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Immobilization of Glucose Isomerase onto Granular Chicken Bone

D. Y. SCHAFHAUSER AND K. B. STOREY*

Institute of Biochemistry, Departments of Biology and Chemistry, Carleton University, Ottawa, Ontario, Canada K1S 5B6

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ABSTRACT

Glucose isomerase was immobilized onto granular chicken bone (BIOBONE™) by adsorption. The amount of activity bound relative to an equal amount of free enzyme was 32±1%, with the estimated specific activity decreasing from 11.1±0.7 to 3.9±0.5 U/mg protein with immobilization. Compared with the free enzyme, immobilized glucose isomerase showed a threefold increase in the K_m for fructose and a fivefold decrease in V_{max}. High operating temperatures were possible (>55°C), but continuous use and long-term storage studies showed gradual losses of activity. Both the binding and the activity of the bone-immobilized enzyme were highly resistant to treatments with detergent, ethanol, and KCl. Studies to determine mass transfer limitation effects on immobilized glucose isomerase showed that these were insignificant for this system.

Index Entries: Glucose isomerase; enzyme immobilization; chicken bone; high fructose syrups.

INTRODUCTION

Over the past two decades, high-fructose syrups (HFSs) have been replacing sucrose as the sweetener of choice for the food industry (*1,2*). HFSs are produced industrially by treating glucose syrups, derived from starch that has been hydrolyzed with soluble amylases, with glucose isomerase (*3*). Critical to the expansion of the industry has been the development of methods for the immobilization of glucose isomerase that

*Author to whom all correspondence and reprint requests should be addressed.

allow the successful recovery and reuse of the enzyme. Numerous techniques have been reported, and a variety of immobilized glucose isomerase preparations are in large-scale commercial use (*2–9*).

Immobilized enzyme preparations can be plagued, however, by high capital costs arising from the use of expensive support materials and from enzyme activity losses caused by harsh immobilization conditions, enzyme instability, or reduction in catalytic efficiency once bound (*10*). BIOBONE™ is a novel support material that is proving to have wide applicability for immobilized enzyme technology (*11,12*). The material is composed of granular chicken bone, a byproduct of the poultry industry, and is plentiful, inexpensive, food grade, and nontoxic—all excellent characteristics for a support material to be used in HFS production (*10,12*). Furthermore, enzyme binding to BIOBONE™ via adsorption is fast, requires no chemicals to promote or stabilize binding, and is resistant to a wide range of harsh conditions (*11,12*). In a previous study, we reported the successful immobilization of amyloglucosidase onto chicken bone and analyzed numerous properties of the bound enzyme (*11*). The present work describes the use of BIOBONE™ as a solid support for the immobilization of glucose isomerase.

MATERIALS AND METHODS

Materials

Glucose isomerase from *Streptomyces rubiginosus* was donated by Finnsugar Chemical Co., Elk Grove Village, IL. All biochemicals were purchased from Sigma Chemical Co., St. Louis, MO, J. T. Baker Chemical Co., Phillipsburg, NJ, or Boehringer Mannheim Corp., Montreal, PQ. Clean granular chicken bone was donated by Protein Foods Research (Guelph, Ontario). The bone had been stripped using hot, aqueous NaOH, followed by washing and sizing to between 10 and 20 mesh. Before use, the bone was washed with phosphate buffer (pH 9.0, 50 m*M*) to remove any particulate matter.

Immobilization of Glucose Isomerase

Before use, glucose isomerase was passed through a column of G-25 Sephadex equilibrated with phosphate buffer to remove any contaminating low molecular wt metabolites. The standard amount of glucose isomerase used for all experiments, unless stated otherwise, was 9.3 U. One unit of activity is defined as the amount of enzyme that will convert glucose to fructose at an initial rate of 1 m*M*/min at pH 7.0 and 60°C with 2.0 m*M* glucose as substrate (Finnsugar Group product information). Standardly, adsorption was achieved by the addition of enzyme to a tube

containing 100±2 mg of prewashed bone and 0.5 mL phosphate buffer (pH 9, 50 m*M*), followed by mixing at 100 rpm for 1 h at 23°C. Excess solution was poured off, followed by four washes with buffer (1 mL each).

Glucose Isomerase Assay

Activity was assayed by measuring glucose production from the isomerization of fructose. Standard reactions were carried out at 55–60°C, using 2.5 M fructose as the substrate, dissolved in 50 m*M* phosphate buffer (pH 8.0) containing 2.0 m*M* bisulfite and 1.5 m*M* $MgCl_2$. To initiate a reaction, 1 mL of substrate was added to the bone-immobilized enzyme followed by mixing for 30 min. The reaction was stopped by removing 0.1 mL of the reaction mixture and adding it to 0.1 mL of 1 *N* NaOH; glucose was determined using a diagnostic kit (Sigma Chemical Co. #510-DA).

