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Immobilization of Amyloglucosidase onto Granular Chicken Bone

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ABSTRACT

Amyloglucosidase was immobilized onto granular chicken bone (BIOBONE™) by noncovalent interactions. The amount of activity bound relative to an equal amount of free enzyme was 13.6±0.4%. The estimated specific activity for amyloglucosidase decreased from 75.3±0.8 to 43.5±9.6 U/mg protein upon immobilization. The K_m value of the bone-immobilized enzyme using glycogen as substrate increased from 3.04±0.38 mg/mL (free) to 9.04±1.51 mg/mL (immobilized), but K_m showed no change upon immobilization when starches were used as substrates. A decrease in V_{max} values occurred upon enzyme immobilization for all substrates, but this largely reflected the percentage of enzyme initially bound to the bone. Immobilization also improved enzyme stability in the presence of various additives (e.g., detergent, KCl, and ethanol) or under low or high pH reaction conditions. Bound amyloglucosidase maintained high activity (>90%) following five cycles of continuous use at moderate (23°C) and high (55°C) temperatures. Data derived from Lineweaver-Burk and Arrhenius plots indicated that substrate and product diffusion limitation were minimal.

Index Entries: Amyloglucosidase; enzyme immobilization; chicken bone; starch degradation.

INTRODUCTION

Amyloglucosidase, or glucoamylase (EC 3.2.1.3), is an exosplitting enzyme that catalyzes the stepwise hydrolysis of α-(1,4) linkages and to a lesser extent α-(1,6) linkages from the nonreducing ends of starch and

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glycogen (*1*). It is one of the most industrially important enzymes and is widely used in soluble form for large-scale saccharification of starch to glucose syrups (*1*). Enzymatic, as opposed to chemical, methods for starch hydrolysis are preferred because of their substrate and product specificity and because of the more moderate reaction conditions of pH and temperature that can be used (*2*).

Amyloglucosidase has been immobilized to a variety of supports in an effort to create a more controlled reusable enzyme system for commercial use (*3–9*). However, as the process of enzyme immobilization is an added expense in the commercial use of the enzyme, many of the existing methods of immobilization are considered unsuitable for large-scale industrial use (*3*). Enzyme activity is often lost during immobilization and is usually attributed to the binding step, which may cause enzyme deactivation, subunit dissociation, and/or loss of allosteric properties (*2*). Furthermore, when immobilized onto porous matrices, enzymes may experience mass transfer limitation problems, particularly with large mol wt substrates (*10*). As a result, diffusion limitation can limit the catalytic efficiency of the enzyme as well as change the apparent kinetic behavior (*11*). Thus, an immobilized amyloglucosidase must offer significant advantages over the soluble enzyme for industrial utilization to occur.

Chicken bone possesses all of the characteristics of an ideal enzyme support matrix. It is a strong porous material composed of relatively inert hydroxyapatite crystals imbedded in a stable protein matrix (*12*). It is believed that bone promotes the formation of a network of cooperative noncovalent interactions with enzyme molecules. Currently recognized as merely a byproduct of the meat processing industry, bone is abundant and inexpensive. Also, as a natural product, bone is food grade and nontoxic (*12*).

The present study describes the immobilization of amyloglucosidase onto granular chicken bone (BIOBONE™). A variety of kinetic properties and stability parameters were compared for free and immobilized amyloglucosidase using glycogen and starch as substrates. Experiments were also done to determine the influence of diffusion limitation on the immobilized system. The use of granular chicken bone shows promise of becoming one of the preferred methods for enzyme immobilization.

MATERIALS AND METHODS

Materials

Amyloglucosidase from *Aspergillus niger*, oyster liver glycogen type II, and Lintner and potato starches were obtained from Sigma Chemical Co. (St. Louis, MO). All biochemicals were purchased from Sigma, J. T. Baker Chemical Co. (Phillipsburg, IL), or Boehringer Mannheim Corp. (Montreal, PQ).

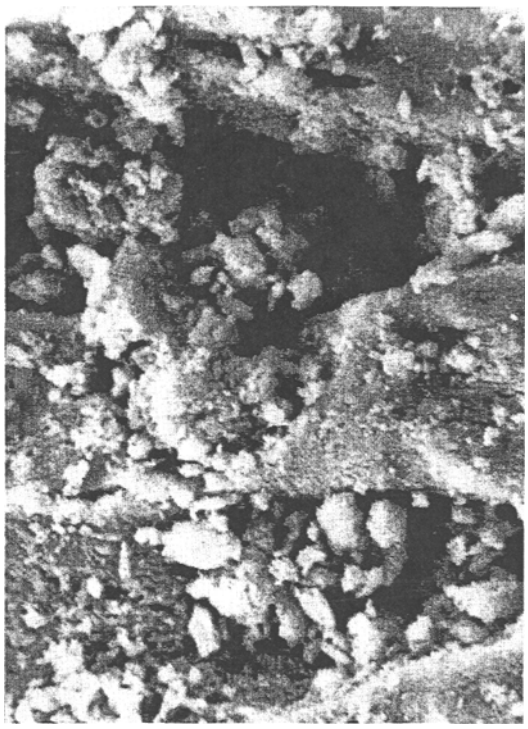

Fig. 1. Scanning electron micrograph (gold coated) of 20–30 mesh BioBone™. This support material is crushed and washed chicken bone, which exhibits a great deal of heterogeneity from particle to particle. Micrographs from 100 to 3000× exhibit the same rough, pitted surface features. The bone fragments are extremely granular but do not contain pores that go completely through individual particles. Micrograph taken by L. Ling, Biology, Carleton University.

Clean, granular chicken bone, which had been previously stripped using hot aqueous NaOH followed by washing and sizing to between 10 and 20 mesh (BIOBONE™), was donated by Protein Foods Research (Guelph, Ont.). Before use, the bone was washed with acetate buffer (pH 4.5, 100 m*M*) to remove any particulate matter (Fig. 1).

