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One-Step Conversion of Cellulose to Fructose Using Coimmobilized Cellulase, β-Glucosidase, and Glucose Isomerase

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ABSTRACT

Glucose isomerase was immobilized by itself and coimmobilized with cellulase and β-glucosidase using a polyurethane foam (Hypol® FHP 2002). Approximately 50% of the enzyme added was immobilized. The immobilized enzyme was active at pH values as low as 6.8. When immobilized alone, the K_m for Mg^{2+} increased by 5.5fold and the K_m for fructose increased 62%. The half-life of the immobilized glucose isomerase was approximately 160 h of continuous hydrolysis, with a substantial (about 35–40%) amount of activity remaining even after 1000 h. When all three enzymes were immobilized together, the system was found capable of functioning at pH 7.0 to produce fructose from both soluble and insoluble cellulose substrates. At this pH, the glucose:fructose ratio was 70:30. The advantageous properties of the foam as a support for enzyme immobilization and the efficiency of the one-step conversion process outlined combine to make this system appear valuable for use in high fructose syrup production.

Index Entries: Glucose isomerase; polyurethane foam; cellulase; enzyme coimmobilization; high fructose syrup; β-glucosidase.

INTRODUCTION

The market for high fructose (corn) syrup (HFS) products has grown dramatically since 1970 (*1*). High fructose sweeteners are valuable because they are 2–3 times as sweet as glucose and possess several other advantageous chemical and physical properties (*2*). The development of immo-

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bilization methods for glucose isomerase has accelerated the growth in the use of these sweeteners (*3*).

Production of HFS is presently undertaken as a two-step process owing to the differing pH optima of the enzymatic processes involved. The first step, involving the saccharification of the starch substrate, is conducted at pH values of 4–5. The second step, isomerization of glucose to fructose catalyzed by glucose isomerase, is conducted at pH values of 7–9 (*1*).

In our previous work with immobilized cellulase, we demonstrated that the immobilized enzyme was a better catalyst (decreased K_m values for cellulose), functioned optimally over a broader pH range, and hydrolyzed insoluble substrates more efficiently compared to the soluble enzyme. Subsequently, we showed that β-glucosidase, coimmobilized with cellulase (*6*), produced a system with a much greater glucose yield than was possible from immobilized cellulase alone. The pH optimum of the coimmobilized enzyme system was also shifted to pH 6.

The present study continues our development toward a one-step immobilized enzyme process for fructose production. We have focused on the use of cellulose as a substrate for two reasons: cellulose is less expensive than starch and cellulose itself cannot be used for human nutrition (*7*). Processes that can permit the human exploitation of this unused resource, which represents half of the photosynthetic biomass produced annually, would be of major economic benefit and, for HFS, would permit low-cost fructose syrup production without consuming valuable starches (*8*).

Previous studies have surveyed the various means for immobilizing glucose isomerase (*2,3*). This study presents a new method of covalent immobilization using a polyurethane foam (Hypol® FHP 2002) (*5,6*). This support has a variety of advantageous mechanical properties (flexibility, high additive loading capacity, high water absorbance, wide porosity range, and adaptability of size and shape) (W. R. Grace Co., product information) (*9,10*).

Therefore, this work represents a new method for approaching the production of high fructose syrups. We coimmobilized cellulase, β-glucosidase, and glucose isomerase to get all three enzymes to work together to achieve what none of them could do alone: the one-step conversion of cellulose to fructose.

MATERIALS AND METHODS

Chemicals

All biochemicals and coupling enzymes were purchased from Sigma Chemical Co., St. Louis, MO, J. T. Baker Chemical Co., Toronto, ON, and Boehringer Mannheim Corp., Montreal, PQ.

Polymer

The foamable hydrophilic prepolymer (Hypol® FHP 2002) was graciously supplied by W. R. Grace and Co., Lexington, MA. This water-activated prepolymer is believed to immobilize enzymes via reactions between primary amino groups on the enzyme with isocyanate groups on the foam during the process of polymerization (W. R. Grace Co., product information) (*5,6,9*).

Immobilization of Enzymes

Glucose isomerase (Spezyme® GI) was a gift from Finnsugar Biochemicals, Inc., Schaumburg, IL, Cellulase (Celluclast®) and β-glucosidase (Novozym™ 188) were kindly provided by Novo Industries, Lachine, PQ. The typical amounts of enzyme immobilized were: 9, 18, or 72 U for glucose isomerase (where 1 U = 1 μmol of fructose/min under specified conditions defined by Finnsugar Biochemicals Inc., Finnsugar product information); 30 U for cellulase and 2 U for β-glucosidase (where 1 U = 1 μmol of glucose produced/min under standard conditions defined by Novo Industries, Novo product information).