Optimum Binding Conditions, Reaction pH and Temperature, Effect of Potential Disrupting Agents, and Storage Half-Life

Experiments to determine the optimal pH for enzyme binding (pH 5.5–9.0 in phosphate buffer), the maximum amount of enzyme that could be immobilized to 100 mg of bone, the optimum reaction pH and reaction temperature, the effects of enzyme disrupting/denaturing reagents, and storage half-life were carried out essentially as described previously for amyloglucosidase but based on modifications of the standard reaction conditions for glucose isomerase described above (*11*).

Kinetic Parameters and Protein Determination

Glucose production after 10 min was determined at varying fructose concentrations from 0.06–3 *M*. K_m and V_{max} values were calculated using Hanes and Hill plots. Protein was determined by the Coomassie Blue method with bone-immobilized enzyme protein calculated as previously described (*11*).

Mass Transfer Limitation

Three tests were used to assess mass transfer limitation:

1. Glucose production after 10 min was determined for reactions using 0.06–3 *M* fructose and comparing mixed and nonmixed tubes;
2. Temperature data for free and immobilized glucose isomerase were compared using the Arrhenius equation; and
3. Kinetic data for fructose using 9 and 18 U of enzyme were compared on Lineweaver-Burk plots.

Data Calculations and Statistics

Data is reported as mean values of triplicate samples ± SEM. Statistical significance was tested by the Student's *t*-test.

RESULTS AND DISCUSSION

Glucose isomerase was readily adsorbed onto BIOBONE™ by simply mixing the enzyme in phosphate buffer with bone for 1 h at 23°C. Thus, immobilization onto bone is fast, simple, and inexpensive—all qualities favorable for an industrially useful system. The optimal incubation pH for binding was pH 9; at pH 8, the amount of enzyme bound was 20% less and binding was reduced further at lower pH values (data not shown). At pH 9 and with the standard 9.3 U of enzyme added, the mean percentage of added glucose isomerase immobilized was 31.8 ± 0.8% compared to an equal amount of the free enzyme assayed under the same conditions. This corresponds to approx 10 mg of enzyme bound/g of bone. Approximately 50.6 ± 0.6% of the activity was recovered in the four washes, and the remaining 17.6% of activity could be attributed to enzyme deactivation upon immobilization. The percentage of enzyme activity immobilized onto BIOBONE™ compares favorably with reports of glucose isomerase binding to other solid supports: polyurethane foam 50% bound, chitin-glutaraldehyde 40% bound, and porous glass 56% bound (*4,7,8*). The resulting specific activities for free and BIOBONE™-immobilized glucose isomerase were 11.1 ± 0.8 and 3.86 ± 0.53 nmol glucose produced/min/mg protein, respectively. The amount of glucose isomerase immobilized onto 100 mg bone rose with increasing amounts of enzyme added (from 1 to 60 U; data not shown); although with 30 or 60 U added, the increase in bound enzyme was only about 1.5- and twofold, respectively, compared with the amount bound when 9.3 U was added.

Both free and immobilized *S. rubiginosus* glucose isomerase were active only at high temperatures, and both showed similar activity vs temperature profiles (Table 1). Most glucose isomerases exhibit high-temperature optima, but industrial bioreactors carry the reaction out at 55–57°C since the enzyme is subject to thermal inactivation with long-term use at higher temperatures (*13*). The enzyme immobilized on BIOBONE™ maintained good operational stability for 2 h of continuous use at 60°C with a linear rate of glucose production over time (Fig. 1). The rate of glucose production was higher for the free enzyme, but much of this difference was due to the lower amount of activity bound to bone.

BIOBONE™-immobilized glucose isomerase also performed well during repeated cycles of use at 60°C (30 min of reaction, followed by washing with buffer and addition of new substrate) (Fig. 2). After five cycles of use, activity remained fairly constant, following an initial 15–20%

Table 1
Effect of Temperature on the Activity
of Free and Immobilized Glucose Isomerase*

Temperature °C	Activity (nmol glucose produced/min/mL) Free	Immobilized
23	0	0
40	14±3 (4%)	4±1 (6%)
60	236±21 (73%)	48±5 (76%)
72	322±16 (100%)	63±3 (100%)
90	333±13 (103%)	57±5 (90%)

*Data are ±SEM; $n=3$. The values in brackets are relative activities compared to 72°C.

