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**CHICKEN BONE AS A SUPPORT MATRIX FOR THE
IMMOBILIZATION OF SOME CARBOHYDRATE DEGRADING
ENZYMES**

by

DORIS YVONNE SCHAFHAUSER, B.Sc. (Hons. Biochemistry)

**A thesis submitted to the faculty of Graduate Studies and
Research in partial fulfillment of the requirements for the
degree of
Master of Science**

Department of Biology

Carleton University

Ottawa, Ontario

September, 1990

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The undersigned hereby recommend to the Faculty of Graduate
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Doris Schafhauser, B.Sc., in partial fulfillment of the
requirements for the degree of Master of Science

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ABSTRACT

Amyloglucosidase, glucose isomerase and β -amylase were immobilized separately and co-immobilized onto chicken bone (Biobone_{TM}). The amount of activity bound relative to an equal amount of free enzyme varied for each enzyme: $13.6 \pm 0.4\%$ for amyloglucosidase, $21.2 \pm 0.4\%$ for β -amylase and $31.8 \pm 0.8\%$ for glucose isomerase. The specific activities for these enzymes all dropped upon immobilization (1.7 fold for amyloglucosidase, 2.3 fold for β -amylase and 2.9 fold for glucose isomerase). The Michaelis constant (K_m) for the enzymes were altered by immobilization. Decreases in V_{max} values occurred for all enzymes but these largely reflected the percentage of enzyme initially bound to bone.

Four chemically derivatized bone surfaces were synthesized and checked for suitability for enzyme binding. Compared to the control bone (not derivatized), 1.3 - 1.7 fold increases occurred in the amount of amyloglucosidase activity bound. All but one method showed higher specific activity values compared to the control bone. However, the K_m values for the enzyme bound to derivatized bone surfaces showed an additional 1.9 - 5.3 fold increase.

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LIST OF ABBREVIATIONS AND SYMBOLS

ADP	- adenosine diphosphate
ATP	- adenosine triphosphate
CaCl ₂	- calcium chloride
°C	- degrees centigrade
EtOH	- ethanol
h	- hours
HCl	- hydrochloric acid
K _m	- Michaelis constant
KCl	- potassium chloride
MgSO ₄	- magnesium sulphate
μg	- microgram
μl	- microlitre
mg	- milligram
ml	- millilitre
mM	- millimolar
min	- minutes
nm	- nanometre
NADH ⁺	- nicotinimide adenine dinucleotide
NADH	- nicotinimide adenine dinucleotide (reduced)
O.D.	- optical density
NaOH	- sodium hydroxide
SEM	- standard error of the mean
Tris	- tris (hydroxymethyl) amino methane
U	- units of enzyme activity
V _{max}	- maximum enzyme velocity
w/v	- weight by volume (g/100 ml)

INTRODUCTION

Biotechnology is best defined as the use of biological organisms, systems or processes within the framework of technical operations and industrial production (Rehm *et al.*, 1989). This application-oriented science has its origins in microbial processes which have been used for hundreds and sometimes thousands of years for the production of beer, wine, bread, cheese products, saurkraut, vinegar and other foods (Rehm *et al.*, 1989). Use of these processes did not change in principle till approximately 100 years ago.

It was not until the second half of the 20th century that the pioneering work of microbiologists and chemists discovered that in microorganisms many important physiological and metabolic reactions take place which could be considered for technical utilization. Following this realization, manufacturing processes for a whole series of primary (lactic acid, citric acid, butanol) and secondary (penicillin) metabolic products were rapidly developed (Rehm *et al.*, 1989). The following industries are only a few of those conducting research in biotechnology today: pharmaceutical, plant and animal agriculture, chemical pollution control, sewage treatment and food production.

Although many of the highlights of biotechnology have occurred in the area of genetic engineering related to human health care products, literature in the last 2 decades has yielded an excessive number of publications which deal with food production. An example of the impact of biotechnology on the food industries is the recent developments in the sweeteners market, which was traditionally the realm of sucrose, derived from cane and beet crops (Coker *et al.*, 1985). Although sucrose had long been the dominant caloric sweetener in the United States, since 1972 it has been losing ground to

glucose syrups. The remarkable growth of the starch based sweetener industry in the last 15 years was triggered by high sugar prices in the early 1970's (Coker *et al.*, 1985). This fueled the drive to produce sugar from temperate zone starchy crops, with a profound and continued impact on national and international economics and politics (Coker *et al.*, 1985).

The development of biomass conversion processes for the commercial use of complex plant polysaccharides remains a major focus of biotechnological research today. By using specific enzymatic degradations, high quality and valuable products can be made from inexpensive and abundant raw materials. Enzymes such as amyloglucosidase, β -amylase, pullulanase and glucose isomerase could be used singly, in sequence or in combination on glycogen and starch substrates to produce a variety of saccharified compounds depending on demand (Coker *et al.*, 1985).

Enzymatic saccharification have a number of additional advantages including: 1) enzymatic methods are highly efficient and environmentally safe procedures of biomass conversion, 2) enzymes exhibit excellent specificity with respect to substrates used and products created, and 3) enzyme conversions can be carried out under conditions of moderate pH and temperature (Chakrabarti, 1988). The discovery, isolation and application of various carbohydrate degrading enzymes (ie. amyloglucosidase, β -amylase) resulted in the development of many new glucose syrups. The high cost of some of these industrially used soluble enzymes makes repeated or continued use desirable in most cases. However, it is generally impractical to reuse suspensions of native enzymes because their recovery from the reaction mixture is difficult and uneconomical (Hartmeier, 1985).

Immobilized Enzymes

Immobilized enzymes offer several improvements over their free counterparts with regards to industrial processes. Immobilized enzymes can be defined as enzymes that are in a state that permits their reuse (Bucholz and Klein, 1987). Five methods of enzyme immobilization may be noted: 1) covalent attachment to water soluble or water insoluble supports, 2) adsorption onto various surfaces, 3) covalent crossing to bi-functional reagents, 4) entrapment in polymeric gels, 5) encapsulation in microcapsules or hollow fibres (Klibanov, 1979). Figure 1 shows the various possible methods of enzyme immobilization in diagrammatic form (Hartmeier, 1985).

Biocatalyst immobilization is not a new technique. In vivo many types of enzymes do not function in a free state but are incorporated into biomembranes or are adsorbed onto their surfaces (Leninger, 1972). Indeed, adsorption is the oldest of the techniques used to immobilize enzymes, for in the early 1800's vinegar was produced by 'acetic acid bacteria' adsorbed onto wood chips (Hartmeier, 1985). In 1916 Nelson and Griffin used both charcoal and aluminum hydroxide to adsorb invertase (Nelson and Griffin, 1916). However, it was not until the 1960's that advances in the elucidation of protein and DNA structure, along with innovations in polymer chemistry, allowed for the practical development of immobilized enzymes (Trevan, 1980).

Ease of enzyme recovery and reuse are the major advantages associated with immobilized enzymes (Klibanov, 1979). Reactions can be stopped by simply removing the insoluble enzyme via filtration or centrifugation (Cheetham, 1985). Use of immobilized enzymes allows for reduced reactor volume and therefore improved volumetric productivity (Hartmeier, 1985). Kinetic properties and pH/temperature optima of immobilized enzymes can also be manipulated to favour specific applications.

Figure 1.

Various methods for immobilizing enzymes.

(C=carrier or support; B=biocatalyst) (Hartmeier, 1985).

Finally, enzyme stability is often enhanced upon immobilization (Klibanov, 1979).

The most widely used immobilized enzyme methods have their own specific advantages and disadvantages. Immobilization by physical adsorption and ionic binding are the easiest and fastest methods (Pitcher, 1980). Because no reactive species are involved there is little or no conformational change in the enzyme on immobilization (Kennedy, 1985). However, because the binding forces involved are relatively weak (hydrophobic, electrostatic, van der Waal's interactions), changes in ionic strength, pH and substrate concentration can all result in enzyme desorption (Kennedy, 1985).

On the other hand, covalently bound enzymes give enhanced stability due to the strength of the bonds created. But, conditions for achieving immobilization are more complicated and harsh, making enzymes more prone to inactivation upon immobilization (Kennedy, 1985). In addition, covalent binding methods have not yet found extensive use in the food processing industry, for the reagents used for covalent bonding are frequently not suitable for applications where they would contact food (Pitcher, 1980). The resulting derivatives are probably quite safe but the testing process to establish this fact may be prohibitively expensive (Pitcher, 1980).

Entrapment methods of enzyme immobilization allow for high concentrations of contained enzyme without any chemical modification or binding that may lead to activity loss. However, enzymes are susceptible to inactivation by the agents used for polymerization (Pitcher, 1980).

Therefore, factors to consider when choosing an appropriate immobilization technique are whether the method results in firm binding, if it is simple, reproducible, mild, cheap, and safe (Kennedy, 1985). The

resulting immobilized enzyme should be capable of use over a wide range of conditions, should not leak, be readily recovered for reuse, be stable and not deteriorate under the reaction conditions (Kennedy, 1985).

Immobilization is often considered to be an added expense and operation for industries. Hence, it must be proven beforehand that the extra costs can be justified by the potential savings of such a system.

Properties of Immobilized Enzymes

The activities and operational properties of enzymes can change quite dramatically upon immobilization. These changes can be attributed to conformational effects, microenvironmental effects and diffusional or mass transfer effects (Kobayashi *et al.*, 1972).

Conformational effects refer to enzyme modifications upon immobilization. Some activity is often lost during immobilization, usually attributed to the binding step which may cause enzyme deactivation, subunit dissociation and/or loss of allosteric properties (Cheetham, 1985). Shifts in substrate affinity may occur with populations of active sites with different enzyme activities found on the matrix (Klibanov, 1979). As a result, specific activities frequently decrease owing to alterations caused by the immobilization procedure (Pitcher, 1980).

One of the principles of enzyme immobilization involves stabilization through multipoint binding of the catalyst molecule to the support. This makes the macromolecule more rigid without necessarily altering it and therefore more stable against unfolding and ultimately inactivation (Klibanov, 1979). As a result, immobilized enzymes may be less accessible to microbial attack or to the action of exogenous proteases relative to the free enzyme. Enhanced stability against the effects of heating, pH shifts,

disrupting agents or organic solvents, also occurs frequently following immobilization.

Once immobilized the enzyme comes under the influence of the microenvironment of the support which may be drastically different from that existing in free solution (Klibanov, 1979). Of particular note are the electrostatic effects and pH shifts that can result when enzymes are immobilized onto charged surfaces. Substrate and matrix surface of opposite charge can result in attractive forces which may enhance mass transfer and increase the local concentrations of substrate (Kobayashi, 1972).