The buffer used for immobilizing glucose isomerase, either by itself or with other enzymes, was 50 m*M* phosphate at pH 7.0. The desired amount of enzyme was dissolved in 2 mL of the phosphate buffer in a petri dish. A 1.0 g aliquot of foam prepolymer was then added. Vigorous agitation was employed to obtain a homogeneous mixture of enzyme, buffer, and prepolymer. Agitation was stopped when extensive polymerization was detected visually and by increased viscosity. The resulting foams were allowed to cure at room temperature for at least 20 min before use.

After polymerization was completed, the foams were washed with 6 mL of the phosphate buffer. The foams were then squeezed repeatedly to ensure complete absorption of the wash into the foam. The amount of enzyme present in the wash could be determined by squeezing the foams to collect the wash and then assaying it for activity.

Glucose Isomerase Experiments

Fructose served as the substrate when glucose isomerase was studied by itself. Both Mg^{2+} (1.5 or 3.0 m*M*), found to be an essential cofactor for glucose isomerase activity, and bisulfite (2.0 m*M*), an anti-oxidant, were included with the fructose (0.50*M*). These experiments were conducted at high (approximately 55°C) temperatures using 50 m*M* phosphate buffer (pH 7.0), unless stated otherwise.

Glucose Isomerase Activity

Direct measurement of glucose isomerase activity was achieved by adding fructose as the substrate and measuring the amount of glucose produced using the colorimetric glucose diagnostic kit from Sigma Chem-

ical Co., St. Louis, MO (No. 510-DA). This assay involves measuring the absorbance at 450 nm of o-dianisidine as it becomes oxidized owing to the actions of glucose oxidase and peroxidase on glucose.

Amount of Enzyme Immobilized

The sum of the glucose production by the immobilized enzymes and those present in the washes was assumed to be equal to the original amount of enzyme added (100%). Hence, glucose production by the immobilized enzymes and the enzymes in the washes was expressed as percentages of that produced by the free enzyme.

Protein Assay

Protein content was determined by the Coomassie Blue dye binding method using the BioRad Laboratories, Montreal, PQ, prepared reagent. Total protein (before addition to the foam) and protein recovered from 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 the dye-reacting materials from the foams were not released into the wash buffer.

Effect of pH on Glucose Isomerase Activity

Glucose isomerase activity was measured at pH values ranging from 4.5 to 9.0. Reaction rates after 60 min at 55°C were compared for enzyme samples in 20 m*M* sodium acetate buffer (pH 4.5 and 6.0), 50 m*M* phosphate buffer (pH 6.0–9.0), and 50 m*M* Tris buffer (pH 8.0). A detailed curve from pH 6.0 to 7.0 was obtained using 50 m*M* phosphate buffer under the same conditions.

Kinetic Parameters

Kinetic parameters (K_m and V_{max}) for fructose and Mg^{2+} were determined in 50 m*M* phosphate buffer (pH 7.0) with a reaction time of 60 min at 55°C and constant substrate concentrations of 3.0 m*M* Mg^{2+} and 0.50*M* fructose, respectively. K_m and V_{max} values were calculated from Lineweaver-Burke (double reciprocal) plots.

Storage Half-Life

Foams containing immobilized glucose isomerase were tested for their ability to convert fructose to glucose over a period of six weeks. Between uses, the foams were stored at 4°C in a substrate solution containing 0.04% sodium azide. For activity testing, foams were warmed to room temperature, thoroughly washed with buffer, and then fresh substrate was added. Glucose production after 2 h at 50°C was subsequently determined.

Coimmobilized Cellulase, β-Glucosidase, and Glucose Isomerase

Cellulose, at a concentration of 1% (w/v), served as the substrate when all three enzymes were studied together. The form of cellulose used was carboxymethylcellulose (CMC), unless otherwise indicated. The CMC (CMC(7LF), a soluble cellulose derivative (mol wt 70,000–110,000) was kindly provided by Hercules Inc., Wilmington, DE. Microcrystalline cellulose (μC) (obtained from Sigma Chemical Co.) served as the insoluble form of cellulose. A small amount of Mg^{2+} (3.0 m*M*) was added to the substrate to enhance glucose isomerase activity. The reactions were performed at 40–50°C using 50 m*M* phosphate buffer (pH 7.0), unless otherwise noted. The reactions were stopped by withdrawing 0.1 mL samples and adding to each 0.1 mL of 100 m*M* Tris buffer (pH 9.5). The pH change that resulted stopped any cellulase and β-glucosidase activity. Glucose isomerase activity was halted by immediately freezing the samples. No glucose isomerase activity was observed after thawing.