Immobilization of Amyloglucosidase

The standard amount of amyloglucosidase used for all experiments, unless stated otherwise, was 1.4 U, where 1 U is defined as the amount of enzyme that liberates 1.0 mg of glucose from starch in 3 min at pH 4.5 and 55°C. Adsorption to bone was achieved by the addition of enzyme to a tube containing 100±2 mg of prewashed bone and 0.5 mL sodium acetate buffer (pH 4.0, 100 m*M*), followed by mixing at 100 rpm for 30 min at 23°C. Excess solution was poured off, followed by successive washes with buffer (4×1 mL).

Protein Assay

Protein content was estimated by the Coomassie blue dye binding method, using the BioRad Laboratories prepared reagent and bovine gamma globulin as the standard (BioRad Inc.). The difference between total protein (an equivalent amount of free enzyme) and protein recovered in the wash was assumed to be the amount bound to bone. Direct measurements of bone-bound enzyme were not possible because the dye reacted with the bone. Any protein found in washings of the bone itself (without enzyme added) was subtracted from the total wash protein determined.

Amyloglucosidase Assay

Amyloglucosidase activity was determined by measuring the glucose liberated from various forms of starch and glycogen. Standard reactions were carried out at 23°C using 12% (w/v) dialysed glycogen or 20% (w/v) whole starch. To initiate a reaction, 0.5 mL of the appropriate substrate was added to the prepared bone. The reaction tube was then shaken at 130 rpm for 30 min at 23°C. To stop the reaction. 0.1 mL of the reaction mixture was mixed with 0.1 mL of 1.0 *N* NaOH. The amount of glucose liberated was then estimated using a glucose diagnostic kit (Sigma Chemical Co. No. 510-DA). Adjustments were made to the glucose standard curve where necessary to account for the effect of various additives on color development.

Determination of Optimum Binding Conditions

Amyloglucosidase was tested for its ability to immobilize to bone in acetate buffer of different pH values (3.0, 4.0, 4.5, 5.0, and 6.0) and buffer concentrations (100 m*M* and 50 m*M*). Amyloglucosidase was also left to immobilize to bone for up to 24 h to determine optimal binding time. Furthermore, increasing concentrations of amyloglucosidase were added to bone to determine the maximum amount of enzyme that could be immobilized.

Optimum pH and Temperature

Free and immobilized amyloglucosidase were preequilibrated with 0.5 mL of the following pH buffers: 0.1 M acetate (pH 3.0, 4.0, 4.5, 5.0, and 5.5) and 0.1 M phosphate (6.5, 7.5, and 8.5). Substrate was added, followed by a 30-min agitation at 130 rpm and 23°C and then quantification of glucose liberated.

Free and immobilized amyloglucosidase were tested for their ability to hydrolyze glycogen at different temperatures. After equilibration at the appropriate temperature, substrate was added to the enzyme for a 30-min incubation. The amount of glucose produced was then determined.

Kinetic Parameters

The kinetic parameters for free and immobilized amyloglucosidase were determined using oyster liver glycogen type II, Lintner starch, or potato starch as substrates. Glucose production after 10 min was determined for reactions using 1–160 mg/mL of glycogen and 4–200 mg/mL of whole starch. Kinetic parameters of the free enzyme in the presence of 10% polyethylene glycol with glycogen as the substrate were also measured. K_m and V_{max} values were calculated using Lineweaver-Burk, Hanes, or Hill plots.

Effects of Enzyme-Disrupting Agents

Free and immobilized amyloglucosidase were equilibrated with one of the following reagents: Triton X-100 (1% w/v), ethanol (30% v/v), or KCl (0.2, 0.5, or 1.0 M) each made up in acetate buffer pH 4.5; or acetate buffer (0.1 M, pH 3) or 0.1 M Tris buffer (0.1 M pH 9.5). After a 30-min incubation, the equilibration washes were poured off. Substrate containing each of the reagents was added to these washes and to the bound and free enzymes, followed by a 30-min agitation at 23 °C and then measurement of glucose production in all fractions.

Storage Half-Life

Between uses, immobilized enzymes were stored at 4°C in buffer with 0.04% (w/v) sodium azide added. After periods ranging up to five wk, immobilized enzyme samples ($n=3$) were allowed to warm to room temperature and thoroughly washed with buffer; then substrate was added. Half-life values were calculated from interpolation or extrapolation directly from plots of relative activity over the storage time period.

Methods Employed for Diffusion Limitation Studies

Four methods were used:

1. Glucose production after 10 min was determined for reactions using 1–160 mg/mL of glycogen, comparing mixed and non-mixed tubes;
2. The activity vs temperature values previously determined for free and immobilized amyloglucosidase were fitted into an Arrhenius equation, and the resulting slopes were compared;
3. Kinetic data for glycogen using 1.4, 5.6, and 11.2 U of enzyme were plotted on Lineweaver-Burk plots, and the resulting slopes were compared; and
4. The activities of immobilized amyloglucosidase were compared on whole (control) and powdered bone. Bone samples con-

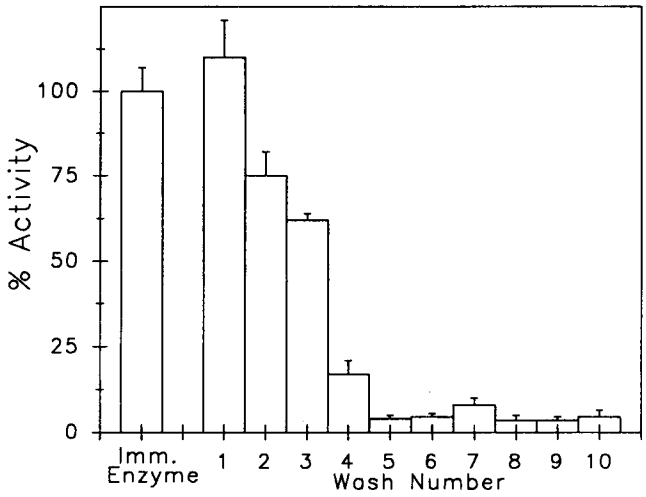

Fig. 2. Effect of repeated washings on the elution of amyloglucosidase activity from bone. After enzyme immobilization, excess solution was poured off, followed by successive washes with buffer (10×1 mL). Activity present in each wash is expressed relative to the amount of activity (with glycogen as substrate) that remained immobilized onto bone after the 10th wash (imm. enzyme). Data are ±SEM; $n=3$.

taining bound enzyme were frozen in liquid nitrogen. Control samples were then allowed to return to room temperature. Other samples were ground into powder under liquid nitrogen using a mortar and pestle and then thawed.