Shifts in pH optimum upon immobilization depend upon the charge of the supporting matrix. A negatively charged matrix results in an alkaline pH shift, due to the creation of a thin film of hydrogen ions around the matrix. These ions make the pH of the microenvironment surrounding the enzyme lower than that of the bulk solution (where the pH is measured). The opposite effect is created by the use of a positively charged matrix (Zaborsky, 1973). The same is true for non polyelectrolytic supports if the partition coefficient for the hydrogen ions in the microenvironment and the bulk solution is not unity (Klibanov, 1979).

Stabilization against pH can occur when the microenvironment of the immobilized enzyme possess buffering properties, due to either the nature of the support surface or an effect of neighbouring protein molecules (Klibanov, 1979).

Since enzymes are frequently immobilized within porous bodies or retained by semipermeable membranes, mass transfer limitation problems, particularly with large molecular weight substrates usually occur. Mass transfer effects in an immobilized enzyme reaction are considered important for two reasons. First they limit the efficiency of the catalytic action of the

enzyme and secondly, they change the apparent kinetic behaviour (Lee *et al.*, 1976).

Diffusion limitation results in the creation of an unstirred layer of solvent called the Nernst layer, which surrounds water-insoluble particles, creating a concentration gradient (Zaborsky, 1973). As a result of this layer, the immobilized enzyme will usually become saturated with substrate at a higher concentration than that normally required for saturation of the free enzyme (Zaborsky, 1973). The result is that enzymes often have a higher K_m value for their substrates.

Diffusion limitation will effect initial rate assays and thus distort the standard plots used to estimate kinetic constants (Pitcher, 1980). Since diffusion limitation problems are more apparent at high enzyme activities, their effects would be readily seen in a graph relating activity and temperature (ie. the Arrhenius plot) (Pitcher, 1980).

External mass transfer resistance depends primarily on particle size and fluid velocity around the particle, which is influenced by mixing conditions in stirred tanks and fluid velocity in fixed beds. Increases in stirrer speed or flow rate have been shown to minimize the problem (Bucholz and Klein, 1987).

Methods of minimizing internal mass transfer or pore diffusion effects and thereby increasing the efficiency of enzyme use include: 1) decrease the diffusion path for the reactants and products, or 2) reduce the activity of the enzyme (lower loading or temperature). The apparent activity of an adsorbed enzyme has frequently been observed to increase as smaller support particles are used, thus decreasing the diffusion path length (Pitcher, 1980).

Similarly, gradients of pH and enzyme disrupting agents can also form around the insoluble enzyme, effectively shielding immobilized enzymes from the full force of these solvents.

Biochemical Applications of Immobilized Enzymes

Enzymes can be co-immobilized to create multienzyme systems or pathways. These are groups of enzymes in close physical proximity connected by the use of a product from one enzyme as a substrate of one of the others (Chakrabarti, 1988). The existence of naturally occurring multienzyme systems (ie. fatty acid synthetase) has been known for some time (Trevan, 1980). The advantages of such systems are that faster reaction rates can often be achieved by the co-immobilized enzymes when compared to the enzymes free in solution. The basis for this occurrence is that the diffusion layers of the enzymes may overlap due to the close physical proximity of the immobilized enzymes (Cheetham, 1985). This in turn may facilitate the step-wise transfer of the product of one enzyme to the substrate site of the next. The one major disadvantage of co-immobilized enzyme systems is that establishing optimum operating conditions (pH and temperature) for all enzymes is often difficult (Cheetham, 1985).

A number of processes using immobilized biocatalysts are now well established on an industrial scale. Among these the conversion of glucose to fructose by glucose isomerase and the hydrolysis of penicillins by penicillin acylase are the most important examples (Klibanov, 1979). Affinity chromatography to isolate enzyme inhibitors and labelled peptides has employed immobilized enzymes (Cheetham, 1985). Immobilized enzymes have also been used in synthetic chemistry, enzyme electrodes, thermistors and biosensors (Cheetham, 1985).

Although immobilized enzymes, in competing with free enzyme systems, may have the advantages of increased stability and catalyst reuse, there are several reasons why immobilized enzymes are still not used in a number of traditional enzyme applications. Where the free enzyme is cheap and the process is already developed, a change to an immobilized system may not be financially beneficial (Hartmeier, 1985). Also, the supports and reagents required for immobilization are normally prohibitively expensive for large scale processing (Kennedy, 1985).

The Substrates

Starch is the principal food reserve polysaccharide in the plant kingdom and forms the major source of carbohydrates in the human diet. It is also a major raw material, the second most abundant renewable biopolymer next to cellulose (Galliard, 1985). The major commercial sources of starch are: maize in the United States, wheat in Canada and Australia, and both grain (wheat and barley) and root (potato) crops in Europe (Kennedy *et al.*, 1987).

Although starch is the same regardless of the source, the starch granules are of a size and shape that is characteristic of the plant (Rothwell and Garner, 1986). The site of the main storage area of starch varies from plant to plant; it may be in the seed (ie. maize) or in the tubers (ie. potato) or in the pith (ie. sago) (Rothwell and Garner, 1986).

Accurate analyses of starch are difficult due to its tenacious hold on water through extensive hydrogen bonding. It is known that all starch granules have the molecular formula $(C_6H_{10}O_5)_n$ and consist of essentially 25% amylose and 75% amylopectin (Rothwell and Garner, 1986). The characteristic features of amylose and amylopectin are their linear chains of (1->4)-linked α -D-glucopyranosyl residues with, in the case of amylopectin,

1,4,6-tri-O-substituted residues acting as branch points (Kennedy *et al.*, 1987).

Significant portions of D-glucose-6-phosphate and lipid material have been found in starches. Up to 1 residue in 6 in amylopectin is phosphorylated whilst the lipid material is believed to be complexed within the amylose structure (Kennedy *et al.*, 1987). Amylose is therefore a linear molecule with a molecular weight in the range 1.6×10^5 to 7.1×10^5 and varies with the source and maturity of the starch (Kennedy *et al.*, 1987). In neutral solutions amylose normally exists as a random coil but in the presence of complexing agents (ie. iodine, fats, polar organic solvents) it will form a regular helical structure fixed via hydrogen bonding. Amylopectin has a highly branched structure with a molecular weight of approximately 10^8 . Branches are believed to be arranged in either a comb-like or tree-like fashion (Kennedy *et al.*, 1987).

Natural starches suffer from poor solubility, inconsistent viscosity and susceptibility to acid degradation. This can result in problems, such as the properties of a food product containing natural starch changing on storage (Rothwell and Garner, 1986). For this reason, most industries make greater use of modified starches. Most processes (ie. Lintnerization) rely on heat and/or acid treatment which results in starches with lower viscosity and an increased number of branched chains. As a result, modified starches are more soluble and have a lesser tendency to "set back" or gelling on standing (Rothwell and Garner, 1986).

Although starches are the substrate of choice for the production of sweeteners, glycogen was used as a model substrate in most of the standard experiments in the work presented. Glycogen, the animal form of storage polysaccharide is very similar in structure to amylopectin. It is a highly

branched glucose polymer but unlike starch, is completely soluble and therefore easier to work with.

Method of Immobilization: Biobone™

The novel technique that was chosen for immobilizing the enzymes studied in the present research was binding onto chicken bone. Figure 2 is a scanning electron micrograph of a sample of bone. Bone is a strong porous substance consisting mainly of collagen protein impregnated with hydroxyapatite crystals (McLean and Urist, 1968). Hydroxyapatite ($\text{Ca}_5(\text{PO}_4)_3\text{OH}$) is the major mineral constituent of bone making up ~60% of the dry weight of bone (McLean and Urist, 1973). It is composed of a definite lattice of calcium hydroxide and phosphate groups, presenting a complex mosaic of charges for binding (Gorbunoff, 1984). The proteinaceous nature of collagen also contributes polar and nonpolar amino acid residues as potential binding sites (Vieth, 1976). Thus it is thought that the hydroxyapatite and collagen together provide a suitable surface for the formation of a network of cooperative non-covalent interactions with enzyme molecules (electrostatic, hydrogen bonds, van der Waal's forces) acting in concert to establish a stable framework (Vieth, 1976).

Chicken bone possesses all of the characteristics of an ideal enzyme support matrix. As the waste product of the meat processing industry, chicken bone is abundant and inexpensive (Findlay et al., 1986). The porous surface provides a high surface area available for enzyme binding. The fibrous nature of collagen makes it well suited in strength and form for enzyme immobilization (Vieth, 1976). Finally, being a natural product, bone is nontoxic and food grade.

Figure 2.

Scanning electron micrograph of a sample of Biobone™, under 60 x magnification.

The Research Project

The research undertaken involved the immobilization onto Biobone™, of a number of enzymes that are used in the commercial production of sweeteners from starch. These were amyloglucosidase, pullulanase, glucose isomerase and β -amylase. Immobilized and free enzymes were compared with respect to a wide variety of parameters. Efforts to enhance enzyme binding by chemical methods of bone derivatization were also employed using amyloglucosidase as the test enzyme. Amyloglucosidase, pullulanase and glucose isomerase were also co-immobilized onto bone in an effort to create a "one-step" reactor for the conversion of starch to fructose.

CHAPTER 1
STUDIES OF FREE AND IMMOBILIZED
AMYLOGLUCOSIDASE

INTRODUCTION

Amyloglucosidase (or glucoamylase)(E.C. 3.2.1.3) is an exo-splitting enzyme that catalyzes the stepwise hydrolysis of $\alpha(1,4)$ linkages and to a lesser extent $\alpha(1,6)$ linkages from the non-reducing ends of starch and glycogen, by a multichain mechanism (Fogarty, 1983). Preparations may be easily obtained from culture filtrates from several fungal species of Aspergillus and Rhizopus groups and from certain yeasts and bacteria (Fogarty, 1983).

Amyloglucosidase is one of the most industrially important enzymes, used for large scale saccharification of starch to glucose syrups for the food industry (Fogarty, 1983). As a result, amyloglucosidase has already been immobilized to a variety of supports, in the hopes of attaining a more controlled reusable system that could be used commercially (Pitcher, 1980).

The present study describes the immobilization of amyloglucosidase onto BioboneTM. Activity, stability, kinetics and temperature and pH optima were determined for the immobilized enzyme systems and compared with soluble enzyme. The use of granular chicken bone shows promise in 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 starch were obtained from Sigma Chemical Co. All biochemicals were purchased from Sigma Chemical Co., J.T. Baker Chemical Co., or Boehringer Mannheim Corp.

Biobone™ (cleaned and sized between 10 and 20 mesh chicken bone) was supplied by Protein Foods Research (Guelph Ont.). All bone samples were prewashed with acetate buffer (pH 4.0, 100mM) prior to use.

All experiments were performed with enzyme from the same bottle, and with the same batch of bone. All trials (ie. n=3) for each experiment were performed on the same day.

