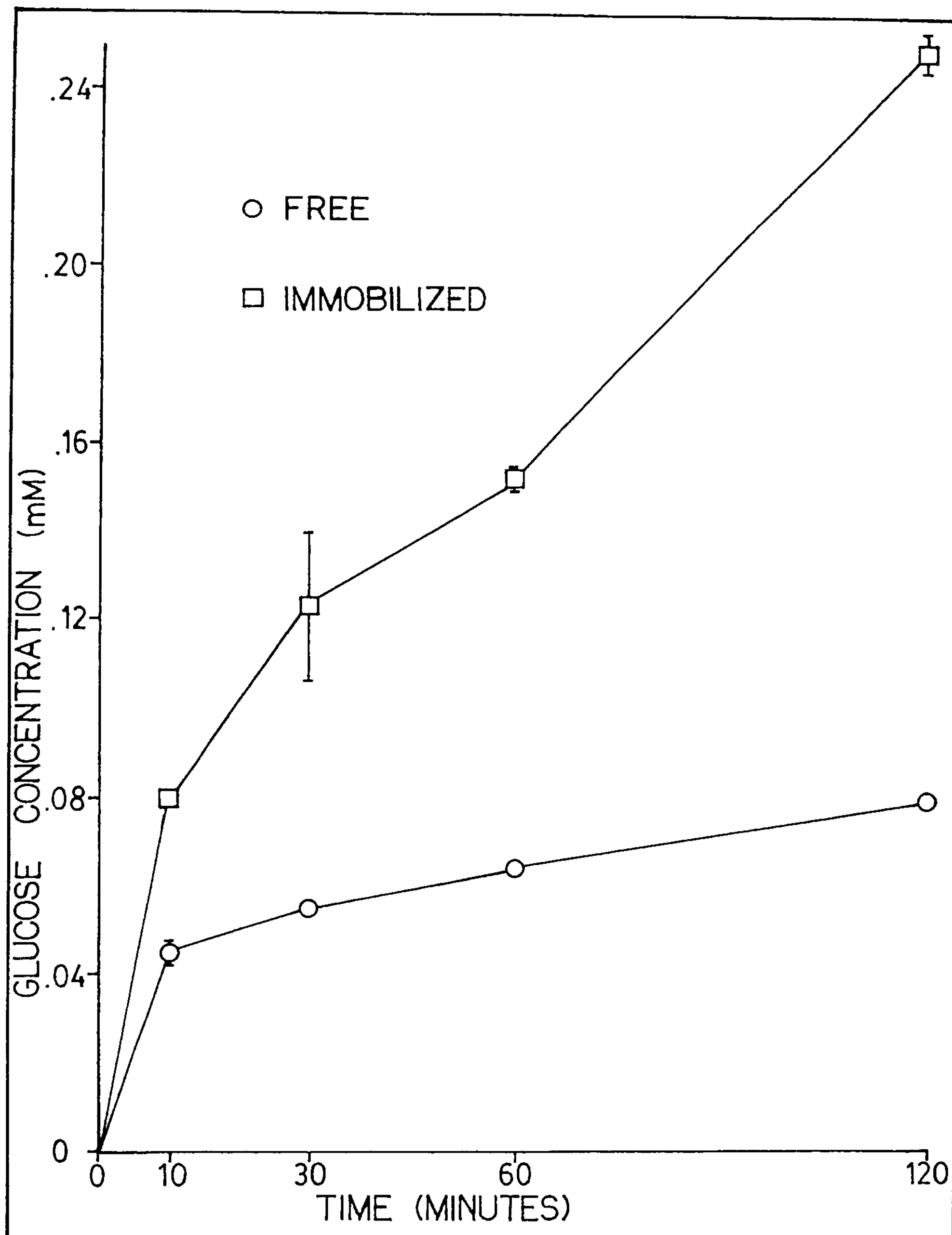


Fig. 4. Time course of glucose production for free and immobilized Novo cellulase at 23°C using 1% (w/v) microcrystalline cellulose as substrate.