Data Calculations and Statistics

Data is reported as mean values of triplicate samples ± SEM. Statistical significance of results was determined using the Students' *t*-test (*13*).

RESULTS

Immobilization of Amyloglucosidase onto BIOBONE™

Figure 2 indicates that amyloglucosidase activity remained immobilized on bone even after 10 repeated washings. It also shows that three or four washes were sufficient to remove any unbound enzyme from the bone.

Incubation times greater than 20 min did not increase the amount of amyloglucosidase bound (Fig. 3). Furthermore, Table 1 shows that the greatest amount of binding occurred with low pH incubations. Thus, the conditions used for optimal binding were incubation for 30 min at pH 3.0 in 100 m*M* acetate buffer; however, subsequent assays of enzyme activity were all done at constant pH 4.5.

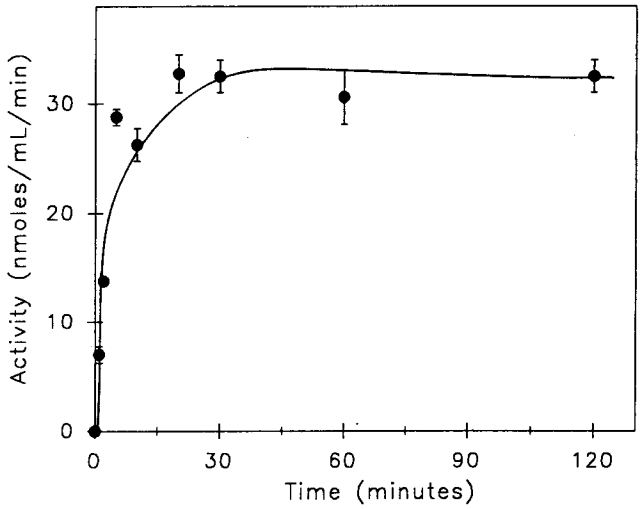

Fig. 3. Effect of increasing binding time on the amount of amyloglucosidase immobilized onto bone. Data are ±SEM; $n=3$ with glycogen as substrate.

Table 1
Effect of Incubation Conditions, pH, and Ionic Strength on the Amount of Amyloglucosidase Immobilized onto Bone*

	Activity (nmol glucose produced/mL/min)	
	Buffer Concentration	
Acetate Buffer (pH)	50 m*M*	100 m*M*
3.0	33.1±0.5	37.5±0.70
4.0	31.0±1.1	34.7±2.70
4.5	30.5±2.5	30.9±4.70
5.5	17.6±0.8	11.3±2.00
7.0	1.0±0.1	0.8±0.05

*Data are ±SEM; $n=3$. To achieve immobilization, the standard amount of enzyme (1.4 U) was added to bone at each pH with a 30-min incubation followed by four washes. Reactions were then run at pH 4.5 23°C for 30 min with glycogen as substrate.

Figure 4 shows the effect of the addition of increasing amounts (0.14–33 U) of amyloglucosidase on the amount of enzyme immobilized to the bone. Although only 1.4 U of enzyme was used in the standard experiments, the bone adsorbed increasing amounts of amyloglucosidase up to 16 U added per 100±2 mg bone. Under standard conditions (1.4 U added), the average amount of amyloglucosidase activity immobilized was 13.6±0.4% with approx 57.8±6.6% being eluted in the washes. The remaining 28.6% of activity was lost and could probably be attributed to enzyme deactivation upon immobilization. The specific activity of free and

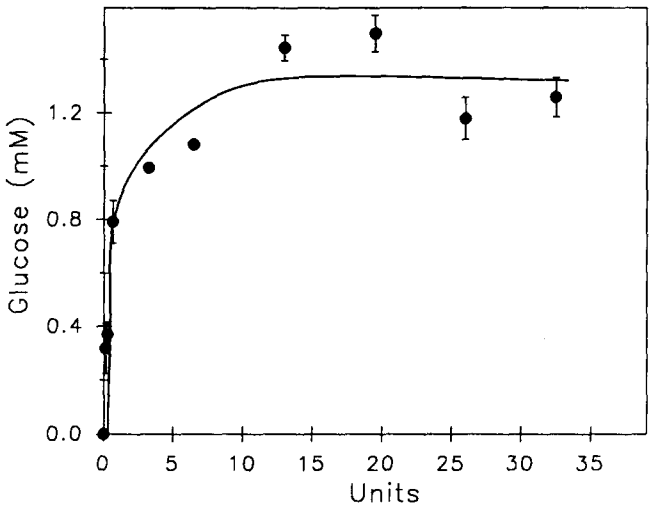

Fig. 4. Effect of attempting to immobilize increasing amounts of amyloglucosidase onto bone on the resulting amount of glucose produced. Data are ± SEM; $n = 3$ with glycogen as substrate. Where error bars are not shown, SEM values are within the dimensions of the symbols used.