Immobilization of Amyloglucosidase

The standard amount of amyloglucosidase (Sigma Chemical Co.) used for all experiments, unless stated otherwise was 1.4U (where 1U is defined as the amount of enzyme that liberates 1.0 mg of glucose from starch in 3 minutes at pH 4.5 and 55°C). Adsorption was achieved through the addition of enzyme to a tube containing 100 ± 2 mg of bone and 0.5 ml sodium acetate buffer (pH 4.0, 100 mM), followed by mixing at 100 rpm for 30 minutes at 23°C. Excess solution was poured off, followed by successive washes with buffer (4 x 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 (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 since the dye reacted with the bone. Any protein found in washings of the bone itself were subtracted from the total wash protein determined.

Amyloglucosidase Assay

Amyloglucosidase 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) of 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 minutes at 23°C. To stop the reaction, 0.1 ml of the reaction mixture was mixed with 0.1 ml of 0.5 N NaOH. Glucose liberated was then estimated using a glucose diagnostic kit (Sigma Chemical Co. No. 510-DA (colorimetric)).

The procedure is based upon the following coupled enzymatic reactions:

Total sample volumes of 0.1 ml were added to a tube containing 1 ml of reagent solution containing ~5 U Glucose oxidase (*Aspergillus niger*) and ~1 U Peroxidase (horseradish) (Keston, 1956). The mixture was left for 45 minutes at room temperature to allow for the brown colour to develop, the

intensity of which, when measured at 450 nm was proportional to the original glucose concentration (Keston, 1956). The amount of glucose was estimated using a glucose standard curve, adjusted where necessary, to account for the effect of various denaturants on colour development by glucose standards.

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, 6.0) and concentrations (100 mM, 50 mM). Amyloglucosidase was also left to immobilize to bone for up to 24 hours to determine optimal binding time. Furthermore, increasing concentrations of amyloglucosidase was added to bone to determine the maximum amounts that could be immobilized.

Optimum pH

Free and immobilized amyloglucosidase were pre-equilibrated with 0.5 ml of the following pH buffers: 0.1 M acetate (pH 3.0, 4.0, 4.5, 5.0, 5.5), 0.1 M phosphate (6.5, 7.5, 8.5), 0.1 M Tris-HCl (pH 7.0) and 0.1 M imidazole (pH 7.0). Substrate was added, followed by a 30 minute agitation at 130 rpm and 23°C.

Optimum Temperature Studies

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 minute incubation.

Kinetic Parameters

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

Effects of Enzyme Disrupting Agents

Free and immobilized amyloglucosidase were equilibrated for 30 minutes with one of the following reagents: TritonX-100 (1%), ethanol (30%), or KCl (0.2, 0.5, 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). Equilibration washes were kept. Substrate containing each of the reagents was added to the washes and to the bound and free enzymes, followed by a 30 minute agitation at 23°C.

Operational Half Life

Between uses immobilized enzymes were stored at 4°C in buffer containing substrate with 0.04% (w/v) sodium azide. Bone was allowed to warm to room temperature, was thoroughly washed with buffer, and then fresh substrate was added. Half life values were calculated from interpolation or extrapolation directly from plots of relative activity over the storage time period (data not shown).

Methods Employed for Mass Transfer Studies

Glucose production after 10 minutes was determined for reactions using 1-160 mg/ml of glycogen, comparing mixed and non-mixed tubes.

The activity versus temperature values previously determined for free and immobilized amyloglucosidase were fit into an Arrhenius equation and the resulting slopes were compared.

Kinetic data for glycogen using 1.4, 5.6 and 11.2 units of enzyme were plotted on Lineweaver-Burk plots. The resulting slopes were compared.

The activities of immobilized amyloglucosidase were compared on whole and crushed bone. Control bound enzymes were frozen in liquid nitrogen and then left to return to room temperature. Immediately following freezing the immobilized enzyme was ground into powder with a mortar and pestle.

Data Calculations and Statistics

Data is reported as mean values of triplicate samples $\pm$ standard error of the mean (SEM). Where experimental data points in figures do not have SEM bars, these values were within the dimensions of the symbols used. Statistical significance of results were determined by the students' t-test where required (Mendenhall, 1983). Values for the kinetic constants were determined by a nonlinear least squares equation, fit to the Michaelis Menton and /or Hill equations. All other lines were generated by the SIGMAPLOTTM graphics program. Lines that did not adequately fit the data were drawn in by hand.

Figure 1-1.

Bone and bone immobilized amyloglucosidase stained with Acid fuchsin and Coomassie blue.

**Far left: Enzyme bound bone stained with Coomassie blue.
Near left: Bone stained with Coomassie blue.
Centre: Unstained bone.
Near right: Bone stained with Acid fuchsin.
Far right: Enzyme bound bone stained with Acid fuchsin.**

Figure 1-2.

Activity present in each wash is expressed relative to the amount of activity immobilized onto bone (ie. imm. activity).

RESULTS

Immobilization of Amyloglucosidase onto Biobone™

Figure 1-1 gives physical evidence of enzyme binding. It is a photograph of Biobone™ in the absence or presence of immobilized amyloglucosidase, each of which has been treated with the protein stains Acid fuchsin and Coomassie blue. Since bone largely consists of collagen, Biobone™ itself stained a light red and blue respectively. Bone with immobilized amyloglucosidase was very distinguishable, being stained much darker due to the presence of bound enzyme.

Figure 1-2 indicates that amyloglucosidase activity remained immobilized on bone even after 10 repeated washings. It also indicates that 3-4 washes were sufficient to remove any unbound enzyme.

Incubation times greater than 20 minutes did not increase the amount of amyloglucosidase bound (Figure 1-3). Also, the greatest degree of binding occurred with low pH incubations (Table 1-1). Thus, the conditions used for optimal binding were incubation for 30 minutes at pH 3.0 in 100 mM buffer (although subsequent assay of enzyme activity were all done at constant pH 4.5).

Figure 1-4 shows the effect of the addition of increasing amounts (0.14-33U) 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 maximum amount of enzyme loading reached ~ 16 U per 100 ± 2 mg bone. Under standard conditions, the average amount of amyloglucosidase activity immobilized was $13.6 \pm 0.4\%$, with approximately $57.8 \pm 6.6\%$ being eluted in the washes (Table 1-2). The 28.6% of lost activity could be attributed to enzyme deactivation upon immobilization. The specific activity of free and

Figure 1-3.

The amount of amyloglucosidase activity immobilized was determined following the stated binding times.

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

Acetate Buffer (pH)	Activity (nmoles glucose produced/ml/min)	
	Concentration	
	50 mM	100 mM
3.0	33.1 $\pm$ 0.5	37.5 $\pm$ 0.7
4.0	31.0 $\pm$ 1.1	34.7 $\pm$ 2.7
4.5	30.5 $\pm$ 2.5	30.9 $\pm$ 4.7
5.5	17.6 $\pm$ 0.8	11.3 $\pm$ 2.0
7.0	1.0 $\pm$ 0.1	0.80 $\pm$ 0.05

Data are means $\pm$ SEM, n=3. To achieve immobilization, the standard amount of enzyme (1.4 U), was added to each with a 30 minute incubation, followed by 4 washes. Reactions were then run at pH 4.5, 23°C for 30 minutes following binding.

Figure 1-4.

EFFECT OF THE ADDITION OF INCREASING CONCENTRATIONS OF
AMYLOGLUCOSIDASE ON THE AMOUNT OF ACTIVITY BOUND

Increasing concentrations of amyloglucosidase were added to bone, and were assayed for immobilized activity following a 30 minute binding period.

Table 1-2
Amount of Amyloglucosidase Activity Retained
During Bone Immobilization Following Ten Washes

	Activity, %	Specific Activity U/mg Protein
Free	100 ± 2	75.3 ± 0.8
Immobilized	13.6 ± 0.4	43.5 ± 9.6
Wash	57.8 ± 6.6	—
Lost	28.6	—

Data are means ± SEM, n=3. Activities are expressed relative to an equal amount of the free enzyme (100%). The amount of protein in free enzyme and bound to 100mg bone was determined by the Biorad protein assay (Biorad Inc.).

Table 1-3
Effect of Temperature on the Activity of Free
and Immobilized Amyloglucosidase

Temperature °C	Activity (nmoles glucose produced/ml/min)			
	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 means ± SEM, n=3. The values in brackets are relative activities, compared to 55°C.

immobilized amyloglucosidase as estimated by the BioRad protein assay was 75.3 ± 0.8 and 43.5 ± 9.6 U/mg protein, respectively.

Optimum Temperature and Temperature Stability Studies

Table 1-3 shows the effects of temperature on free and immobilized amyloglucosidase. Both maintained $V_{\max}$ activities at high temperatures (80°C).

Figure 1-5a,b shows the time course of glucose production at high (80°C) and moderate temperatures (23°C), for free and immobilized amyloglucosidase. Bound enzyme demonstrated slow but fairly linear rates of glucose production over the 2 hour period at both temperatures, indicating constant activity with time. The free enzyme maintained a linear rate for only 60 minutes at 80°C and 23°C , later leveling off with time. This suggests either a gradual loss of activity, or a slower activity due to the increased concentration of exposed (1-6) branched points.

Figure 1-6 indicates that the amount of adsorbed enzyme activity remains fairly constant after repeated use (5 times), even at high temperature (55°C). Although an additional 6.4% of activity is eluted into the substrate following the first cycle only, this did not have a significant effect on the activity or the stability of the enzyme.

Optimum pH

Figure 1-7 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. Compared to phosphate buffer at pH 7.0, imidazole buffer enhanced enzyme activity by 20%, and Tris-HCl buffer inhibited enzyme activity by 36%.

Figure 1-5a.

Amyloglucosidase was immobilized under standard conditions. After the addition of substrate, the system was incubated for the required period of time at 23°C.

Figure 1-5b.

Amyloglycosidase was immobilized under standard conditions. After the addition of substrate, the system was incubated for the required period of time at 80°C.

Figure 1-6.

Amyloglucosidase was immobilized under standard conditions. After each 30 minute reaction period, bone was thoroughly washed with buffer, and then fresh substrate was added. Spent substrate was left to incubate for an additional 30 minutes for the determination of eluted activity. This cycle was repeated 5 times.

Figure 1-7.

Effects of Enzyme Disrupters on Amyloglucosidase Activity

Table 1-4 shows the effect of some disrupting agents (conditions that an enzyme may encounter with crude substrates in industrial scale reactions) on the activity of free and immobilized amyloglucosidase. Also shown is the amount of enzyme released from the bone when incubated with the disrupters. The results show that immobilized amyloglucosidase maintained greater activity in the presence of the disrupters than did the free enzyme, indicating 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. These experiments do not distinguish between the effects of these disrupters on enzyme stability or the strength of enzyme binding.

Kinetic Properties

Table 1-5 outlines the kinetic properties of free and immobilized amyloglucosidase. The K_m value for glycogen increased by 3 fold for the immobilized enzyme, whereas the values for Lintner and potato starch remained essentially the same. V_{max} values for the immobilized enzyme were reduced compared to the free enzyme by 78%, 82% and 60%, respectively, for glycogen, Lintner and potato starch. The decrease 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 (Atha and Ingham, 1981). Table 1-6 indicates that 10% PEG had no significant effect on either the K_m or V_{max} of the enzyme using glycogen as substrate. This suggests that the effects of immobilization on the kinetic parameters with glycogen are not simply due to enzyme crowding in the immobilized state.