Table 6
Effect of Increasing Surface Area
on the Apparent Activity of Immobilized Novo Cellulase

	Relative amount of glucose produced
Control[a]	100% ± 4%
Chopped foam	118% ± 11%
Powdered foam	240% ± 20%

[a] Activity for control (intact) foam was set at 100%. Values are means ±SEM, $n = 3$, with CMC as the substrate.

with a mortar and pestle) had a substantial effect on glucose production, increasing relative activity by 2.4-fold. Results for the powdered foam are also important in showing that the immobilized enzyme is stable to freezing in liquid nitrogen and to physical disruption of the foam.

Cellulase Localization and Permeation of High Molecular Weight Compounds into the Foam

Experiments were conducted to determine whether the immobilized cellulase was isolated in or restricted to one specific region of the foam. Foams were cut so that only surface layers or centers (interiors) of the foams remained. When tested for cellulase activity, however, both the interior and exterior portions of the foam produced glucose in similar amounts, indicating an even distribution of the enzyme throughout the foam.

Two high molecular weight compounds (Blue Dextran, mw 2,000,000 and hemoglobin, mw 64,000) were tested to observe how they would partition when mixed with the prepolymer or when introduced to foams that had already completed polymerization. Blue Dextran was tested both free and bound to beads. Both types of Blue Dextran as well as hemoglobin were found to be evenly distributed throughout all areas of the foams when the compounds were mixed with prepolymer. When Blue Dextran was introduced to polymerized foams, the compound also spread throughout the foam. Results indicate that cellulose substrates (CMC is 70–110,000 mw) would also have free mobility within the foam.

PEG Experiments

Polyethylene glycol (PEG) is a large polymer that acts to crowd enzymes together by restricting free water availability (*8*). PEG effects on the kinetic properties of free cellulase are shown in Table 7. PEG had no significant effect on either the K_m or V_{max} of the enzyme suggesting that the effects of immobilization on these parameters (Table 1) are not simply owing to enzyme crowding in the immobilized state.

Table 7
Effect of Polyethylene Glycol
on the Activity of Novo Cellulase in Solution

	K_m, mg/mL	V_{max}, nmol glucose min^{-1} mL^{-1}
Cellulase	14.6	83.0
Cellulase + 10% PEG	14.7	73.0

Salt (KC1) Effects

High salt concentrations have inhibitory effects on cellulase activity. Table 8 shows that immobilization does not protect the enzyme from the inhibitory actions of high salt concentrations. The immobilized cellulase actually proved to be more susceptible to such inhibition (activity dropped by 55%) when compared with the free enzyme (activity dropped 32%). This may have been caused by a significant shrinkage of the foam in the presence of 2 *M* KCl, which could restrict substrate penetration into the foam.

Long-Term Cellulase Activity

Figure 5 shows the decline in cellulase activity noted over a 6-wk period. A large decline was noted after 1 w. The remaining activity (about 75% of the original) remained fairly constant from 1 to 3 w, declining only slightly after the 6-wk time period had elapsed (down to 60% of the original). The half-life of the immobilized cellulase can therefore be assumed to be slightly greater than 6 w.

Diffusion Limitation of the Immobilized System

In our experiments, no differences were observed for the free and immobilized enzymes with respect to the Arrhenius plot (even at high temperatures), nor were there differences in either slopes or intercept characteristics of the Lineweaver-Burke plots. Hence, it can be concluded that diffusional limitations play an insignificant role in the system presented (*15*).

Table 8
Effect of Salt (KC1) on Cellulase Activity

Sample	Control,[a] No KC1	2 *M* KCl
Free cellulase	100% ± 1%	69% ± 1%
Immobilized cellulase	100% ± 3%	46% ± 3%

[a] Activities are given relative to enzyme velocities in the absence of KCl. Data are means ± SEM, $n = 3$, with CMC as the substrate.