Cellulase and β-Glucosidase Activity

Glucose concentrations of these samples were calculated by monitoring the reduction of NAD^+ at 340 nm using a hexokinase/glucose-6-phosphate dehydrogenase coupled enzyme assay described by Lowry and Passonneau (*11*). Hexokinase (0.28 U) was added to a 1 mL solution of buffer (100 m*M* Tris, pH 8.0), NAD^+ (0.50 m*M*), $MgSO_4$ (5.0 m*M*), ATP (0.50 m*M*), glucose-6-phosphate dehydrogenase (0.10 U), and the test sample (0.100 mL).

Low concentrations of fructose were also determined using this coupled enzyme method. Phosphoglucose isomerase (0.07 U/assay) was added to the reaction cuvet after completion of the glucose assay to determine the fructose concentration.

Data Calculations and Statistics

Data is reported as means ± standard error of the mean (SEM), n = 3. Where SEM bars are not shown in figures, these values were within the dimensions of the symbol used.

RESULTS

Immobilization of Glucose Isomerase Alone

Studies of glucose isomerase alone were conducted first, followed by studies on all three enzymes together. Free enzymes were compared to the immobilized enzymes to assess the effects of immobilization and to point out advantages of the process.

Table 1
Effect of Temperature on Glucose Isomerase Activity[a]

Temperature	Free enzyme	Immobilized enzyme
	nmol glucose/min/mL	
23°C	<1.0	<1.0
40°C	33±3	<1.0
60°C	417±40	52±8
65°C	568±37	70±10

[a]Data are means ± SEM, $n = 3$. The substrate used was 0.50*M* fructose (containing 1.5 m*M* Mg^{2+} and 2.0 m*M* bisulfite) incubated for 1 h in the presence of 36 U glucose isomerase.

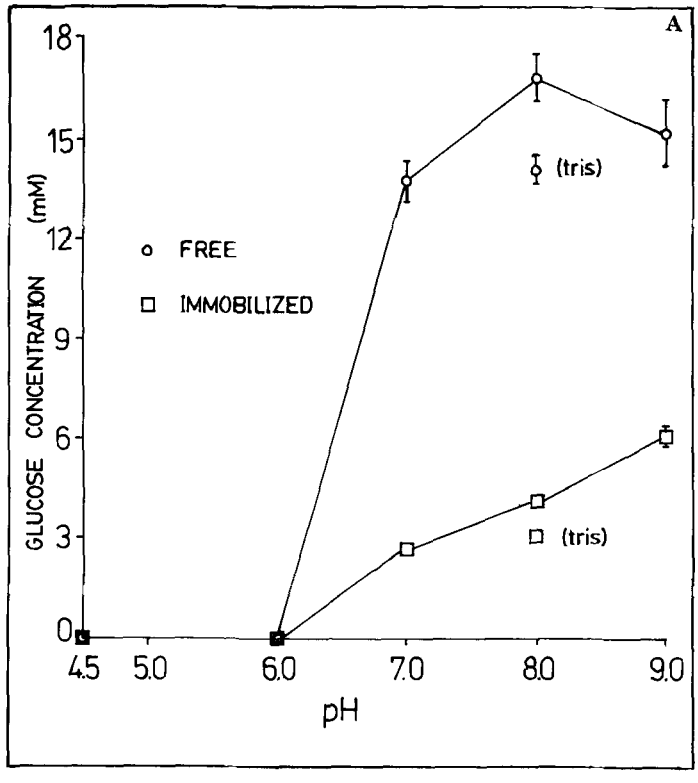

Fig. 1A. Effect of pH on the activity of free and immobilized glucose isomerase (18 U) over a wide pH range. Reaction conditions: 0.50*M* fructose, 1.5 mM Mg^{2+}, and 2.0 m*M* bisulfite at 55°C for 1 h. At pH 8, activity in both tris and phosphate buffers are shown. Data are means ± SEM, $n = 3$. Where error bars are not shown, SEM values were within the dimensions of the symbol.

The distribution of glucose isomerase activity was 55.5±7.7% immobilized within the foam and 44.5±3.3% present in the wash when glucose isomerase was studied alone. Protein immobilized within the foam was 47±2% of the total amount of protein added.

Table 1 shows the effects of temperature on the activity of glucose isomerase. It is important to note that glucose isomerase has very low activity at room temperature. Optimum temperatures for both the free and immobilized enzymes lie in the range of 60–65°C. Hence, all experiments involving glucose isomerase alone were conducted at elevated temperatures.

Figure 1A shows the broad pH profile for glucose isomerase by itself. Neither the free nor immobilized enzymes showed activity below pH 6.0. The pH optimum for free glucose isomerase was 8.0, whereas the pH optimum for the immobilized enzyme was 9.0 or higher.