Table 2
Effect of Temperature on the Activity of Free and Immobilized Amyloglucosidase*

	Activity (nmol glucose produced/mL/min)	
Temperature (°C)	Free	Immobilized
0	52± 6 (11%)	13± 2 (9%)
15	214±24 (45%)	23± 3 (16%)
23	238±13 (50%)	33± 4 (24%)
32	357±39 (75%)	95± 9 (68%)
45	381±40 (80%)	129± 8 (93%)
55	476± 8 (100%)	138±15 (100%)
70	462±14 (97%)	138±15 (97%)
80	490±38 (103%)	115±16 (83%)

*Data are ± SEM; $n = 3$ with glycogen as the substrate. The values in brackets are relative activities compared to values at 55°C that were set at 100%.

immobilized amyloglucosidase as estimated by the BioRad protein assay was 75.3±0.8 and 43.5±9.6 nmol·min^{-1}·mL^{-1}·mg^{-1} protein, respectively.

Optimum Temperature and Temperature Stability Studies

Table 2 shows the effects of temperature on free and immobilized amyloglucosidase. Both maintained near V_{max} activities over a wide range

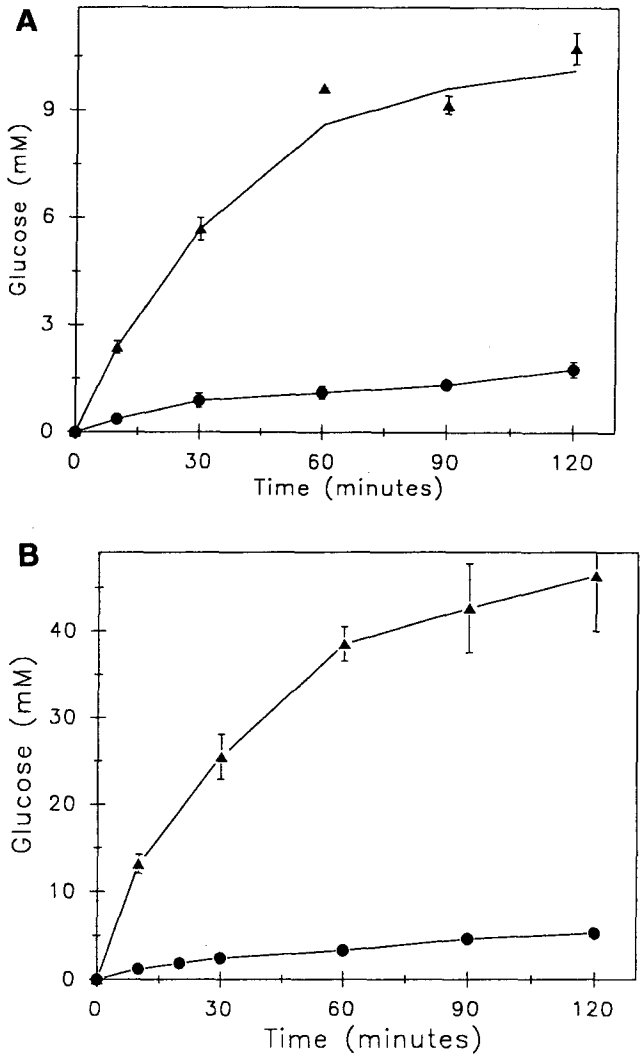

Fig. 5. Time course of glycogen hydrolysis at 23°C (A) and 80°C (B) by free and immobilized amyloglucosidase. Data are ± SEM; $n = 3$. Where error bars are not shown, SEM values are within the dimensions of the symbols used. ▲ Free; ● Adsorbed.

from about 45 to 80°C. At 23°C, the relative decrease in activity was greater for the bound vs free enzyme.

Figures 5A and B show the time course of glucose production at high (80°C) and moderate temperatures (23°C) for free and immobilized amyloglucosidase, respectively. Bound enzyme showed slow but fairly linear rates of glucose production over the 2-h period at both temperatures, indicating constant activity with time. The free enzyme maintained a linear rate for only 60 min at 80°C and 23°C. This suggested either a gradual loss of activity or a limitation of substrate supply.

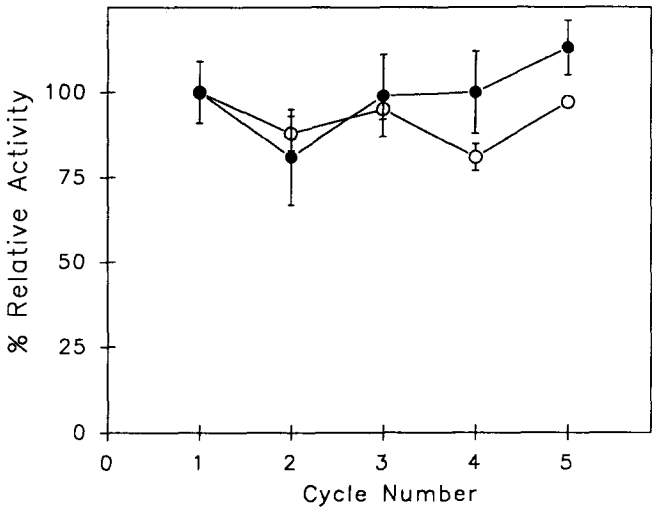

Fig. 6. Effect of continuous enzyme use on immobilized amyloglucosidase activity at 23 and 55°C. Following each 30-min reaction period, bone was washed with buffer, and then fresh glycogen was added. Spent substrate was left to incubate for an additional 30 min for determination of eluted activity. This cycle was repeated five times. Activities are expressed as percentages relative to the first cycle of use. Data are ±SEM; $n=3$. Where error bars are not shown, SEM values are within the dimensions of the symbols. ● reuse at 23°C; ○ reuse at 55°C.

Figure 6 shows that the amount of adsorbed enzyme activity remained fairly constant after repeated use (five times) even at high temperature (55°C). Although an additional 6.4% of activity was eluted into the substrate following the first cycle, this did not have a significant effect on the activity or stability of the enzyme.

Optimum pH

Figure 7 shows the effect of pH on the activities of free and immobilized amyloglucosidase. Upon immobilization, the pH optimum broadened from 4.5 to 3.0–4.5, with a significant drop at pH 7.