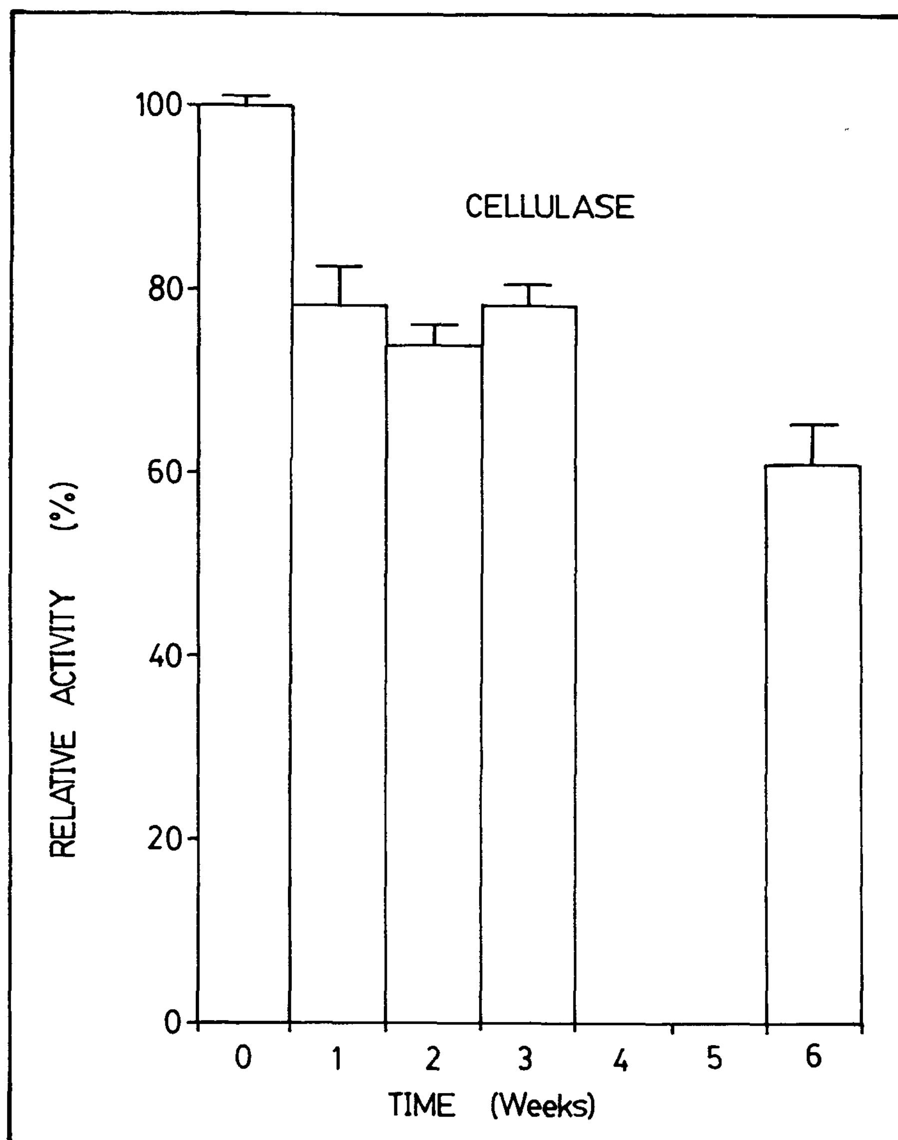
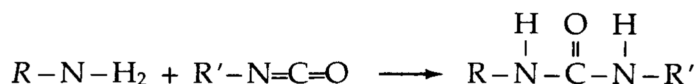


Fig. 5. Cellulase activity over a 6-wk period using immobilized Novo cellulase. Activity was determined at 23°C using 1% (w/v) carboxymethylcellulose as substrate. Foams were stored at 4°C in substrate containing 0.04% sodium azide between activity measurements.

DISCUSSION

We believe that cellulase was immobilized by covalent binding to the foam prepolymer. The reactive group used for the chain extension and crosslinking reactions in the prepolymer is an isocyanate group (W. R. Grace Co. product information). The reaction is as follows:



The polymer will generate both the amine and isocyanate groups required on introduction of water. The reactivity of isocyanate groups with other compounds is as follows: $RNH_2 > R_2NH > RCH_2OH > H_2O > RCOOH$ (*9*). We believe that immobilization occurred when an enzyme primary amino group reacted with the polymer to produce an amide linkage, interrupting crosslinking reactions during polymerization (*2*) Our main evidence supporting such covalent attachment comes from the fact that the enzymes of the cellulase complex (with mw ranging from 40–75,000) were not removed from the foam by repeated washings (beyond the first wash) yet high mw molecules (the Blue Dextrans used ranged up to 2,000,000 mw) moved freely throughout the entire foam matrix (*2*). That cellulase is bound and not physically entrapped was also supported by the observation that cellulase was not released after the foams had been powdered under liquid nitrogen.