A more detailed pH curve in the range 6.0–7.0 (Fig. 1B) was constructed since the ultimate goal of the research was to run all three enzymes at a

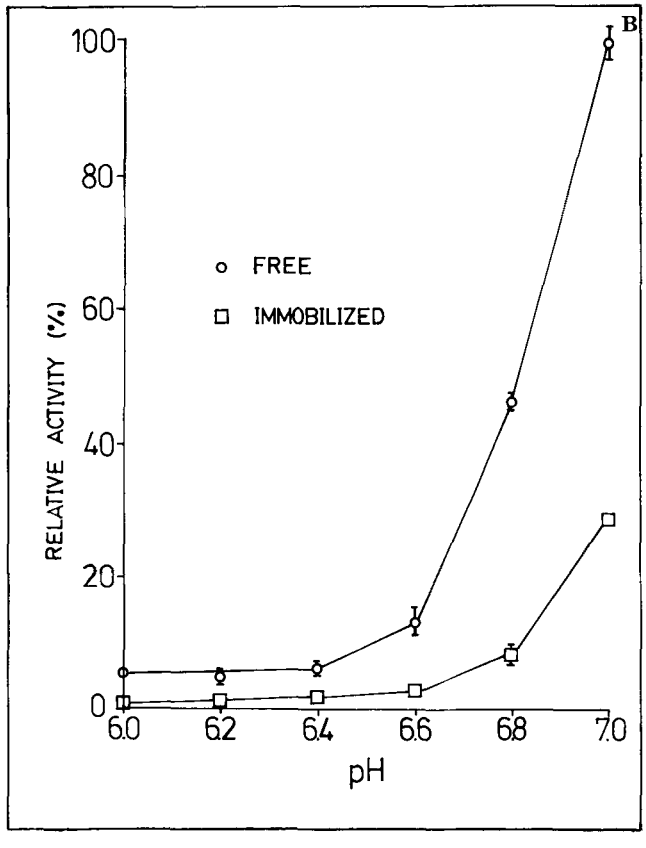

Fig. 1B. Effect of pH on the activity of free and immobilized glucose isomerase (18 U) over a narrow pH range. Reaction conditions: 0.50*M* fructose, 1.5 m*M* Mg^{2+}, and 2.0 m*M* bisulfite at 55°C for 1 h. Data are means±SEM, $n=3$. Where error bars are not shown, SEM values were within the dimensions of the symbol.

Table 2
Effect of Sodium Bisulfite on Glucose Isomerase Activity[a]

	Free enzyme	Immobilized enzyme
Bisulfite Concentration, mM	nmol glucose/min/mL	
0	260±20	58±4
0.1	252±33	91±18
2.0	307±3	91±15

[a]Data are means±SEM, $n=3$. The substrate used was 0.50*M* fructose (containing 1.5 mM Mg^{2+}) for 1 h at 55°C. The amount of glucose isomerase used was 18 U.

Table 3
Kinetic Properties of Fructose and Mg^{2+} for Glucose Isomerase[a]

	Free enzyme	Immobilized enzyme
K_m fructose, *M*	0.411±0.091	0.666±0.129
K_m Mg^{2+}, m*M*	0.248±0.036	1.44±0.38
V_{max} (nmol glucose/min/mL)	220±27	103±19

[a]Data are means±SEM, $n=3$. The substrate used was 0.50*M* fructose containing 0.1 m*M* bisulfite to determine the K_m for Mg^{2+} and 3.0 m*M* Mg^{2+} with 0.1 m*M* bisulfite to determine the K_m for fructose. Reactions were run for 60 min at 55°C using 9 U glucose isomerase.

single pH value, which gave adequate activity for each. The detailed pH curve showed that the activity of glucose isomerase dropped precipitously at pH values below 7. Thus, the minimum pH value useful for glucose isomerase experiments was pH 7.

Bisulfite is recommended as an additive for stabilizing glucose isomerase and preventing oxidation (*12*). Table 2 shows the effects of varying concentrations of bisulfite on free and immobilized glucose isomerase. Low concentrations (0.1 m*M*) increased the activity of the immobilized enzyme significantly (by 57%), whereas much higher concentrations (2.0 m*M*) resulted in only a slight increase (18%) in the activity of the free enzyme. It was decided that a small amount of bisulfite (0.1 m*M*) would be added to all subsequent substrate mixtures to enhance glucose isomerase activity.

Magnesium ion (Mg^{2+}) is required for glucose isomerase activity. Mg^{2+} serves as an activator for the enzyme and a competitor for Ca^{2+}, a glucose isomerase inhibitor. The suggested level of Mg^{2+} was 1.5 m*M* (*12*).

Table 3 shows the effect of immobilization on the kinetic parameters for Mg^{2+} and fructose of glucose isomerase. The K_m for Mg^{2+} increased by 5.8fold upon immobilization. Thereafter, the concentration of Mg^{2+} added to the substrate solutions was increased to 3.0 m*M* so that the Mg^{2+} concentration was roughly two times that of K_m for the immobilized enzyme.