Effects of Enzyme Disrupters on Amyloglucosidase Activity

Table 3 shows the effect of some disrupting agents on the activity of free and immobilized amyloglucosidase; these represent some potential conditions that the enzyme might be exposed to when dealing with crude substrates in industrial-scale reactions. Also shown is the amount of enzyme released from the bone when incubated with the disrupters. The results show that immobilized amyloglucosidase maintained greater rela-

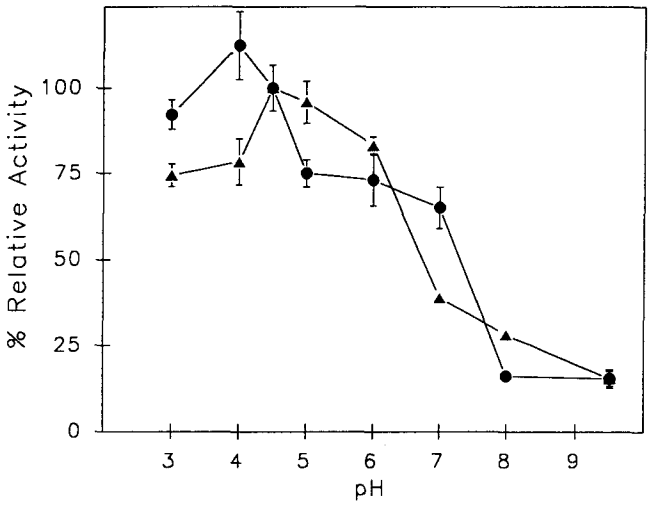

Fig. 7. Effect of pH on the activity of free and immobilized amyloglucosidase with glycogen as the substrate. Activity at pH 4.5 was set to 100% for comparison. The following pH buffers were used: 0.1 M acetate (pH 3.0, 4.0, 4.5, 5.0, and 5.5) and 0.1 M phosphate (6.5, 7.5, and 8.5). Data are ±SEM; $n=3$. Where error bars are not shown, SEM values are within the dimensions of the symbols used. ▲ Free; ● adsorbed.

Table 3
Effect of Disrupting Agents
on Free and Immobilized Amyloglucosidase Activity*

	Activity (nmol glucose produced/min/mL)		
Treatment	Free	Immobilized	Wash
Control	179±10 (100%)	24±5 (100%)	3±1 (2%)
Triton X-100	127± 1 (70%)	24±3 (98%)	8±2 (4%)
Ethanol (30%)	123± 2 (69%)	25±2 (102%)	4±1 (2%)
KCl:			
1 M	109± 2 (61%)	21±1 (86%)*	6±1 (4%)
0.5M	125± 4 (69%)	24±1 (100%)	6±1 (3%)
0.2M	146± 7 (82%)†	20±1 (82%)	3±1 (2%)
pH 3.0, 0.1 M	115± 8 (64%)	22±1 (93%)	3±1 (2%)
pH 9.5, 0.1 M	23± 2 (13%)	7±1 (27%)	3±2 (13%)

*Data are±SEM; $n=3$ with glycogen as the substrate. Relative activities are given in brackets; control values (using standard conditions) are set to 100%. Activity in the equilibration washes was set relative to free values under the given conditions. Reactions were conducted at 23°C for 30 min.

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

Table 4
Kinetic Parameters for Free and Immobilized Amyloglucosidase*

Substrate	K_m (mg/mL)	V_{max} (nmol glucose/mL/min)
Glycogen		
Free	3.0±0.4	210±11
Immobilized	9.0±1.5‡	47.1±1.8‡
Lintner Starch†		
Free	80±5.0	70.2±5.1
Immobilized	76±2.4	12.5±0.5‡
Potato Starch		
Free	101±4	64.3±6.8
Immobilized	95±2.2	26.1±0.4‡

*Data are ± SEM; $n=5$.

†Lintner starch is a potato starch that has been modified by the Lintner method (heat and/or acid treatment) to create a starch of lower viscosity with an increased number of branched chains (*16*).

‡Significantly different from the corresponding value for the free enzyme ($p<0.05$).

tive activity in the presence of the disrupters than did the free enzyme, indicating a greater stability of the immobilized enzyme. With the exception of high-alkaline pH, treatment with disrupters also removed relatively little enzyme activity from the bone, indicating that enzyme binding is strong.

Kinetic Properties

Table 4 shows some kinetic parameters of free and immobilized amyloglucosidase. The K_m value for glycogen increased by threefold for the immobilized enzyme, whereas the K_m values for Lintner or potato starches were the same for free and immobilized enzymes. V_{max} values for the immobilized enzyme were reduced compared with the free enzyme by 78%, 82%, and 60%, for glycogen, Lintner starch, and potato starch, respectively. These decreases in V_{max} largely reflect the percentage of enzyme initially immobilized onto bone.

Polyethylene glycol (PEG) is a large polymer that acts to crowd enzymes together by restricting free water availability (*14*). When 10% w/v PEG was added to the free enzyme, neither the K_m nor the V_{max} changed. K_m was 3.41±0.4 mg/mL without PEG and 3.62±0.4 mg/mL ($n=3$) with PEG; corresponding values for V_{max} were 195±10 and 203±3 nmol/min/mL. This suggests that the effects of immobilization on the kinetic parameters with glycogen were not simply caused by enzyme crowding in the immobilized state.

Table 5
Comparison of Glycogen, Lintner, and Potato Starches as Substrates for Free and Immobilized Amyloglucosidase*

Substrate	Activity (nmol glucose produced/mL/min)	
	Free	Immobilized
Glycogen		
Dialyzed	191±4.0 (100%)	28±2.0 (100%)
Crude	241±9.0 (126%)	41±2.0 (151%)
Lintner Starch		
Whole	123±3.0 (100%)	28±1.0 (100%)
Soluble	19±1.0 (16%)	11±1.0 (41%)
Potato Starch		
Whole	62±4.0 (100%)	19±2.0 (100%)
Soluble	12±0.2 (19%)	7±0.3 (38%)

*Data are ± SEM; $n = 3$. The relative activities are given in brackets compared to the corresponding value for dialyzed glycogen or whole starches. All substrates were subjected to overnight dialysis using dialysis tubing with a mol wt cutoff of 50,000. Soluble starch was obtained by allowing the insoluble portions of whole starch to settle.