The results of the present study indicate that cellulase is evenly distributed throughout the polyurethane foam (i.e., inner and outer areas of the foam had equivalent enzyme activities) and that the foam presents little or no diffusional barrier to penetration by high mw cellulose substrates (Blue Dextran and hemoglobin were evenly distributed in the foam), particularly when foam and substrate are continuously mixed. In line with this, enzyme activity was improved somewhat (2.4-fold) by powdering the foam, but this improvement was less than would be expected in proportion to the enormous increase in surface area of the powder.

Physical advantages of cellulase immobilization in these polyurethane foams were readily apparent. The immobilized enzyme had a broader and slightly more basic pH optimum (Fig. 2), a particular advantage when considering the next step, coimmobilization of glucose isomerase, in composing a complete system for the degradation of cellulose to high fructose product. The immobilized enzyme also had a somewhat greater thermal stability than the free enzyme (Table 4, Fig. 1) and was protected from freezing denaturation when exposed to liquid nitrogen. Immobilized cellulase did not, however, offer an advantage over the free enzyme with respect to the inhibitory actions of high salt on enzyme activity; such high salt concentrations are often encountered in cellulose products from industrial processing.

The decrease in K_m values observed on the immobilization of cellulase indicated that the affinity of the cellulase for CMC had increased. The three constituent enzymes of the cellulase complex are: endoglucanase (1,4 β-D-Glucanohydrolase); cellobiohydrolase (1,4 β-D-Glucan cellobiohydrolase), and β-glucosidase (β-D-Glucoside glucanohydrolase) (*2*). Endoglucanases, which account for the vast majority of the enzymes present within the cellulase complex, attack substituted celluloses like CMC (*10*). Immobilization may have altered the conformation of the active site or regulatory and effector sites, increasing the affinity of endo-

glucanase for cellulose. Effects of immobilization on the properties of other enzymes of the cellulase complex are also possible, such as reduced feedback inhibition of cellobiohydrolase by accumulating cellobiose, or an improved function of β-glucosidase that reduces cellobiose accumulation. The polyethylene glycol studies established that the decrease in K_m values observed were not primarily because of a crowding of the various component enzymes of the cellulase complex following immobilization.

We are not the first to have shown that cellulase immobilization can lead to a more efficient catalyst (*4,11*). However, the reduction in K_m values we observed (approx 7.5- and 3-fold) were greater than those reported previously.

Other investigators have observed that immobilized cellulases not only utilize insoluble forms of cellulose, but in fact make more efficient use of them when compared to free cellulases (*4,11*). Our time course studies using microcrystalline cellulose as the insoluble substrate confirmed these observations. Only the immobilized enzyme could carry out long term high rates of hydrolysis of microcrystalline cellulose (Fig. 4). Cellobiohydrolase is the enzyme responsible for attacking crystalline cellulose. Immobilization may have served to modify the enzyme so as to improve its efficiency as a catalyst and/or increase the strength of its binding to cellulose (*10*).

The initial rate of adsorption of cellulase to a cellulose substrate is dependent on a number of factors, the most important of which may be the structural properties of the cellulose adsorbent (*12,13*). Cellulose has crystalline and amorphous regions (*13*). The crystalline regions are considered to be inflexible, impeding the access of cellulase to cellulose and hence slowing the rate of hydrolysis (*13*). The flexible amorphous regions are thought to be the ones degraded by cellulase (*13*). The form of CMC (CMC(7LF)) used had seven carboxymethyl substitutions for every ten anhydroglucose residues (*14*). Crystalline regions are very unlikely to be present in CMC because of the absence of unsubstituted regions (*14*). The free endoglucanases of cellulase use CMC more easily than insoluble forms of cellulose (*14*). Thus, immobilization may act to reduce the rate of hydrolysis of soluble substrates by creating barriers that slow diffusion, while speeding the rate of hydrolysis of insoluble substrates by increasing the affinity of endoglucanases for them (*14*).