Table 1-4
Effect of Disrupting Agents on Free and Immobilized
Amyloglucosidase Activity

Treatment	Activity (nmoles glucose produced/min/ml)					
	Free		Immobilized		Wash	
Control	179	+ 10 (100%)	24	+ 5 (100%)	3	+ 1 (2%)
TritonX-100	127	+ 1 (70%)	24	+ 3 (98%)	8	+ 2 (4%)
EtOH (30%)	123	+ 2 (69%)	25	+ 2 (102%)	4	+ 1 (2%)
KCl 1M	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 0.1M	115	+ 8 (64%)	22	+ 1 (93%)	3	+ 1 (2%)
pH 9.5 0.1M	23	+ 2 (13%)	7	+ 1 (27%)	3	+ 2 (13%)

Data are means + 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 the given conditions. Reactions were conducted at 23°C for 30 minutes.

Table 1-5
Kinetic Parameters for Free and Immobilized
Amyloglucosidase

Substrate	K _m (mg/ml)	V _{max} ^a
Glycogen:		
Free	3.04 + 0.38	210 + 11
Immobilized	9.04 + 1.51	47.1 + 1.8
Lintner Starch:		
Free	80.2 + 5.0	70.2 + 5.1
Immobilized	75.7 + 2.4	12.5 + 0.5
Potato Starch:		
Free	101 + 4	64.3 + 6.8
Immobilized	95.4 + 2.2	26.1 + 0.4

Data are means + SEM, n=5.

^a nmole glucose/ml/min

Table 1-6
Effects of 10% Polyethylene glycol on the Kinetic
Parameters of Free Amyloglucosidase

	K _m (mg/ml)	V _{max} ^a
no PEG	3.41 + 0.4	195 + 10
10% PEG	3.62 + 0.4	203 + 3

Data are means + SEM, n=3. Standard solutions of 120mg/ml of dialyzed glycogen were used.

^a nmoles glucose/min/mL.

Table 1-7
Comparison of Glycogen, Lintner and Potato Starches
as Substrates for Free and Immobilized Amyloglucosidase

Substrate	Activity (nmoles glucose produced/ml/min)	
	Free	Immobilized
Glycogen:		
Dialysed	191 + 4 (100%)	28 + 2 (100%)
Crude	241 + 9 (126%)	41 + 2 (151%)
Lintner Starch:		
Whole	123 + 3 (100%)	28 + 1 (100%)
Soluble	19 + 1 (16%)	11 + 1 (41%)
Potato Starch:		
Whole	62 + 4 (100%)	19 + 2 (100%)
Soluble	12 + 0.2 (19%)	7 + 0.3 (38%)

Data are means + SEM, n=3. The relative activities are given in brackets. Control values were set to 100%. Substrates were subjected to overnight dialysis in membrane with a molecular weight cutoff of ~50,000. Soluble starch was obtained by allowing the insoluble portions of whole starch to settle.

Substrate Comparison

Table 1-7 shows amyloglucosidase activation with various substrates. The free enzyme utilized glycogen to a far greater extent than it did the starches (Lintner and potato). The differences in activities were less pronounced upon immobilization, for amyloglucosidase was a better catalyst for starch breakdown when immobilized. Both the free and immobilized enzymes utilized crude glycogen more efficiently than they did substrates that had been dialysed to remove low molecular weight fragments. Furthermore, 80-85% of glucose production from free enzyme activity against samples of whole starch came from the hydrolysis of insoluble portions of the substrate, compared to only 60% for immobilized amyloglucosidase.

Storage Stability

After a period of 5 weeks, immobilized amyloglucosidase maintained ~83% activity. This results in an immobilized enzyme half life of approximately 144 days.

Diffusion Limitation of the Immobilized System

Since internal mass transfer limitation is a common problem with many immobilized enzyme systems with porous carrier surfaces, experiments were done to specifically address this subject. Table 1-8 shows the results from studies to determine whether mixing the reaction tubes helps to alleviate diffusion limitations. Results show that agitating the reaction tubes in a shaker has no significant effect on the K_m or V_{max} values for free and immobilized amyloglucosidase with glycogen as substrate. For Lintner starch as substrate, mixing also had no significant effect on the kinetic values for

Table 1-8
Effects of Mixing on the Kinetic Parameters of
Free and Immobilized Amyloglucosidase

	K_m (mg/mL)		V_{max}^a	
	Mixed	Nonmixed	Mixed	Nonmixed
Glycogen:				
Free	3.4 + 0.4	3.3 + 0.4	195 + 10	200 + 12
Immobilized	9.6 + 0.9	12.8 + 1.6	43.5 + 4.2	34.5 + 3
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 means + SEM, n=3. Glycogen and Linter starch were used as substrates. Tubes were mixed by agitating in a rotary shaker. Nonmixed tubes were left motionless.

^a nmoles glucose/ml/min

Figure 1-8.

ARRHENIUS PLOT FOR FREE AND IMMOBILIZED AMYLOGLUCOSIDASE

The results from the temperature profile (Table 1-3) for free and immobilized amyloglucosidase, were fit to an Arrhenius equation (which relates temperature and reaction velocity). The resulting slopes were compared for evidence of diffusion limitation.

the free enzyme. But, immobilized amyloglucosidase showed a substantial 3.3 fold increase in the $V_{\max}$ value with mixing (K_m could not be determined).

If a limitation due to substrate diffusion exists, one would notice deviations in the Arrhenius relationship (relating temperature and reaction velocity) at high temperatures. Whereas if an Arrhenius plot for the immobilized enzyme gives a straight line with a slope equal to that of a similar plot for the soluble enzyme it usually implies that no internal diffusion limitations exist (Pitcher, 1980). The results in Figure 1-8 indicate that both immobilized and free amyloglucosidase have similar slopes, both at low and high temperatures.

Furthermore, if higher enzyme loading onto a support matrix results in steeper slopes for Lineweaver-Burk plots, this also indicates an increase in diffusion effect (Pitcher, 1980). The results from Figure 1-9 shows that the slope decreases with increased enzyme concentrations with Oglycogen as substrate. Similar results were obtained with Lintner starch as substrate (data not shown).

One other method of evaluating mass transfer limitations due to internal diffusion resistance is to decrease matrix particle size. Following freezing in liquid nitrogen, the immobilized enzymes were ground into powder with a mortar and pestle. The control enzymes were also frozen. The results from Table 1-9 indicate that no change in activity occurred with an increase in surface area for glycogen as substrate. However, a 1.5 fold increase in activity resulted for powdered bone using Lintner starch as the substrate.

Figure 1-9.

Kinetic studies for free and immobilized amyloglucosidase were performed with 3 different concentrations of enzyme. The data was fit to Lineweaver-Burke plots, and the resulting slopes were compared for evidence of diffusion limitation.

Table 1-9
Effects of Increasing Surface Area on the Apparent
Activity of Immobilized Amyloglucosidase

Treatment	Activity (nmoles 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 means + SEM, n=3. Relative activities are given in brackets. Control values were set to 100% for comparison. 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 N₂; the powdered enzyme was then thawed.

DISCUSSION

The application of immobilized enzyme technology has the potential for improving the industrial use of amyloglucosidase. Production of high glucose syrups (ie. 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 confectionary industry (Fogarty, 1983).

Compared to the large scale degradation of starch to glucose by soluble enzyme (48-72h, at pH 4.5 and 55-60°C), immobilization reduces the conversion time because very high enzyme concentrations can be used and recovered (Pitcher, 1980). Furthermore, the shorter residence times would decrease the number of side reactions, thus lowering refining costs (Fogarty, 1983). These considerations amply justifies the efforts that have been made to immobilize amyloglucosidase. Immobilization of amyloglucosidase onto Biobone™ represents an improved method of binding over other methods described.

Amyloglucosidase binding to bone is firm and immediate. Approximately 14% of activity remains bound following 10 washes of the bone, with maximum binding occurring after only 20 minutes incubation with the enzyme (Figure 1-2, 1-3). Also, binding appears to be most favourable at pH 3.0, 100 mM buffer, which appears to take advantage of the net positive charge of amyloglucosidase at low pH (due to a low isoelectric point ie. pH 3.5-4.0) (Pazur *et al.*, 1971).

Although 14% enzyme bound is a small amount, Nithiandam *et al.* (1981) immobilized only 19% of amyloglucosidase activity on a copolymer of

methyl methacrylate, Storey and Chakrabarti (1990a) immobilized 25% of the activity onto polyurethane polymers and Cabral *et al.* (1981) bound only 12% onto metal activated inorganic supports.

The enhanced stability of immobilized amyloglucosidase was repeatedly demonstrated during the experiments. First of all, the immobilized enzyme remained very active, displaying a half life of approximately 144 days following 5 weeks of storage and use.

Secondly, a high temperature optimum (55°C) was maintained upon immobilization (Table 1-3). Also, long term glycogen degradation remained fairly constant with time, unlike the free enzyme, even at high operating temperatures (Figure 1-5a,b). Furthermore, activity remained fairly constant following 5 repeated cycles of operation, at moderate and high temperatures (Figure 1-6).

Immobilized amyloglucosidase acquired a broader range of pH values over which activity was retained (pH 3.0-4.5), with an optimum at pH 4.0 (Figure 1-7). This is in contrast to the free enzyme, which shows an optimum at pH 4.5. Tomar *et al.* (1985) and Slininger *et al.* (1988) also observed an acidic shift in the pH optimum upon amyloglucosidase immobilization.

Shifts in the pH optima are largely dependent on the type of carrier matrix used. Positively charged matrices usually result in acidic shifts in pH optimum, due to the formation of a pH gradient in which a depletion of hydrogen ions occurs in the vicinity of the enzyme, compared to that of the bulk solution (Zaborsky, 1973). However, since the bone matrix is believed to be essentially neutral in nature, this drop in pH optima can not be explained by charge effects. Tomar *et al.* (1985) concluded that the shift in pH optima to the acidic region was due to the loss of basic groups by the enzyme upon immobilization.

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 the disrupting agents. Immobilization protected the enzyme from the inhibitory effects of Triton X-100, ethanol, KCl, and pH extremes (Table 1-4). Coulet *et al.* (1974) maintained that the high hydrophilicity of collagen helps to prevent denaturation. The minor amounts of activity present in the washes following denaturant pretreatments further supports the firmness with which amyloglucosidase bound to bone (Table 1-4). 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 free enzyme activity) largely accounted for the low activity immobilized. The pH 9.5 buffer may have upset the binding interactions between the enzyme and bone matrix.