Substrate Comparison

Table 5 shows amyloglucosidase activity with various substrates. The free enzyme showed a much higher activity with glycogen as its substrate than with the starches (Lintner and potato). The differences in activities were less pronounced upon immobilization since amyloglucosidase was a relatively better catalyst of starch breakdown when immobilized. Both the free and immobilized enzymes utilized crude glycogen more efficiently than they did substrates that had been dialyzed to remove low mol wt fragments. The effectiveness of the enzyme with soluble vs insoluble starch was also compared. Approximately 83% of glucose production from free enzyme activity using whole starch came from hydrolysis of the insoluble portions of the substrate. However, only 60% of glucose production from immobilized amyloglucosidase came from the breakdown of insoluble starch.

Storage Stability

Amyloglucosidase was stored at 4°C in buffer containing 0.04% sodium azide and tested for activity after 1, 2, and 4 d and 1, 2, 3, 4, and 5 wk. After a 5-wk immobilized amyloglucosidase still maintained about 83% of its original activity. The estimated half-life of the immobilized enzyme was approximately 144 d.

Table 6
Effects of Mixing on the Kinetic Parameters of Free and Immobilized Amyloglucosidase*

	K_m (mg/mL)		V_{max} (nmol glucose/mL/min)	
Substrate	Mixed	Nonmixed	Mixed	Nonmixed
Glycogen				
Free	3.4±0.4	3.3±0.4	195.0±10.0	200.0±12.0
Immobilized	9.6±0.9	12.8±1.6	43.5± 4.2	34.5± 3.0
Starch				
Free	80.2±5.0	91.4±7.0	70.0± 5.2	65.1± 5.1
Immobilized	75.7±2.4	—	11.5± 0.5	3.4± 0.1†

*Data are ± SEM; $n=3$. Glycogen and Lintner starch were used as substrates. Tubes were mixed by agitating in a rotary shaker. Nonmixed tubes were left motionless.
†Significantly different from the corresponding mixed value ($p<0.01$).

Diffusion Limitation of the Immobilized System

Because mass transfer limitation is a common problem with many immobilized enzyme systems on porous carrier surfaces, experiments were done to specifically address this subject. Mass transfer resistance caused by diffusional resistance through the external boundary layer was tested by comparing enzyme activity in mixed vs nonmixed samples (Table 6). Results showed that agitating the reaction tubes in a shaker had no significant effect on the apparent K_m or V_{max} values for either free or immobilized amyloglucosidase with glycogen as the substrate. When Lintner starch was the substrate, mixing had no significant effect on the kinetic parameters of the free enzyme, but immobilized amyloglucosidase showed a 3.3-fold increase in the V_{max} for starch hydrolysis as a result of mixing.

Deviations in the Arrhenius relationship (relating temperature and reaction velocity), particularly at high temperatures, can also indicate a limitation of enzyme rate because of substrate diffusion. However, if an Arrhenius plot for the immobilized enzyme gives a straight line with a slope equal to that of the same plot for the soluble enzyme, it usually implies that no internal diffusion limitations exist (3). The results in Fig. 8 show that the Arrhenius plots for both immobilized and free amyloglucosidase had similar slopes at low and high temperatures.

Furthermore, if higher enzyme loading onto a support matrix results in steeper slopes for Lineweaver-Burk plots, this also can indicate an increase in diffusion effect (3). The results from Fig. 9 show, in fact, that the opposite occurred. The slope decreased with increased enzyme concentrations with glycogen as substrate. Similar results were obtained with Lintner starch as substrate (data not shown).

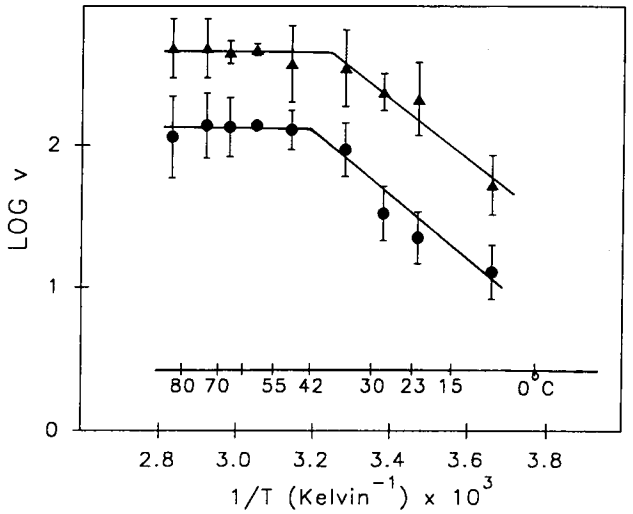

Fig. 8. Arrhenius plots for free and immobilized amyloglucosidase. Data from the temperature profile (Table 2) were fitted to the Arrhenius equation, which relates temperature and reaction velocity; and the resulting slopes were compared. Data are ±SEM; $n=3$. ▲ Free; ● adsorbed.