The long term activity curve (Fig. 5) indicates that there may be two subsets of immobilized cellulase: a labile group that is susceptible to denaturation or release and a strongly immobilized group that may be more resilient to long term storage. The location of binding within the foam (interior vs exterior) and/or the degree of binding (single vs multiple bonds) may partially account for these differences.

Previous studies have reported the immobilization of cells and enzymes using hydrophilic polymers (*5,6,9*). These immobilizations have involved entrapment and covalent binding of cellulase to polyurethane particles and immobilization of whole cells within foams similar to our own. However, covalent immobilization of cellulase within polyurethane foams is described here for the first time. This new method of immobiliza-

tion presents many unique advantages for immobilizing any enzyme, and for immobilizing cellulase in particular. Thus, the foams can be made into a variety of sizes and shapes. They are also flexible, stable, and nonreactive, once polymerized. The homogeneous distribution of enzyme within the foam means that large amounts of enzyme can be immobilized and then subdivided according to need. This represents a great advantage over immobilization techniques involving surface attachment of enzymes to polymers, resins, or beads. Powdered foams could be used to pack columns, whereas intact foams could be polymerized so as to coat reaction vessels or stirring bars. Intact foams could also be added directly to batch reactors.

The key advantage of immobilizing cellulase using this system is the dramatic decrease in K_m values observed, making the enzyme a much better catalyst. Another important advantage is the increased efficiency of hydrolysis seen for the insoluble forms of cellulose. A relatively high percentage (33%) of the cellulase activity added to the prepolymer can be immobilized using this system. The pH optimum of immobilized cellulase is also broadened and shifted toward more basic values (Fig. 4), allowing for cellulose hydrolysis at more neutral pH values. A potential improvement in this system could involve the immobilization of cellulase obtained from thermophilic bacteria (functional at 75°C), thus increasing the temperature stability of the enzyme considerably (*1*).

The main thrust of future research will involve introducing additional enzymes to be coimmobilized with cellulase. β-Glucosidase, which acts to hydrolyze the cellobiose produced by the cellulase, could be added to enhance rates of glucose production (*2*). Glucose isomerase could also be added to allow a one-step production of high fructose syrups from cellulose substrates. Overall, further developments of the immobilization system outlined in this paper could help to improve the hydrolysis of cellulose and other carbohydrate polymers for industrial purposes.

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REFERENCES

1. Emsley, J. (1987), *New Scientist* **39,** October 8.
2. Cheetham, P. S. J. (1985), in *Handbook of Enzyme Biotechnology,* 2nd ed. (A. Wiseman, ed.), Harwood, UK.
3. Tjerneld, F., Persson, I., Albertsson, P.-A., and Hahn-Hagerdal, B. (1985), *Biotechnol. Bioeng.* **27,** 1044.
4. Fadda, M. B., Dessi, M. R., Maurici, R., Rinaldi, A., and Satta, G. (1984), *Appl. Microbiol. Biotechnol.* **19,** 306.

5. Drioli, E., Iorio, G., Santoro, R., De Rosa, M., Gambacorta, A., and Nichlaus, B. (1982), *J. Mol. Catal.* **14,** 247.
6. Kumakura, M. and Kaetsu, I. (1982), *Biosci. Reports* **4,** 181.
7. Lowry, O. H. and Passonneau, J. V. (1972), *A Flexible System of Enzymatic Analysis*, Academic, NY.
8. Atha, D. H. and Ingham, K. C. (1981), *J. Biol. Chem.* **256,** 12108.
9. Kumakura, M. and Kaetsu, I. (1983), *Helv. Chim. Acta.* **66,** 2778.
10. Klyosov, A. A. (1986), *Appl. Biochem. Biotech.* **12,** 249.
11. Woodward, J. and Zachary, G. S. (1982), *Enzyme Microb. Technol.* **4,** 245.
12. Ryu, D. D. Y., Kim, C., and Mandels, M. (1984), *Biotechnol. Bioeng.* **26,** 488.
13. Puri, V. P. (1984), *Biotechnol. Bioeng.* **26,** 1219.
14. Henrissat, B., Driguez, H., Viet, C., and Schulein, M. (1985), *Bio/Technology* **3,** 722.
15. Pitcher, W. H. (1980), in *Immobilized Enzymes for Food Processing* (W. H. Pitcher, Jr., ed.), CRC Press, Cleveland, OH.