The changes in kinetic parameters noted for immobilized amyloglucosidase included a 3 fold increase in K_m for glycogen as substrate, with a virtually unchanged K_m for the starches (Table 1-5). These values are within reason with respect to results previously reported. Nithiandam *et al.* (1981) found that the K_m value of their immobilized enzyme showed a 10 fold increase compared to the free enzyme. Slininger *et al.* (1988) found a 5.5% increase in K_m upon immobilization and Storey and Chakrabarti (1990a) showed a 14% increase.

The kinetic data, together with the results from Table 1-7, indicate that immobilized amyloglucosidase has a comparatively higher catalytic activity with the starches than with glycogen. However, the overall rate of starch hydrolysis is considerably smaller compared to glycogen. Rates of hydrolysis are affected by both the molecular size and structure of the substrate as well as the next bond in the sequence (Fogarty, 1983). Thus,

although the initial rate of substrate hydrolysis is rapid, it subsequently drops due to the lower affinity of the enzyme for the $\alpha(1,6)$ links (Fogarty, 1983).

The great difference in amyloglucosidase affinity for glycogen over starch is likely due to the limited solubility of starch. Although both glycogen and the amylopectin portion of starch are highly branched polymers with similar molecular weights ($\sim 10^8$), amylopectin has longer chain lengths (20-25 glucose units) compared to glycogen (10-12 glucose units) (Banks, 1975). This, together with the amylose content of starch, which is essentially a linear glucose polymer, result in a very insoluble system (Banks, 1975).

Results from Figure 1-4 indicate that large enzyme loadings can be achieved per 100 mg bone. However, relative activities at high enzyme loadings suffer adverse affects, partly due to steric effects due to enzyme crowding on the bone and partly as a result of diffusion limitation in the substrate supply and product elimination (Caldwell *et al.*, (1976). The amounts of enzyme used in experiments here (1.4 U), represent 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. Furthermore, the PEG experiments done with free enzyme designed to mimic steric effects due to immobilization, show that none existed (Table 1-6). Enzyme loading must be optimized considering the tradeoff between less efficient enzyme use at high loadings and larger carriers and immobilization cost at low enzyme loadings (Pitcher, 1980). Thus it would be feasible to run reactions at maximum enzyme efficiency due to the negligible cost of BioboneTM.

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 (Zaborsky, 1973). Using Lintner starch as substrate, the 3 fold increase in $V_{\max}$ and a now detectable K_m value due to mixing indicated that such gradients occurred for the immobilized amyloglucosidase and could be alleviated by mixing the reaction tubes, where none existed for the free enzyme or for both immobilized and free enzyme using glycogen (Table 1-8).

Diffusion limitations will affect initial rate assays and thus distort the standard plots used to estimate kinetic constants (Pitcher, 1980). The slopes of the lines of Lineweaver-Burk plots become larger as the effect of intraparticle diffusion increases (Kobayashi *et al.*, 1972). Such plots done at increasing enzyme concentrations show no significant diffusional limitation (Figure 1-9). Also, data from the Arrhenius plots predicts similar slopes for the free and immobilized enzyme systems (Figure 1-8).

Despite efforts to minimize substrate diffusion limitation, sufficient evidence exists to conclude that it was still a problem for amyloglucosidase immobilized onto bone, particularly with starch as a substrate. Although the reductions in enzyme activity upon immobilization were substantial (14%), they could be largely attributed to the low amount of activity bound. But, the 2.7 fold decrease in specific activity upon immobilization confirms a high diffusion resistance as well as an inefficient coupling method (Nithiandam *et al.*, 1981). In fact, the 29% loss of enzyme activity (possibly due to enzyme inactivation upon immobilization) was likely accounted for here (Table 1-2).

The results from the bone grinding experiment, designed to increase surface area and free the enzyme from any restrictions brought on by small pore size, further supported the existence of diffusional limitations with

starch as substrate. The resultant 1.5 fold increase in activity following grinding was not due to enzyme leakage and subsequent increase in amyloglucosidase activity while in the free state, for no increase in activity was obtained with glycogen under similar conditons (Table 1-9).

Substrate comparison further illustrates the limitations imposed on the immobilized amyloglucosidase by the insoluble portions of starch. Whereas 80-85% of free enzyme activity can work on insoluble starch, only ~60% of immobilized activity hydrolyses insoluble starch (Table 1-7). Low product yields can occur if high molecular weight 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 maltotriose (Slininger *et al.*, 1988). The drop in activity with crude versus dialysed glycogen could be simply due to smaller molecular weight molecules that are easier to hydrolyse.

Biobone™ has been previously used by Findlay *et al.* (1986) to immobilize invertase, pepsin, pectinase, lactase and catalase. Amyloglucosidase has also previously been immobilized onto collagen and hydroxyapatite (Coulet *et al.*, 1974; Fukui, 1972). This work however, represents preliminary efforts to immobilize amyloglucosidase onto granular chicken bone.

Other attempts to immobilize amyloglucosidase by adsorption have been plagued with enzyme leakage. Tomar *et al.* (1985), found that amyloglucosidase bound to a DEAE-cellulose support suffered from leakage with 100 mM acetate buffer washings at pH 3.0-6.6. Activity also decreased in subsequent cycles of operation. However, the results from washing experiments, long term enzyme storage, enzyme recycling and denaturant

studies indicate that the immobilization of amyloglucosidase onto Biobone™ is firm.

Even though the immobilized amyloglucosidase is plagued by substrate diffusion limitation, low enzyme loadings as well as vigorous mixing help to minimize the problem. Also, broad ranges of pH and temperature stability, as well as an increased affinity for starch, are attractive features when considering industrial adaptation.

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CHAPTER 2
STUDIES OF FREE AND CO-IMMOBILIZED
AMYLOGLUCOSIDASE AND PULLULANASE

INTRODUCTION

Pullulanase (pullulan 6-glucanhydrolase EC 3.2.1.41) attacks $\alpha(1-6)$ -linkages in the amylopectin and β -limit dextrin portions of starch and glycogen in a random endo fashion (Solomon, 1985). End products are maltotriose and maltotriosyl oligosaccharide side chains of varying length (Fogarty, 1983).

Because amyloglucosidase has only a limited ability to hydrolyse $\alpha(1-6)$ linkages, an amyloglucosidase/pullulanase complex is of interest to industry, for any improvements in glucose yield result in significant improvements in the overall process economics (Ram, 1982). Furthermore, since purified pullulanase is expensive, an immobilized form would be of considerable value in continuous reactions and in the ease of enzyme recovery (Ram, 1982).

Both pullulanase and amyloglucosidase were immobilized onto BioboneTM in an effort to enhance starch and glycogen degradation. Activity profiles were determined analyzing the effects of temperature, pH and potential enzyme disrupters on the immobilized and free enzyme systems. The increase in catalytic activity against glycogen and potato and Lintner starches was also compared with amyloglucosidase activity alone.

Materials and Methods

Materials

Pullulanase from Bacillus sp. was kindly donated by Novo Industries. The enzyme was first purified by passing through a column of G-25 Sephadex equilibrated with pH 4.5 buffer. 1 unit of pullulanase is defined as the amount of enzyme that hydrolyses pullulan under standard conditions, releasing reduced carbohydrates equivalent to 1 μ mole of glucose per minute (Novo Industries).

Co-immobilization of Amyloglucosidase and Pullulanase

Standard co-immobilization experiments were carried out using a combination of 0.7 U amyloglucosidase and 10 U pullulanase. Amyloglucosidase contamination of the pullulanase accounted for 0.15 U of the total amount of amyloglucosidase present (Novo Industries). Adsorption was achieved through the addition of enzymes to a tube containing 100 ± 2 mg of pre-washed bone and 0.5 ml acetate buffer (pH 4.5, 100 mM), followed by agitation at 100 rpm for 30 minutes at 23⁰C. Excess solution was poured off, followed by successive washes with buffer (4 x 1ml).

Co-immobilized Experiments

The experimental protocol for analysis of free and immobilized amyloglucosidase and pullulanase followed exactly that used for amyloglucosidase (see materials and methods, Chapter 1).

RESULTS

Co-immobilization of Amyloglucosidase and Pullulanase onto Biobone™

Figure 2-1 shows that the optimal level of pullulanase for pairing with 0.7 U amyloglucosidase was 10 U. Table 2-1 indicates that the buffer conditions for optimal co-immobilization were acetate buffer 100 mM at pH 4-4.5. Under these conditions, the average amount of co-immobilized activity was $21.3 \pm 0.2\%$, with approximately $47.4 \pm 3.8\%$ being eluted in the washes. The 31.3% of lost activity could be attributed to enzyme deactivation upon immobilization. The specific activity of free and co-immobilized amyloglucosidase/pullulanase (with protein content estimated by the BioRad protein assay) was 14.1 ± 0.2 and 5.6 ± 1.1 U/mg respectively (Table 2-2).

Optimum Temperature and Stability Studies

Table 2-3 shows that both free and co-immobilized amyloglucosidase/pullulanase showed a high temperature optimum of 55-70°C. Co-immobilization also broadened the temperature range at which high activity was maintained.

Figures 2-2a,b show the time course of glycogen degradation at moderate (23°C) and high (80°C) temperatures, for free and co-immobilized amyloglucosidase/pullulanase. Bound activity was fairly linear over the 24 hour period at 23°C. The free enzyme system also showed linear rates, but showed a decrease in slope after 4 hours. At 80°C, both the free and immobilized systems show linear rates of hydrolysis, with changes in slope occurring 10 and 30 minutes, respectively. The appearance of such 2 phase reaction rates implies initial rapid reactions, which slow down to a reduced but still fairly linear rate.

Figure 2-1

EFFECT OF THE ADDITION OF INCREASING CONCENTRATIONS OF PULLULANASE TO SET AMOUNTS OF AMYLOGLUCOSIDASE ON THE FINAL OUTPUT OF GLUCOSE

Increasing concentrations of pullulanase were added to set amounts of amyloglucosidase for immobilization onto bone. Following a 30 minute binding period, bone was assayed for immobilized activity.

Table 2-1
Effect of pH and Ionic Strength of the Incubation Mixture
During Binding on the Amount of Amyloglucosidase
and Pullulanase Co-immobilized onto Bone

Acetate Buffer (pH)	Activity (nmoles glucose produced/ml/min)	
	Concentration	
	50 mM	100 mM
3.0	6.9 $\pm$ 0.7	20.9 $\pm$ 1.7
4.0	32.9 $\pm$ 1.2	44.4 $\pm$ 1.8
4.5	31.1 $\pm$ 1.2	46.0 $\pm$ 2.8
5.0	16.7 $\pm$ 1.3	18.9 $\pm$ 4.3
7.0	3.6 $\pm$ 0.2	0.7 $\pm$ 0.1

Data are means $\pm$ SEM, n=3. After binding, bone was washed times and then activity was measured by reaction with glycogen at pH 4.5, 23°C for 30 minutes.