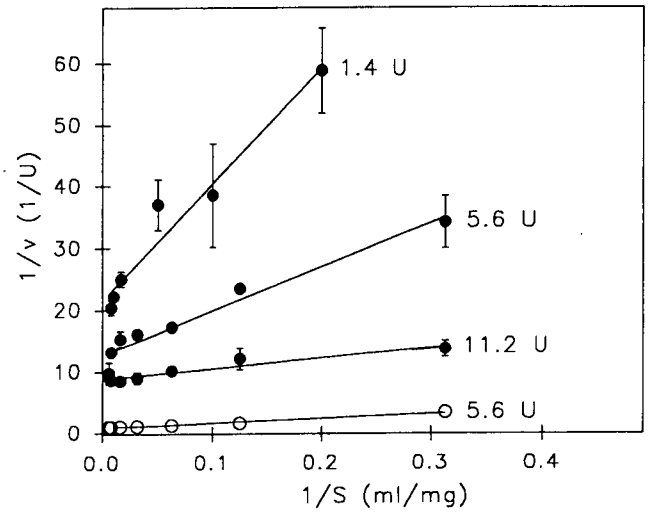

Fig. 9. Lineweaver-Burk plots for free and immobilized amyloglucosidase. Data were obtained from kinetic experiments using 1.4, 5.6, and 11.2 U of added enzyme and a 160 mg/mL glycogen stock solution as substrate. Data are ±SEM; $n=3$. Where error bars are not shown, SEM values are within the dimensions of the symbols used. ○ Free; ● adsorbed.

Table 7
Effects of Increasing Surface Area on the Apparent Activity of Immobilized Amyloglucosidase*

Treatment	Activity (nmol glucose/mL/min)
Glycogen	
Control bone	42.2±2.1 (100%)
Powdered bone	45.8±6.0 (109%)
Lintner Starch	
Control bone	27.2±0.5 (100%)
Powdered bone	40.3±1.9 (148%)†

*Data are ± SEM; $n=3$. Relative activities are given in brackets. After the immobilized enzymes were frozen with liquid nitrogen, control samples were left to return to room temperature. The other samples were ground to a fine powder in a mortar and pestle chilled with liquid N_2; the powdered enzyme was then thawed.

†Significantly different from the corresponding control ($p<0.01$).

Another aspect of mass transfer limitation is internal diffusion resistance caused by the pore size of the matrix particles. To test whether pore diffusion influenced immobilized amyloglucosidase activity, bone samples containing immobilized enzymes were ground into a powder under liquid nitrogen using a mortar and pestle. The powdered and control (frozen but not powdered) bone samples were then thawed and activities of the two compared. Table 7 shows that no change in activity occurred with the increase in surface area caused by powdering when glycogen was the substrate. However, a 1.5-fold increase in activity resulted for powdered bone using Lintner starch as the substrate.

DISCUSSION

The application of immobilized enzyme technology has the potential for improving the industrial use of amyloglucosidase. Production of high-glucose syrup (i.e., containing 96–98% *D*-glucose) is the most important use of amyloglucosidase. High-glucose syrups are used either for the production of crystalline *D*-glucose or as a starting material for the production of high-fructose syrups. High-glucose syrups also find extensive use in the brewing, baking, soft drink, and confectionery industries (*1*).

Compared with the large-scale degradation of starch to glucose by soluble amyloglucosidase (48–72 h at pH 4.5 and 55–60°C), immobilization reduces the conversion time, because very high enzyme concentrations can be used and recovered (*3*). Furthermore, the shorter residence times

would decrease the number of side reactions, thus lowering refining costs (*1*). These considerations amply justify the efforts that have been made to immobilize amyloglucosidase.

Immobilization of amyloglucosidase onto BIOBONE™ represents an improved method of binding over other methods described. The binding step requires no reactive species and is simple, fast, and inexpensive. Bone itself is a low-cost, natural product; this eliminates the necessity for its regeneration or the potential problems associated with product contamination by bone. BIOBONE™ has been previously used by Findlay et al. to immobilize invertase, pepsin, pectinase, lactase, and catalase (*12*). Amyloglucosidase has also previously been immobilized onto collagen (*5*).

Amyloglucosidase binding to bone is firm and immediate. Approximately 14% of activity remained bound following 10 washes of the bone, with maximum binding occurring after only 20 min incubation with the enzyme (Figs. 2,3). The calculated productivity of the immobilized enzyme was 365 ng glucose/mL reactor vol/h. Binding was most favorable at pH 3.0 and 100 m*M* buffer, which appears to take advantage of the net positive charge of amyloglucosidase at low pH because of a low isoelectric point, i.e, pH 3.5–4.00 (*15*). Although 14% bound is a relatively low value, it compares well with other attempts to immobilize amyloglucosidase: 19% bound onto a copolymer of methyl methacrylate, 25% bound onto polyurethane foam, and 12% bound onto metal-activated inorganic supports (*4,6,8*).

The enhanced stability of immobilized amyloglucosidase was repeatedly demonstrated during the experiments. First, the immobilized enzyme remained very active, displaying a storage half-life of approx 144 d following repeated tests of activity over 5 wk of storage at 4°C. Secondly, a high-temperature optimum (55°C) was maintained upon immobilization (Table 2). Also, long-term glycogen degradation remained fairly constant with time, unlike the free enzyme, even at high operating temperatures (Fig. 5A,B). Furthermore, activity remained fairly constant following five cycles of continuous operation at both moderate and high temperatures (Fig. 6). All of these factors indicate that BIOBONE™-bound amyloglucosidase has a high potential for excellent performance and stability over long cycles of continuous operation, such as are required for industrial applications.

Immobilized amyloglucosidase showed a broader pH optimum (pH 3.0–4.5) compared to the free enzyme (a sharp peak at pH 4.5) (Fig. 7). Other authors have also observed an acidic shift in the pH optimum of amyloglucosidase upon immobilization (*7,9*). Shifts in pH optima are largely dependent on the type of carrier matrix used. Positively charged matrices usually result in acidic shifts in pH optimum and are caused by the formation of a pH gradient in which a depletion of hydrogen ions occurs in the vicinity of the enzyme compared with that of the bulk solution (*10*). However, since the bone matrix is believed to be essentially neutral in nature, this drop in pH optimum cannot be explained by charge

effects. The shift in pH optima to the acidic region was because of the loss of basic groups by the enzyme upon immobilization (*9*).