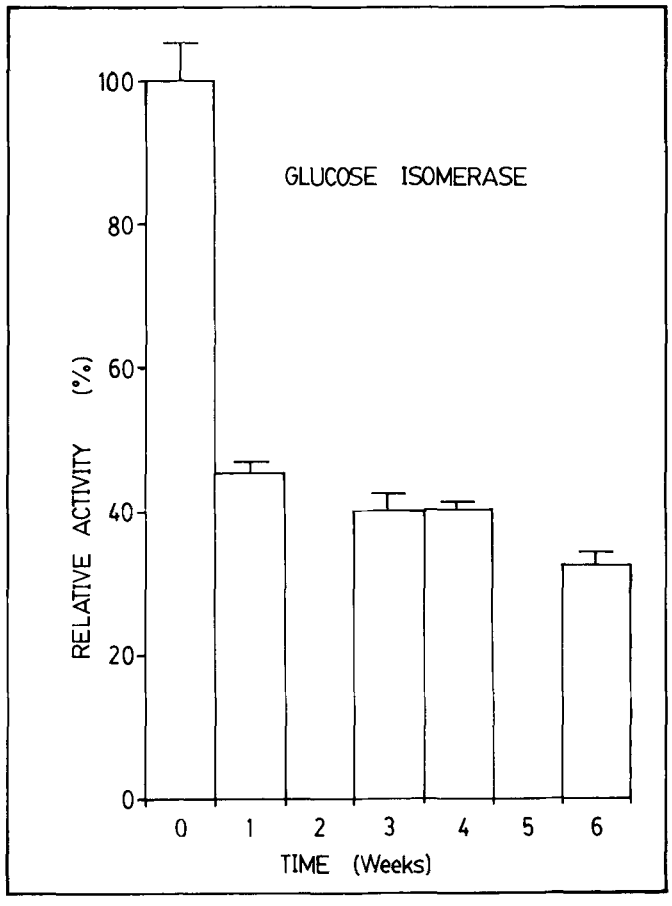

Fig. 2. Decline in immobilized glucose isomerase (18 U) activity over a 6-wk period. Procedures were followed as outlined in the "Materials and Methods" section. Data are means ± SEM, $n = 3$.

The K_m for fructose increased 62% upon immobilization (Table 3). The V_{max} decreased by approximately 53% when immobilized. The net result of these changes was that the k_{cat} ($= V_{max}/K_m$) value dropped by about 1.9-fold (taking into account that only 55.5% of the enzyme was immobilized). Glucose isomerase, therefore, was adversely affected by the process of immobilization, but remained functional.

Figure 2 shows the storage half-life for glucose isomerase alone while incubated with substrate. The half-life was approximately 160 h. However, a substantial (35–40%) amount of activity remained even after 1000 h of storage.

Coimmobilization of Cellulase, β-Glucosidase, and Glucose Isomerase

The subsequent experiments involved studying glucose isomerase in association with cellulase and β-glucosidase. The distribution of the sum

of the activities of coimmobilized cellulase, β-glucosidase, and glucose isomerase (measured as the total amount of glucose and fructose produced) was 49.4±7.9% immobilized within the foam and 50.6±3.5% present in the wash. The individual activities of the enzymes were impossible to determine owing to the interconversion of the glucose and fructose produced. Protein immobilized within the foam was 59±2% of the total amount of protein added (cellulase, β-glucosidase, and glucose isomerase).

Preliminary experiments showed that addition of bisulfite, for the glucose isomerase reaction, had no effect on the cellulase or β-glucosidase reactions. The optimal amount of glucose isomerase for the free and immobilized enzymes was found to be 18 U. The increases in fructose concentration, obtained by using larger amounts of glucose isomerase, were not proportional to the increased concentration of glucose isomerase.

Figure 3 shows the effect of varying the pH on the relative production of glucose and fructose. Comparison was made between the free enzyme system, which had been dialyzed to remove contaminating free glucose, and immobilized enzymes. Increased pH resulted in a decline in the total hexose sugar yield (glucose and fructose) of the system, but the percentage of fructose produced was increased. Decreasing pH had the opposite effect. A 50:50 ratio of glucose to fructose was achieved at pH 7.5 for the free enzyme system, whereas the highest percentage of fructose produced for the immobilized enzyme was 30% at pH 7.5. The absolute amounts produced and product ratios at pH 7.0 seemed to be optimal. Therefore, pH 7.0 was chosen for all future experiments involving the three enzymes.

The time course of glucose and fructose production by free and coimmobilized cellulase, β-glucosidase, and glucose isomerase, using carboxymethylcellulose or microcrystalline cellulose as substrates, are given in Fig. 4 and 5, respectively. Glucose production far exceeds that of fructose over the initial time points, but the net amounts of fructose produced become substantially larger over longer incubation times. Fructose concentrations produced by the immobilized enzyme system were much higher in all cases than those seen in the soluble enzyme system.