Table 2-2
Amount of Amyloglucosidase and Pullulanase Activity
Retained During Bone Immobilization Following Ten Washes

	Activity, %	Specific Activity U/mg Protein
Free	100 $\pm$ 9	14.1 $\pm$ 0.2
Immobilized	21.3 $\pm$ 0.2	5.6 $\pm$ 1.1
Wash	47.4 $\pm$ 3.8	—
Lost	31.3 $\pm$ 4.1	—

Data are means $\pm$ SEM, n=3. Activities are expressed relative to an equal amount of the free enzyme (100%). The amount of protein in free enzyme and bound to 100mg bone was determined by the Biorad protein assay (Biorad Inc.). Units for specific activity expressed as nmoles glucose produced/min/ml.

Table 2-3
Effect of Temperature on the Activity of Free
and Immobilized Amyloglucosidase and Pullulanase

Temperature °C	Activity (nmoles glucose produced/ml/min)			
	Free		Immobilized	
0	34	+ 2 (8%)	7.8	+ 0.3 (15%)
15	77	+ 3 (18%)	15.7	+ 2.2 (16%)
23	144	+ 3 (34%)	29.6	+ 6.6 (24%)
37	201	+ 19 (47%)	45.9	+ 4.5 (68%)
45	221	+ 21 (52%)	59.4	+ 6.0 (93%)
55	427	+ 33 (100%)	63.8	+ 6.4 (100%)
70	423	+ 38 (99%)	61.4	+ 10.0 (97%)
80	294	+ 20 (69%)	52.9	+ 0.4 (83%)

Data are means, + SEM, n=3. The values in brackets are relative activities, compared to 55°C.

Figure 2-2a.

TIME COURSE OF GLYCOGEN DEGRADATION BY FREE
AND CO-IMMOBILIZED AMYLOGLucOSIDASE AND
PULLULANASE AT 23°C

Figure 2-2b.

TIME COURSE OF GLYCOGEN DEGRADATION BY FREE
AND CO-IMMOBILIZED AMYLOGLYCOSIDASE AND
PULLULANASE AT 80°C

For comparison, Figure 2-2a also shows a time course for free and immobilized amyloglucosidase alone. The addition of pullulanase not only increases the overall rate of hydrolysis for both bound and free enzymes, but also maintains the high rate of hydrolysis for a longer period of time.

Figure 2-3 indicates that although co-immobilized enzyme activity remains constant after repeated use (5 times) while under moderate temperatures (23⁰C), it undergoes gradual yet constant loss of activity when functioning at higher temperatures (55⁰C). No detectable activity was eluted into the substrate following successive cycles.

Optimum pH

Figure 2-4 shows that co-immobilized amyloglucosidase/ pullulanase maintains at least 80% of $V_{\max}$ activity over a broad pH range (pH 3.0-6.5), whereas the free enzyme system maintains at least 90% of $V_{\max}$ activity over a slightly narrower range (pH 3.0-5.0). The slight optimum at pH 4.0 is conserved upon immobilization.

Effects of Disrupters on Amyloglucosidase/Pullulanase Activity

Table 2-4 compares the activity and stability of the free and immobilized enzymes in the presence of disrupting agents. Both enzyme systems showed similar activities with 1 M KCl and pH 9.5 buffer. Although the immobilized amyloglucosidase/pullulanase system showed significant activity loss in the presence of 0.2 and 0.5 M KCl compared to the free, it maintained maximum activity in the presence of pH 3.0 buffer. Both free and immobilized enzymes suffered no activity loss in the presence of TritonX-100 or ethanol. The free enzyme actually showed activity enhancement in the presence of TritonX-100 and 0.5 M KCl. Relatively little (0.5-3%) activity was

Figure 2-3.

Amyloglucosidase and pullulanase were co-immobilized under standard conditions. After each 30 minute reaction period, bone was thoroughly washed with buffer, and then fresh substrate was added. Spent substrate was left to incubate for an additional 30 minutes for the determination of eluted activity. This cycle was repeated 5 times.

Figure 2-4.

EFFECT OF pH ON THE ACTIVITY OF FREE AND CO-IMMOBILIZED AMYLOGLUCOSIDASE AND PULLULANASE

detectable in the washes after disrupter pretreatments, indicating that enzyme binding is strong.

Substrate Comparison

Table 2-5 compares amyloglucosidase activity alone with the combined activities of amyloglucosidase and pullulanase. Overall, the absolute rates are higher for glycogen than the starches. The presence of pullulanase in the free coupled enzyme system results in greater hydrolysis of starch than glycogen. A 1.5, 2.5 and 5 fold increase in free activity occurred over that for amyloglucosidase alone, for glycogen, potato and Lintner starch, respectively.

Immobilized amyloglucosidase and pullulanase shows greater activity against glycogen than the starches. A 2 fold increase in activity resulted over that for amyloglucosidase alone, using glycogen as substrate, while no significant increase in activity resulted with potato and Lintner starches.

Table 2-6 shows amyloglucosidase/pullulanase activities with various substrates. Although the free system showed no significant increase in activity with crude over dialysed glycogen, the co-immobilized system showed a 2 fold increase. Furthermore, the approximately 55% of coupled enzyme activity that hydrolyses the insoluble portions of Lintner starch increased to 75% upon immobilization. The approximately 50% of activity that degrades the insoluble portions of potato starch is maintained upon immobilization.

Storage Stability

After a period of 5 weeks, co-immobilized amyloglucosidase/pullulanase maintained approximately 75% activity. This corresponds to a half life of approximately 70 days.

Table 2-4
Effect of Disrupting Agents on Free and Co-immobilized
Amyloglucosidase and Pullulanase Activity

Treatment	Activity (nmoles glucose produced/ml/min)		
	Free	Immobilized	Wash
Control	135 ± 11 (100%)	27 ± 3 (100%)	3 ± 0.3 (2%)
TritonX(1%)	200 ± 15 (148%)	30 ± 4 (111%)	4 ± 0.3 (2%)
EtOH (30%)	150 ± 20 (111%)	27 ± 2 (99%)	2 ± 0.2 (1%)
KCl 1 M	80 ± 9 (60%)	16 ± 1 (59%)	2 ± 0.3 (3%)
0. 5M	176 ± 9 (130%)	12 ± 1 (43%)	4 ± 0.3 (2%)
0. 2M	19 ± 15 (88%)	11 ± 1 (42%)	2 ± 0.3 (2%)
pH 3.0	97 ± 7 (72%)	27 ± 3 (98%)	1 ± 0.04 (1%)
pH 9.5	31 ± 4 (23%)	6 ± 0.5 (27%)	0.3 ± 0.03 (1%)

Data are means ± 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 the given conditions. Reactions were conducted at 23°C for 30 minutes.

Table 2-5
Effect of Free and Co-immobilized
Amyloglucosidase/Pullulanase on the Hydrolysis of
Glycogen, Lintner and Potato Starches

Substrate	Activity (nmoles glucose/ml/min)	
	Free	Immobilized
Glycogen:		
Amyloglucosidase	140 ± 14 (66%)	24.6 ± 0.9 (48%)
Amyloglucosidase/ Pullulanase	210 ± 13 (100%)	51.2 ± 4.7 (100%)
Potato Starch:		
Amyloglucosidase	45 ± 5 (40%)	14.4 ± 1.2 (82%)
Amyloglucosidase/ Pullulanase	142 ± 18 (100%)	17.6 ± 1.8 (100%)
Lintner Starch:		
Amyloglucosidase	33 ± 2 (20%)	8.5 ± 0.8 (83%)
Amyloglucosidase/ Pullulanase	168 ± 7 (100%)	10.3 ± 1.1 (100%)

Data are means ± SEM, n=3. Relative activities are given in brackets. Control values were set to 100% for comparison.

Table 2-6
Comparison of Glycogen, Lintner and Potato Starches
as Substrates for Free and Co-immobilized
Amyloglucosidase/Pullulanase

Substrate	Activity (nmoles glucose produced/ml/min)	
	Free	Immobilized
Glycogen:		
Dialysed	181 + 1 (100%)	42.6 + 5.1 (100%)
Crude	202 + 11 (112%)	88.2 + 5.5 (207%)
Lintner Starch:		
Whole	243 + 18 (100%)	19.3 + 1.8 (100%)
Soluble	110 + 3 (45%)	4.9 + 0.2 (25%)
Potato Starch:		
Whole	227 + 8 (100%)	20.9 + 2.2 (100%)
Soluble	111 + 3 (49%)	10.9 + 1.3 (52%)

Data are means + SEM, n=3. The relative activities are given in brackets. Control values were set to 100%.

DISCUSSION

Pullulan (poly- α -1,6-maltotriose), although not degraded by amylases, is the ideal substrate for the establishment of the activity of pullulanase (Solomon, 1985). However, studies focusing on immobilized pullulanase alone were not done, since the goal of these experiments was to improve the efficiency of glycogen and starch hydrolysis and not just the hydrolysis of α -1,6 glycosidic bonds in a "nonamylaceous" glucan environment (Lee, 1971). Furthermore, with respect to the known similarities in pH and temperature optimum between amyloglucosidase (pH 4.0-4.5, 55-70°C, Chapter 1) and pullulanase (pH 4.5-4.7, 60°C, Novo Industries product information), great deviations in the parameters for immobilized pullulanase itself were not expected.

These similarities are what made the joint use of amyloglucosidase and pullulanase a very promising approach to the production of high glucose syrups. Other practical uses for the coupled enzyme system include the structural elucidation of poly and oligosaccharides. This could be of importance in the diagnosis of the 2 types of glycogen storage diseases III and IV, that lead to abnormal glycogen structure (Martensson *et al.*, 1972).

While a few reports exist of the immobilization of pullulanase alone (Martensson *et al.*, 1972; Ohba *et al.*, 1978), only two reports on the co-immobilization of amyloglucosidase and pullulanase were found (Chakrabarti and Storey, 1990b; Ram *et al.*, 1982).

Co-immobilization of amyloglucosidase and pullulanase onto BioboneTM resulted in $21.3 \pm 0.2\%$ of activity bound, with optimum binding occurring in incubations with 100 mM acetate buffer, pH 4-4.5 (Table 2-1 and 2-2). Enough pullulanase was used to ensure that the hydrolysis reaction was

not limited by pullulanase action (0.7 U/10 U amyloglucosidase/ pullulanase) (Figure 2-1). More was not added so as not to overload the bone and therefore cause limitations to bound activity that can result from overcrowding (Caldwell, 1976). Indeed, the 31% lost activity, a 10% increase from the amount of activity lost upon amyloglucosidase immobilization (Chapter 1), may be due to enzyme overload onto bone. The 2.5 fold drop in specific activity upon immobilization further implies a less efficient system (Table 2-2).