The enhanced stability of immobilized amyloglucosidase activity as well as the firmness with which it binds to bone was also apparent in the studies with disrupting agents. Immobilization protected the enzyme from the inhibitory effects of Triton X-100, ethanol, KCl, and pH extremes (Table 3). Coulet et al. maintain that the high hydrophilicity of collagen helps to prevent denaturation (*5*). The minor amounts of activity present in the washes following denaturant pretreatments further support the firmness with which amyloglucosidase bound to bone (Table 3). The only exception appeared to be with high-alkaline pH treatments. The relatively high amounts of activity eluted into the wash (13% with respect to the corresponding free enzyme activity) largely accounted for the low activity left immobilized after treatment. The pH 9.5 buffer may have upset the binding interactions between the enzyme and bone matrix.

Immobilized amyloglucosidase showed a threefold increase in the K_m for glycogen compared to the free enzyme, but affinity for the starches was unchanged (Table 4). These values are similar to previously reported values. Nithiandam et al. found a tenfold increase in the K_m for starch of the immobilized enzyme, whereas Slininger et al. found no change in K_m upon immobilization using a starch substrate (*6,7*). Storey and Chakrabarti, using glycogen as a substrate, found no change in K_m when amyloglucosidase was immobilized onto a polyurethane foam polymer but a 3.5-fold increase in K_m when a gel polymer was used instead (*8*).

The kinetic data, together with the results from Table 5, indicate that as compared with free enzyme activity, immobilized amyloglucosidase shows relatively higher catalytic activity with the starches than with glycogen. However, the overall rate of starch hydrolysis is considerably lower compared to glycogen. The great difference in amyloglucosidase affinity for glycogen over starch is likely caused by the limited solubility of starch. Although both glycogen and the amylopectin portion of starch are highly branched polymers with similar mol wt ($\sim 10^8$ daltons), amylopectin has longer chain lengths (20–25 glucose U) compared with glycogen (10–12 glucose U) (*16*). This and the amylose content of starch, which is essentially a linear glucose polymer, result in a very insoluble system (*16*).

Results from Fig. 4 indicate that large enzyme loadings can be achieved per 100 mg bone. However, relative activities at high enzyme loadings may suffer adverse affects partly because of steric effects caused by enzyme crowding on the bone and partly as a result of diffusion limitation in the substrate supply and product elimination (*17*). The amount of enzyme used in experiments here (1.4 U) represents a fraction of the amount of enzyme used to achieve maximum enzyme activity per 100 mg bone. Thus, since the maximum binding capacity is not reached at this point, the immobilized enzyme is likely not suffering from steric hindrance. Further-

more, the PEG experiments done with free enzyme, designed to mimic enzyme crowding because of immobilization, showed that none existed. Enzyme loading must be optimized by considering the tradeoff between less-efficient enzyme use at high loadings and larger carriers and immobilization cost at low enzyme loadings (*3*). Thus, it would be feasible to run reactions at maximum enzyme efficiency because of the negligible cost of BIOBONE™. Substrate concentration gradients are usually established around insoluble enzymes, resulting in enzyme saturation occurring at higher substrate concentrations than is normally required for saturation of the soluble enzyme (*10*). We found some evidence of a mass transfer resistance due to diffusional resistance through the external boundary layer when starch was the substrate; thus, the mixing experiments revealed a threefold increase in enzyme V_{max} when samples were mixed compared to nonmixed (Table 6). However, no such effect occurred for the immobilized enzyme using glycogen as its substrate or for the free enzyme (Table 6).

Diffusion limitations can also affect initial rate assays and distort the standard plots used to estimate kinetic constants (*3*). The slopes of the lines of Lineweaver-Burk plots become larger as the effect of intraparticle diffusion increases (*18*). However, such plots done at increasing enzyme concentrations showed no significant diffusional limitation of bone-bound amyloglucosidase (Fig. 9). Also, data from the Arrhenius plots showed similar slopes for free and immobilized enzyme systems, again indicating a lack of diffusion limitation (Fig. 8).

The possibility of mass transfer resistance caused by pore diffusion effects was also tested by comparing substrate use by powdered bone vs that of normal 10–20 mesh pieces of bone. The increase in the surface area of the support brought about by powdering the bone had no effect on the enzyme activity with glycogen as the substrate (Table 7). Powdering, however, did increase enzyme activity with starch by 1.5-fold, indicating that pore sizes can restrict the penetration of starch substrates into some areas of the bone support. Indeed, scanning electron micrographs of BIOBONE™ show highly irregular, heterogeneous pore sizes that represent the holes and pits left in crushed bone after all cells and organic material have been removed (Fig. 1).

The immobilized enzyme was relatively poorer at hydrolyzing the insoluble portions of starch compared with the free enzyme. Thus, activities toward the soluble portions of starch were only 16–19% of the corresponding activity with whole starch for the free enzyme, but the immobilized enzyme showed that a higher portion of its activity (38–41%) was caused by hydrolysis of soluble portions of the starch substrates (Table 5). Low product yields can occur if high mol wt starch fractions are excluded from small pores or if glucose, being slow to diffuse out of pores, forms reversion products such as maltose, isomaltose, and maltrotriose (*7*). The

drop in activity with dialyzed vs crude glycogen could simply be caused by the loss during dialysis of smaller mol wt molecules that are easier to hydrolyze.

Other attempts to immobilize amyloglucosidase by adsorption have been plagued with enzyme leakage. Amyloglucosidase bound to a DEAE-cellulose support suffered from leakage with 100 m*M* acetate buffer washings at pH 3.0–6.6 (*9*). Activity also decreased in subsequent cycles of operation. However, the results from washing experiments, long-term enzyme storage, enzyme recycling, and denaturant studies indicated that the immobilization of amyloglucosidase onto BIOBONE™ is firm.

In summary, the broad ranges of pH and temperature stability, together with the simple and inexpensive method of enzyme immobilization, are attractive features when considering the industrial applications of amyloglucosidase bound to granular chicken bone. Any potential diffusion limitation with starch substrates may be minimized by low enzyme loadings as well as vigorous mixing.

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