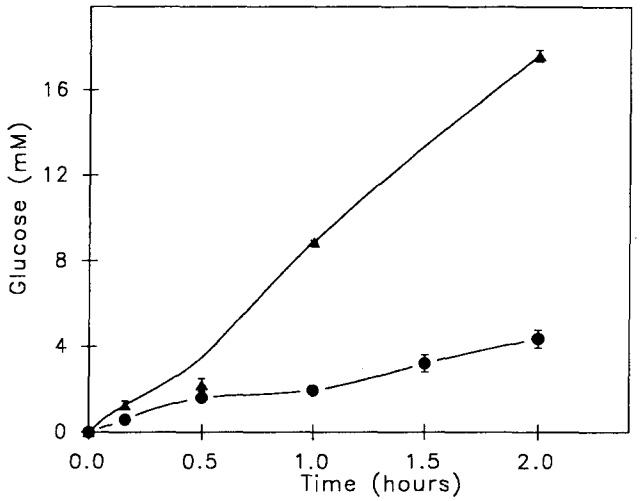

Fig. 1. Time course of glucose production at 60°C by free and immobilized glucose isomerase using fructose as substrate. Data are ±SEM; $n=3$. ▲Free; ● Adsorbed.

activity loss after the first cycle. This initial drop in activity was explainable by the approx 21% of activity that was eluted in the spent substrate during the first three cycles. Storage stability of bone-immobilized glucose isomerase was also quite good. The enzyme was assayed at intervals of up to 5 wk at 4°C (stored in buffer with sodium azide added). Approximately 50% of the immobilized activity was lost after 1 wk of storage, but this rose only slowly thereafter to 70% lost after 5 wk. Other studies with glucose isomerase showed similar results with half-lives of 6–15 d for other immobilized preparations (4,7).

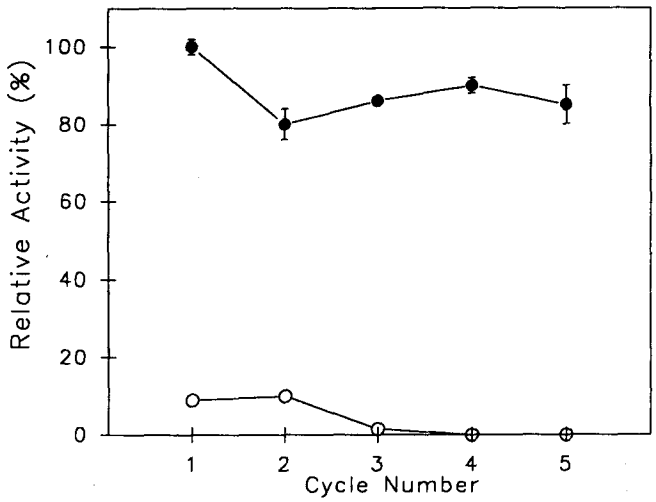

Fig. 2. Effect of continuous enzyme use on immobilized glucose isomerase activity at 60°C. After each 30-min reaction with substrate, bone was washed with buffer, and then fresh substrate was added. The spent substrate was left to incubate for an additional 30 min to determine eluted activity. This cycle was repeated five times. Data are ± SEM; $n=3$. ● Immobilized activity; ○ Eluted activity.

Immobilization of glucose isomerase did not affect the pH optimum; both the free and immobilized enzymes showed optima at pH 8–9 (Fig. 3). Neither enzyme form retained much activity at pH 6 and below, but both forms showed good activity at pH 7. Enzyme function at pH 7 has been exploited in developing coimmobilized enzyme systems that combine cellulase, β-glucosidase, and glucose isomerase on a single, solid support for a one-step conversion of cellulose to fructose (*7*).

For practical use in industrial processes, an immobilized enzyme should be resistant to disruption or denaturation by the presence of various chemical agents that could be present in commercial substrate materials, as well as remain firmly bound to the support under a wide variety of conditions. The results in Table 2 show that both immobilized and free glucose isomerase had high resistance to treatment with detergent (Triton X-100), organic solvent (ethanol), or salt (KCl). Not only did the immobilized enzyme retain high activities in the presence of these agents, but none of these treatments eluted significant amounts of activity from the bone (compared with control washes), demonstrating the stability of glucose isomerase binding to bone.

Table 3 shows kinetic properties for free and immobilized glucose isomerase. The K_m value for fructose increased 2.8-fold upon immobilization, whereas the V_{max} value showed a 4.4-fold decrease. The drop in V_{max} largely reflects the percentage (31.8%) of enzyme immobilized onto bone.