However, the ability of pullulanase to act on its substrates, particularly glycogen branch points was evident by the rate increases for both free and immobilized enzymes compared to amyloglucosidase alone (Table 2-5). The debranched products of pullulanase are more accessible to amyloglucosidase which has higher reaction rates on linear glucose polymers (Ram *et al.*, 1982). Also, the close proximity of the two enzymes on a single matrix would tend to increase stability as well as favour higher reaction rates (Ram *et al.*, 1982; Martensson *et al.*, 1974). The results from Table 2-5 suggested that free coupled enzyme activity showed a greater affinity for the starches than glycogen. However, upon immobilization, glycogen hydrolysis was favoured.

It was Lee (1971) who deduced that some of the tertiary features of polysaccharides (ie. the length of inner and outer chains, and whether $\alpha(1,6)$ links constitute a branch point) could effect the degradation ability of pullulanase. It is also an absolute requirement for $\alpha(1-4)$ links to be present in the α -D-glucan chains, with pullulanase acting most readily when the $\alpha(1-6)$ links are separated by at least 2 $\alpha(1-4)$ linked glucose units (Kennedy, 1985). Glycogen consists of chains so closely packed that it may limit access by pullulanase. In contrast, the lower density of branch points and therefore

chain ends in the amylopectin portions of starch implies that the gaps between chains are wider than in glycogen. The debranching enzyme is therefore more easily able to approach these branched linkages (Lee, 1971).

Also, enzymes from different microbial sources differ in their rates of hydrolysis of the substrates and in the composition of the degradation products (Solomon, 1985). For example, pullulanase from Anterobacter aerogenes are able to debranch parent polymers, whereas pullulanase from Bacillus cereus var. mycoides, shows a higher affinity towards the shorter branches in α -limit dextrin, and a smaller affinity towards the longer parent branches (Solomon, 1985).

The considerable drop in immobilized pullulanase activity against the starches compared with glycogen (Table 2-5), is most likely due to diffusion limitation of the largely insoluble substrate, as was seen with immobilized amyloglucosidase (Chapter 1).

The increased ability of the co-enzyme system to degrade crude glycogen, particularly when immobilized, is likely due to the presence of lower molecular weight molecules with either fewer or more easily accessible branch points (Table 2-6). Insoluble starch degradation, although likely diffusion limited, was maintained upon co-immobilization (Table 2-6).

Immobilization of amyloglucosidase and pullulanase appeared to have had very little effect on pH and temperature properties of the enzyme reactions. The fairly broad pH optimum as well as the high temperature optimum was maintained upon immobilization (Figure 2-4, Table 2-4).

Long term glycogen degradation remained steady over 24 hours at 23°C, for both free and immobilized amyloglucosidase and pullulanase. The enhanced hydrolysing activity imparted by pullulanase was readily apparent, particularly with the immobilized system after 4-8 hours of hydrolysis

(Figure 2a). Ram *et al.* (1982) who immobilized amyloglucosidase and pullulanase onto collagen membranes via azide linkages, also demonstrated that pullulanase was especially effective during long periods of hydrolysis.

Although the co-immobilized system maintained long term stability (75% after 5 weeks) at moderate temperatures (23°C), little improvement in high temperature stability was seen compared to the free system. Free amyloglucosidase/ pullulanase showed faster rates of glycogen hydrolysis (ie. steeper slope), than when immobilized (Figure 2b). After only 5 cycles of operation, the co-immobilized system showed a 24% loss of activity at 55°C, but remained 100% active at 23°C (Figure 2-3). Since no detectable activity was eluted into the substrate following subsequent cycles, the high temperature activity loss was not due to elution of bound enzyme. Both Ram *et al.* (1982) and Ohba *et al.* (1978) encountered similar temperature instabilities following immobilization of pullulanase onto collagen membranes and TEAE-cellulose, respectively.

The co-immobilized amyloglucosidase and pullulanase showed improved enzyme stability at low pH (Table 2-4). Both free and immobilized enzyme systems showed comparable stabilities in the face of several disrupting agents. Only when treated with 0.2 and 0.5 M KCl did the co-immobilized enzymes prove less stable. These activity losses due to high temperature and denaturant treatment are likely due to the presence of pullulanase, since amyloglucosidase remained stable under similar conditions (Chapter 1).

Pullulanase did substantially improve the efficiency of starch and glycogen conversion into glucose. Higher substrate conversion is important when one considers the addition of glucose isomerase to the immobilized

enzyme complex as the next progressive step in starch processing. Such an enzyme system would allow for the complete conversion of starch or glycogen to fructose (an important sweetening agent). The enriched glucose production afforded by co-immobilized amyloglucosidase and pullulanase activity leads to higher conversion efficiency and reduced reaction time for the isomerization step. The fact that the coupled enzyme system maintains 80% of activity at pH 6.5 and has a high temperature optimum, further supports the feasibility of this next step.

CHAPTER 3
STUDIES OF FREE AND IMMOBILIZED
GLUCOSE ISOMERASE

INTRODUCTION

Glucose isomerase or xylose isomerase (EC 5.3.1.5), catalyses the reversible isomerization of xylose to xylulose (Bucke *et al.*, 1983). However, its commercial importance lies in its ability to catalyse the aldose-ketose interconversion of glucose - fructose for the production of high fructose (corn) syrup (abbreviated HFCS or HFS) (Antrim *et al.*, 1985). Glucose isomerase is an intracellular enzyme produced by organisms from a range of genera, principally *Streptomyces*, *Bacillus* and *Arthrobacter* (Bucke *et al.*, 1983). Catalytic action requires the transfer of 2 hydrogen atoms to interconvert the aldose and ketose, either through an ene-diol intermediate, or hydride ion transfer (Collyer and Blow, 1990). Furthermore, the enzyme requires 1.5 mM Mg^{2+} for the full expression of catalytic activity as well as 2 mM bisulfite to maintain stability (Antrim *et al.*, 1985).

Before 1970, the principal source of food sweeteners was sugar cane and sugar beets (Verhoff *et al.*, 1985). However, with 1.7 times more sweetening ability, fructose was considered a good alternative food sweetener (Chen, 1980). Unfortunately, the cost of conversion of glucose to fructose was huge due to the expense of using fresh glucose isomerase for each step (Verhoff *et al.*, 1985). Alkaline isomerization was possible, but produced excessive colour and side products (Cheetham, 1985).

Enzymatic fructose production was made economically feasible by the development of immobilized glucose isomerases, enabling the enzyme to be re-utilized and therefore able to convert much more glucose per unit of enzyme activity (Verhoff *et al.*, 1985). The literature now contains numerous methods for glucose isomerase immobilization, many of which are now commercially available (Chen, 1980; Verhoff *et al.*, 1985). Glucose isomerase

is now widely used in the commercial production of high fructose corn syrups, with an estimated 50 tons of pure glucose isomerase produced annually (Al-Tai *et al.*, 1987).

Corn is the major source of glucose in the United States (Verhoff *et al.*, 1985). Glucose syrups, produced from corn starch which has been treated with soluble amylases, is further treated with glucose isomerase to produce syrup with a significant fructose content (42%)(Verhoff *et al.*, 1985).

Since the interconversion of glucose to fructose is limited to approximately equal concentrations of the 2 sugars at equilibrium, carrying out the conversion to completion is rarely economical (Verhoff *et al.*, 1985). However, iso-syrups containing 55, 60 and 90% fructose are being manufactured from corn syrup, but at much lower quantities than the 42% fructose products (McAllister, 1980). With production levels reaching several million tonnes annually, HFCS has been replacing sucrose as a food sweetener in the soft drink and candy industries (Bucke, 1983).

The immobilization of glucose isomerase onto BioboneTM is presented. Studies to determine temperature, pH and kinetic parameters, as well as long term stability and use, were performed in an effort to determine the effects of immobilization on the enzyme. The feasibility of using immobilized glucose isomerase in combination with immobilized amyloglucosidase and pullulanase for the production of high fructose syrups is also discussed.

MATERIALS AND METHODS

Materials

Glucose isomerase from Streptomyces rubiginosus was donated by Finnsugar Chemical Co. The enzyme was passed through a G-25 Sephadex

column equilibrated with 50 mM phosphate buffer pH 8.0, before use. One unit of activity is defined as the amount of enzyme that will convert glucose to fructose at an initial rate of one micromole per minute under specified conditions, which include 2.0 mol/L glucose and a pH of 7.0 at 60°C (Finnsugar Group product information).

Glucose Isomerase Experiments

The experimental protocol for analyzing free and immobilized glucose isomerase was followed exactly as that found for amyloglucosidase (Chapter 1), except for the following changes:

Immobilization of Glucose Isomerase

The standard amount of glucose isomerase used for all experiments, unless stated otherwise was 9.3 units. Phosphate buffer (pH 9.0, 50 mM) was used to immobilize the enzyme. Agitation was for 1 hour at 23°C.

Glucose Isomerase Assay

The standard assay conditions, unless stated otherwise, consisted of a 30 minute reaction time at high temperature (55-60°C), using 50 mM phosphate buffer pH 8.0, containing 2.0 mM bisulfite and 1.5 mM Mg²⁺. Fructose (3 M), was used as the substrate. To stop the reaction, 0.1 ml of the reaction mixture was mixed with 0.1 ml of 10 mM HCl. Direct measurement of glucose isomerase activity was achieved by measuring the amount of glucose produced. Glucose liberated was estimated using the glucose diagnostic kit as outlined previously (Chapter 1). For the sake of convenience in handling the large number of analyses involved in enzyme activity

measurements, the reaction involving fructose as substrate was followed, in order that an easily quantifiable product, glucose, was produced.

Determination of Optimum Binding pH

For the determination of optimum binding pH, phosphate buffer 50 mM, containing 1.5 mM Mg^{2+} and 2 mM bisulfite was used, with pH adjusted to 5.5, 6.0, 7.0, 8.0 and 9.0.

Optimum pH

Optimum reaction pH was determined using acetate (50 mM, pH 4.0, 5.0), phosphate (50 mM, 6.0, 7.0, 8.0, 9.0) and Tris-HCl (50 mM, pH9.0) buffers. All included 1.5 mM Mg^{2+} and 2 mM bisulfite

Kinetic Parameters

Glucose production after 10 minutes was determined for reactions using 0.06-3 M of fructose substrate. Kinetic parameters in the presence of 10% polyethylene glycol (PEG) were also determined.

Effect of Potential Denaturing Agents

For the studies to determine denaturant effects, all denaturants were made up in phosphate buffer, pH 8.0 containing 1.5 mM Mg^{2+} and 2 M bisulfite.

Methods Employed for Mass Transfer Studies

Glucose production after 10 minutes was determined for reactions using 0.06-3 M fructose, comparing mixed and non-mixed tubes.

The temperature profile values for free and immobilized glucose isomerase were fit into an Arrhenius equation and the resulting slopes were compared.

Kinetic data for fructose using 9 and 18 units of enzyme were plotted on Lineweaver-Burk plots. The resulting slopes were compared.

The activities of immobilized glucose isomerase were compared on whole bone and bone powdered under liquid nitrogen.