DISCUSSION

Immobilization of glucose isomerase can be divided into two broad categories: immobilization of the isolated enzyme and immobilization of cell-associated glucose isomerase (*1*). Our method involved the immobilization of a pure (cell-free) form of glucose isomerase supplied by Finnsugar Biochemicals Inc. (*12*). It was produced as a glucose isomerase concentrate for the reactivation of their SPEZYME® IGI immobilized glucose isomerase (Finnsugar product information). That immobilization system and several other commercially available systems utilize DEAE-cellulose as the solid support, with glucose isomerase being electrostatically ad-

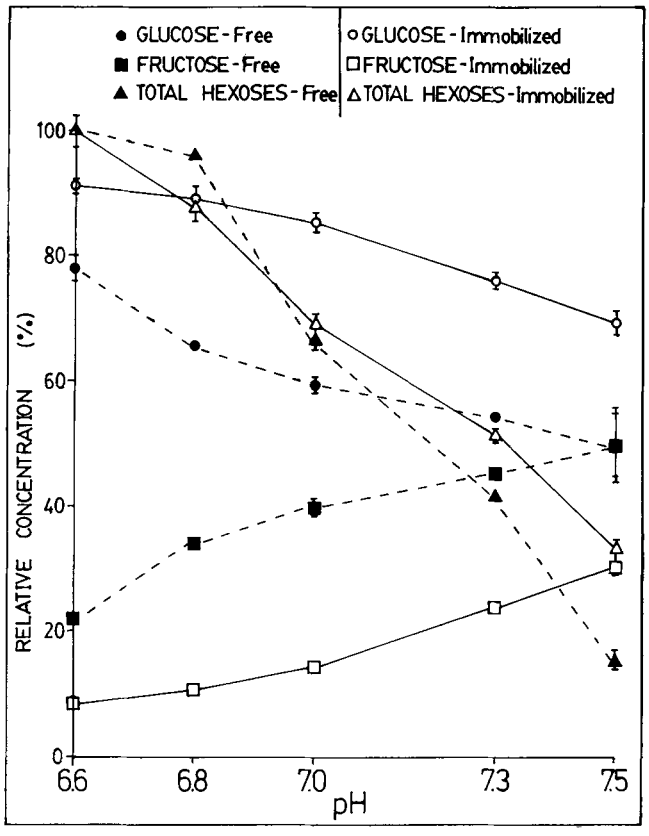

Fig. 3. Effect of varying the pH on the relative production of glucose and fructose by free and coimmobilized cellulase (60 U), β-glucosidase (4 U), and glucose isomerase (72 U), using 1% carboxymethyl cellulose (containing 3.0 m*M* Mg^{2+}) at 50°C for 4 h. Total amount of hexoses produced were defined relative to the amount present at pH 6.6. Glucose and fructose concentrations were expressed as percentages of the total amount of hexose sugars present at each pH (e.g., always added up to 100%). Comparison was made between immobilized enzymes and free enzymes that had been dialyzed to remove background glucose contamination. Data are means ± SEM, $n = 3$.

sorbed onto the support (*2,3,12*). Such DEAE-cellulose supports are obviously unsuitable for cellulose hydrolysis. In addition, adsorbed enzyme systems are extremely susceptible to changes in the conditions used for isomerization (e.g., pH) (*5,6*). The polyurethane foam-immobilized system developed here provides several important advantages over the methods that have been outlined previously for the immobilization of glucose isomerase (*1-3,12*).

Immobilization using the foams can be achieved quickly (within several minutes) and without the use of harsh pH or temperature conditions. The polyurethane foam is resistant to enzyme or microbial attack, unlike most previously used supports (*1-3,12*). The homogeneous distribution of enzyme within the foam means that large amounts of enzyme can be im-