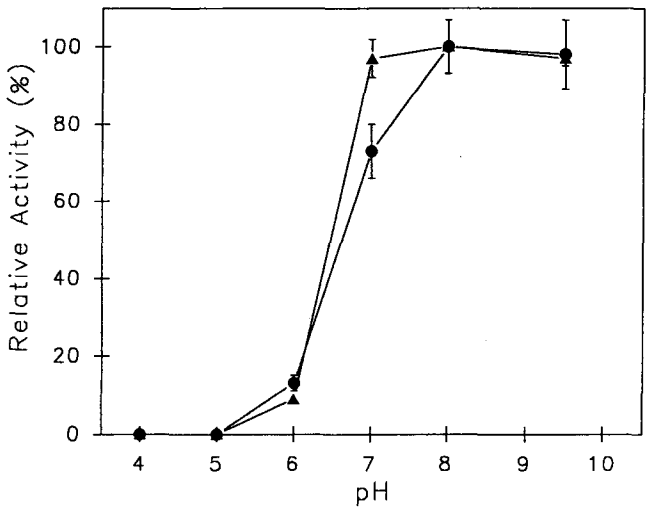

Fig. 3. Effect of pH on the activity of free and immobilized glucose isomerase compared with activity at pH 9. Buffers and pH values tested were 50 m*M* sodium acetate (pH 4.0 and 5.0) and 50 m*M* sodium phosphate (pH 6.0, 7.0, 8.0, and 9.0); reactions were run for 30 min at 55°C. Data are ± SEM; $n=3$. ▲ Free; ● Adsorbed.

Table 2
Effect of Denaturing Agents
on Free and Immobilized Glucose Isomerase Activity*

	Activity (nmol glucose produced/min/mL)		
Treatment	Free	Immobilized	Wash
Control	217 ± 25 (100%)	49 ± 4 (100%)	18 ± 2 (8%)
Triton X100 (1% v/v)	182 ± 15 (84%)	37 ± 2 (75%)†	15 ± 2 (8%)
Ethanol (30% v/v)	260 ± 13 (120%)	39 ± 3 (81%)	18 ± 1 (7%)
KCl			
1.0M	215 ± 8 (99%)	46 ± 1 (95%)	22 ± 1 (10%)
0.5M	234 ± 3 (108%)	48 ± 5 (99%)	21 ± 2 (9%)
0.2M	213 ± 8 (98%)	51 ± 1 (104%)	18 ± 2 (8%)

*Data are ± SEM; $n=3$. Relative activities are given in brackets. Control values, using standard conditions, were set to 100% for comparison. Activity in the pretreatment washes was set relative to free values under given conditions. Reactions were conducted at 60°C for 30 min.

†Significantly different from the corresponding control value $p<0.05$.

Table 3
Kinetic Parameters for Free and Immobilized Glucose Isomerase Using Fructose as Substrate*

Treatment	K_m (mg/mL)	V_{max} (nmol glucose/mL/min)
Mixed		
Immobilized	1.02±0.12	52±1
Free	0.37±0.03	251±9
Nonmixed		
Immobilized	1.16±0.11	58±4
Free	0.40±0.05	256±22

*Data are±SEM; $n=3$ for reactions at 60°C.

However, the increased K_m of the immobilized enzyme indicated that the bound enzyme was a somewhat less effective catalyst. Such effects of immobilization on K_m are not unusual and have been noted before for glucose isomerase (*7,9*). Changes in K_m may be caused by enzyme immobilization in an unfavorable configuration or by steric hindrance from overcrowding of the immobilized enzyme (*10*).

Since immobilization onto porous carriers can result in repressed substrate and product diffusion, experiments were done to determine whether immobilized glucose isomerase was limited in this way. External mass transfer or film diffusion resistance of a system can be alleviated by an increase in stirrer speed (*13*). However, Table 3 shows that mixing the tube containing the immobilized enzyme+substrate had no effect on either the K_m or V_{max} values, indicating that there was no significant diffusion limitation in this system. This was not surprising because of the small molecular mass of both the substrate and product of the enzyme. Additional analyses that can indicate internal mass transfer limitations are:

1. deviations from the Arrhenius relationship at high temperatures (assessed for data in Table 1) (*10*); and
2. steeper slopes for Lineweaver-Burk plots at higher enzyme loading (9 vs 18 U were compared) (*14*).

Neither of these produced evidence of diffusion limitation of the BIOBONE™-immobilized glucose isomerase reaction (data not shown) (*10,14*).

In summary, the successful immobilization of both amyloglucosidase and glucose isomerase to BIOBONE™ indicates the promise of this system as a suitable support matrix for commercially important carbohydratases (*11*).

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