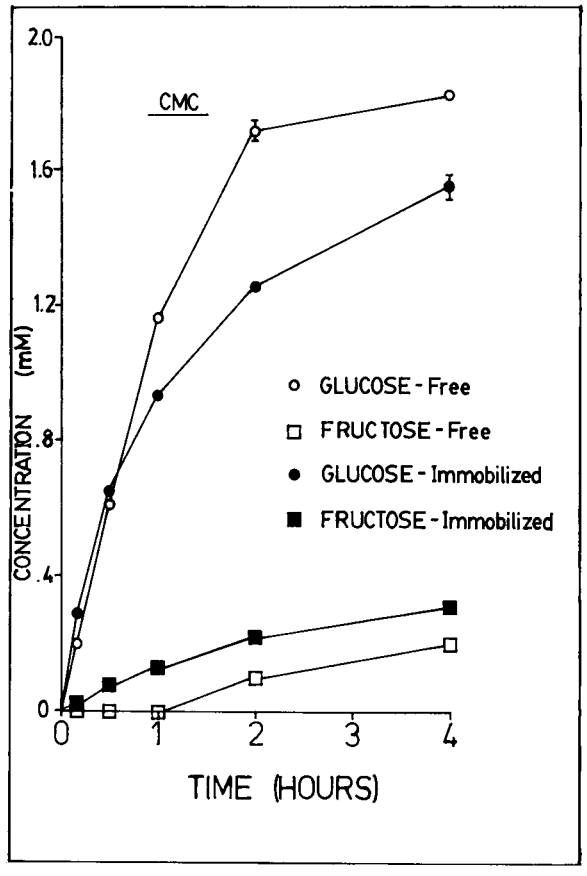

Fig. 4. Time course of glucose and fructose production by free and co-immobilized cellulase (30 U), β-glucosidase (2 U), and glucose isomerase (18 U) at 50°C using 1% carboxymethyl cellulose (CMC) (containing 3.0 m*M* Mg^{2+} and 0.1 m*M* bisulfite) as substrate. Data are means ± SEM, $n=3$.

mobilized and then subdivided according to need (5). This represents a significant advantage over immobilization techniques involving surface attachment to polymers, resins, and beads (4).

The experiments involving glucose isomerase by itself established that about 50% of the enzyme added to the prepolymer was successfully immobilized; both activity and protein measurements support this conclusion, as well as calculations of enzyme V_{max} values (Table 3).

Immobilization caused a slight increase in the K_m values for fructose and indicated that the enzyme had become a slightly less effective catalyst either through immobilization in an unfavorable configuration or through covalent modification during immobilization. The K_m value obtained for the foam-immobilized glucose isomerase (0.666*M*) is not significantly different from those obtained for free glucose isomerases (0.09–0.92*M*) (2).

Coimmobilization of all three enzymes also resulted in 50% of the total activity being immobilized. The amount of protein bound within the foams

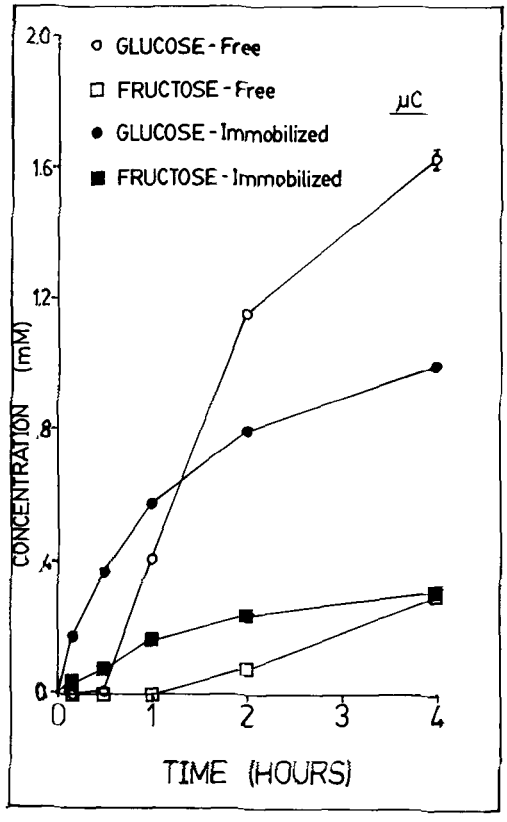

Fig. 5. Time course of glucose and fructose production by free and co-immobilized cellulase (30 U), β-gluicosidase (2 U), and glucose isomerase (18 U) at 50°C using 1% microcrystalline cellulose (μC) (containing 3.0 mM Mg^{2+} and *0.1 mM bisulfite*).

was almost 60%. Hence, a small percentage of the enzymes may have been immobilized in configurations unfavorable to catalysis.

Our previous work with cellulase and β-glucosidase had established that the foam matrix did not impose barriers to diffusion of high molecular weight substrates (mol wt of cellulose used was 70,000–110,000) (*5,6*). Glucose isomerase, therefore, was not expected to suffer from any barriers to substrate diffusion based on substrate size alone (mol wt of glucose or fructose is 180). Lineweaver-Burke plots for immobilized glucose isomerase confirmed this conclusion since no deviation from linear behavior was observed at high substrate concentrations (*16*).

The glucose isomerase used (isolated from *Streptomyces rubiginosus*) was a thermostable enzyme with high activity up to 65°C (*12*). However, we have previously shown that such temperatures are capable of denaturing cellulase (*5*). A reaction temperature of 50°C, hence, was chosen for the coimmobilized system. A lower than optimal temperature is also routine for the industrial use of glucose isomerase, because it lowers the net cost of conversion by prolonging the enzyme half-life (3).

Figure 1A indicates that immobilization did not serve to significantly alter the pH vs relative activity profile of glucose isomerase (compared to the soluble enzyme) over the pH 6.0–9.0 range. Immobilization may have served to increase the activity of glucose isomerase at very basic pH values (e.g., pH 10–12). These values, however, were too extreme to be of use in coimmobilizing cellulase/β-glucosidase. Hence, glucose isomerase activity at pH values above 9.0 was not examined.

Previous experiments had already established that cellulase and β-glucosidase had a pH optimum of 6 when coimmobilized (compared to the pH optimum of 5 when free), as well as showing significant activity at pH 7 and 8 (*6*). Immobilized glucose isomerase showed sufficient activity at pH 7 to justify the coimmobilization of all three enzymes. The use of a neutral pH for the isomerization reaction offers an additional advantage because it decreases the formation of color and byproducts, such as D-psicose (*2,3,13*).

The specific pH used when all three enzymes are coimmobilized strongly influences the yield and ratio of hexose sugars produced (Fig. 3). Lowering the pH increases the yield of total hexose sugars by increasing the activity of cellulase and β-glucosidase, but lowers the fructose yield by inhibiting glucose isomerase activity. Increasing the pH has the opposite effect. A two-phase reactor could be envisioned where the pH initially is low (to enhance glucose production by cellulase) and is slowly raised as the reaction proceeds (to enhance glucose isomerase activity).

It has been established that glucose isomerase in the free form requires both cobalt (Co^{2+}) and magnesium (Mg^{2+}) for maximal activity (*1*). Unfortunately, cobalt is a toxic metal that must be removed following isomerization (*1,3*). Hence, it was decided that testing of cobalt would be needless since adequate glucose isomerase activity was observed using magnesium as the sole metal cofactor.

The time course of cellulose hydrolysis and glucose conversion by all three enzymes (Figs. 4 and 5) shows that the immobilized enzymes produced slightly less glucose than the free enzymes, but produced more fructose initially. Previous work with coimmobilized cellulase and β-glucosidase found that, when utilizing microcrystalline cellulose, the free enzymes initially gave higher glucose yields (*6*). However, the glucose produced by the immobilized enzymes eventually (>24 h) surpassed that of the free enzymes (*6*). Glucose and fructose produced by the immobilized enzymes, therefore, would be expected to continue increasing for both forms of cellulose (especially microcrystalline cellulose) over longer time courses (>4 h) (*5,6;* Figs. 4 and 5). Thus, the three enzymes, when coimmobilized, appear to become superior catalysts to their free forms.

The half-life obtained for glucose isomerase immobilized by itself (about 160 h; Fig. 2) is quite similar to those obtained for several other immobilized glucose isomerase preparations (*3*). Various parameters, such as optimum pH and temperature for enzyme stability, could be altered to increase the catalytic "lifespan" of the enzyme.

It is important to note for the half-life curve (Fig. 2) that after the initial sharp decline in activity, there exists a residual activity that declines at a much slower rate. Thus, there may be two subsets of immobilized enzymes within the foams: a labile group that is more susceptible to denaturation or release and a tightly immobilized group that may prove more resilient to long-term storage. The location of binding within the foam (interior vs exterior) and/or the degree of binding (multiple vs single bonds) may partially account for these differences.

The half-life observed for cellulase and β-glucosidase, coimmobilized in foams, was approximately 500 h (*6*). Hence, all three enzymes coimmobilized together should result in an adequate overall half-life.

Although no experiments were conducted concerning reactor design, it is felt that the three coimmobilized enzymes could be used in either batch or column processes. Intact foams (for batch processes) or powdered foams (for column processes) could be used to break down various forms of cellulose to produce glucose/fructose mixtures (*5*).

The immobilized glucose isomerase developed here has some unique advantages, in addition to those previously listed. This new immobilized glucose isomerase could function at pH values as low as 6.8. More importantly, this enzyme could convert the very low glucose concentrations produced by the coimmobilized cellulase and β-glucosidase into fructose at ratios (about 50%) expected for industrial conversions (where the glucose syrups are much more concentrated).

A final unique advantage of the coimmobilized enzyme system outlined in this work is that the mixture of cellulase, β-glucosidase, and glucose isomerase will allow for a greater degree of cellulose conversion to take place (*14,15*). This increased utilization would take place because fructose, unlike glucose, is a poor inhibitor of β-glucosidase (*14*). Hence, feedback inhibition of β-glucosidase, and possibly cellulase, by glucose would be reduced.

Overall, the increased stability, fructose and glucose yield, substrate affinity, and potential for enzyme reuse combine to make this method for cellulose hydrolysis and simultaneous fructose syrup production appear potentially valuable to the industries involved in HFS production. The coimmobilized enzyme system developed here not only has potential for developing cellulose saccharification on an industrial scale, but could also prove applicable to various analogous "one-step" reaction processes, such as the saccharification of starch via coimmobilized amyloglucosidase, pullulanase, and glucose isomerase.

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