RESULTS

Immobilization of Glucose Isomerase onto Biobone™

Tables 3-1 and 3-2 indicate that when under optimum pH conditions (pH 9.0), the average amount of glucose isomerase activity immobilized was $31.8 \pm 0.8\%$, with approximately $50.6 \pm 0.6\%$ being eluted into the washes. The $17.6 \pm 1.4\%$ of lost activity could be attributed to enzyme deactivation upon immobilization. The specific activity of free and immobilized glucose isomerase (with the amount of protein estimated by the Biorad protein assay) was 11.1 ± 0.8 and 3.86 ± 0.53 U/mg protein, respectively. Figure 3-1 shows that very large amounts of enzyme (in excess of 60 units), can be immobilized onto 100mg bone.

Optimum Temperature and Temperature Stability Studies

Table 3-3 shows that both free and immobilized glucose isomerase were active at high temperatures only. Thus all experiments were run at 55-60°C or greater.

Figure 3-2 shows the time course of glucose production at 60°C, for free and immobilized glucose isomerase. The rate of glucose production was

Table 3-1
Effect of Incubation pH During Binding on the Amount of
Glucose Isomerase Immobilized onto Bone

Activity (nmoles glucose produced/ml/min)	
Acetate Buffer (pH)	
5.5	27.8 $\pm$ 2.3
6.0	27.6 $\pm$ 0.9
7.0	39.2 $\pm$ 4.0
8.0	41.2 $\pm$ 1.4
9.0	50.2 $\pm$ 1.3

Data are means $\pm$ SEM, n=3. After binding, bone was washed 4 times and then activity was measured in reactions with fructose at pH 8.0 and 60°C for 30 minutes.

Table 3-2
Amount of Glucose Isomerase Activity Retained
During Bone Immobilization Following Ten Washes

	Activity, %	Specific Activity U/mg Protein
Free	100 $\pm$ 3	11.1 $\pm$ 0.7
Immobilized	31.8 $\pm$ 0.8	3.86 $\pm$ 0.53
Wash	50.6 $\pm$ 0.6	—
Lost	17.6 $\pm$ 1.4	—

Data are means $\pm$ SEM, n=3. Activities are expressed relative to an equal amount of the free enzyme (100%). The amount of protein in free enzyme and bound to 100 mg bone was determined by the BioRad protein assay (BioRad Inc.). Units are expressed as nmole glucose/ml/min.

Figure 3-1.

EFFECT OF THE ADDITION OF INCREASING CONCENTRATIONS
OF GLUCOSE ISOMERASE ON THE AMOUNT OF
ACTIVITY IMMOBILIZED

Increasing concentrations of glucose isomerase were added to bone, and were assayed for immobilized activity following a 30 minute binding period.

Table 3-3
Effect of Temperature on the Activity of Free
and Immobilized Glucose Isomerase

Temperature °C	Activity (nmoles glucose produced/ml/min)	
	Free	Immobilized
23	0	0
40	14.6 ± 3 (4%)	4.2 ± 1.1 (6%)
60	236 ± 21 (73%)	48.0 ± 5.2 (76%)
72	322 ± 16 (100%)	63.1 ± 3.6 (100%)
90	333 ± 13 (103%)	57.3 ± 5.2 (90%)

Data are means ± SEM, n=3. The values in brackets are relative activities, compared to 72°C.

much higher for free versus bound but much of this difference is due to the lower amount of activity bound to bone.

Figure 3-3 shows the effects of continuous use of immobilized glucose isomerase on the amount of activity retained on the bone. Activity remained fairly constant with reuse, following an initial 15-20% activity loss after the first cycle. This appears to correlate with the approximately 21% activity present in the spent substrate following the first 3 cycles.

Optimum pH

Figure 3-4 shows that both free and immobilized glucose isomerase show minimal activity below pH 6, but show optimum activity at pH 8-9. The free enzyme maintains maximal activity over a slightly broader pH range (pH 7-9).

Effects of Disrupters on Glucose Isomerase Activity

The results from Table 3-4 indicate that the effects of the disrupters on both free and immobilized glucose isomerase were minor. Both showed no significant differences in activity from the controls in the presence of varying salt concentrations, and slightly reduced activity in the presence of Triton X-100. However, unlike bound glucose isomerase, the free enzyme showed enhanced activity in the presence of ethanol. The relatively constant amount of activity present in the disrupter and control washes (~8%) implies that the disrupters had minimal effect on enzyme binding.

Kinetic Parameters

Table 3-5 outlines the kinetic properties of free and immobilized glucose isomerase. The K_m value for fructose increased by 3 fold upon

Figure 3-2.

TIME COURSE OF GLUCOSE PRODUCTION BY FREE
AND IMMOBILIZED GLUCOSE ISOMERASE AT 60°C

2

of/de

2

MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS
STANDARD REFERENCE MATERIAL 1010a
(ANSI and ISO TEST CHART No. 2)

Figure 3-3.

EFFECT OF CONTINUOUS USE ON IMMOBILIZED
GLUCOSE ISOMERASE ACTIVITY

Glucose isomerase was immobilized under standard conditions. After each 30 minute reaction period, bone was thoroughly washed with buffer, and then fresh substrate was added. Spent substrate was left to incubate for an additional 30 minutes for the determination of eluted activity. This cycle was repeated 5 times.

Figure 3-4.

EFFECT OF pH ON FREE AND IMMOBILIZED
GLUCOSE ISOMERASE ACTIVITY

Table 3-4
Effect of Disrupting Agents on Free and Immobilized
Glucose Isomerase Activity

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

Data are means + 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 the given conditions. Reactions were conducted at 60°C for 30 minutes.

immobilization, whereas the $V_{\max}$ value showed a 5 fold decrease. The drop 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 (Atha and Ingham 1980). The 1.5 fold increase in K_m of the free enzyme in the presence of 10% PEG indicates a certain degree of enzyme constraint, suggesting similar enzyme restriction when in the immobilized state.

Mass Transfer Limitation Studies

Mixing the tube containing the enzyme-substrate system had essentially no effect on the K_m or $V_{\max}$ values for either free or immobilized glucose isomerase (Table 3-5). Therefore, film diffusion effects were minimal for this enzyme.

If internal mass transfer limitations exist due to substrate diffusion, one would notice deviations in the Arrhenius relationship (relating temperature and reaction velocity) at high temperatures (Kennedy, 1985). Arrhenius plots for free and immobilized glucose isomerase were similar over the temperature range tested, indicating no diffusional limitation (data not shown).

Lineweaver-Burk plots using 9 and 18 units of enzyme show a decrease in slope with increased enzyme loading onto bone, indicating an absence of diffusional limitation. Since results were similar to those obtained for amyloglucosidase (Chapter 1), data are not shown.

Table 3-6 illustrates the effects of decreasing matrix particle size on immobilized glucose isomerase activity. The resulting increase in surface

Table 3-5
Kinetic Parameters for Free and Immobilized
Glucose Isomerase Using Fructose as Substrate

Treatment	Km (mg/ml)		Vmax ^a	
Mixed:				
Immobilized	1.02	+ 0.12	52	+ 1
Free	0.367	+ 0.029	251	+ 9
Free + 10% PEG	0.560	+ 0.067	261	+ 8
Not Mixed:				
Immobilized	1.16	+ 0.11	58	+ 4
Free	0.399	+ 0.048	256	+ 22

Data are means + SEM, n=3. Reactions were carried out at 60°C using 3M stock solution of fructose.

^a nmole glucose/ml/min

^b nmole glucose/min/mg

Table 3-6
Effects of Increasing Surface Area on the Apparent
Activity of Immobilized Glucose Isomerase

	Activity (nmoles glucose/min/ml)
Control bone	42.8 + 6.7 (100%)
Powdered bone	47.4 + 4.2 (110%)

Data are means + SEM, n=3. Relative activities are given in brackets. Control values were set to 100% for comparison. 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 N₂; the powdered enzyme was then thawed.

area after the bone was powdered had little effect on isomerase activity, providing further evidence that diffusion limitation is not a problem.

Storage Stability

After 5 weeks of storage, approximately 70% of the initial enzyme activity was lost, with 50% of activity being lost after one week. Thus, operational half life was approximately 7 days.

DISCUSSION

The enzymatic conversion of glucose to fructose by immobilized glucose isomerase on a large industrial scale has been the first successful application of immobilized enzyme biotechnology (Chen, 1980). Although many methods have been reported for the immobilization of glucose isomerase, only a few have the properties necessary for the commercial production of HFCS; in particular, operational stability at high temperature (Verhoff *et al.*, 1985). Thus, there is always enthusiasm for new methods of immobilization which may result in a more stable glucose isomerase. Even a modest increase in half life of the enzyme will substantially decrease the cost of the HFCS product (Volkin and Klibanov, 1988).

The immobilization of glucose isomerase was successful, achieving $31.8 \pm 0.8\%$ of activity bound to Biobone™ at high alkaline pH. However, the amount of bound activity was lower than values reported elsewhere. Chakrabarti (1988) obtained 50% bound activity following glucose isomerase immobilization onto polyurethane foam. Stanley *et al.* (1976) retained 40% of the activity onto a chitin-glutaraldehyde system, and Lee *et al.* (1976) bound 56% of activity onto porous glass.

Both free and immobilized glucose isomerase displayed high pH optima (pH 8-9), with good operating activities still maintained between pH 6 and 7. Such results were encouraging, for although co-immobilized amyloglucosidase and pullulanase show a low pH optima, roughly 80% of $V_{\max}$ activity is still retained at pH 7. Thus, the potential of co-immobilizing all 3 enzymes in a one step system to produce HFCS from starch does show promise.

The glucose isomerase used (isolated from *S. rubiginosus*), is a thermophilic enzyme, with both free and immobilized glucose isomerase maintaining good operational stability over the 2 hour period at high temperature (Figure 3-2). Also, the immobilized enzyme maintained stability and activity following 5 cycles of continuous reuse (Figure 3-3). Although high operating temperatures were required for maximum activity (Table 3-3), standard reactions were run at 55-60°C. Although most glucose isomerases exhibit high temperature optimums, industrial bioreactors carry the reaction out at 55-57°C, as the enzyme is subject to thermal inactivation with long term use at high temperatures (Volkin and Klibanov, 1988). Indeed, one of the primary causes of enzyme activity loss is thermal inactivation (Antrim, 1985). Lower reaction temperatures may also be more beneficial when combining glucose isomerase with amyloglucosidase and pullulanase, which show comparatively less stability at high temperature (Chapter 2).

With the exception of ethanol, free and immobilized glucose isomerase showed similar responses to the effects of the disrupters. The disrupters had little effect on enzyme binding, for the amount of enzyme eluted into the washes was the same as that for the controls (~10%). Because much more activity was eluted into the washes than for amyloglucosidase under similar

conditions (~3%, Chapter 1), the glucose isomerase binding onto bone appears to be less firm.

Similarly, the cycling experiment showed a decrease in immobilized activity with continued reuse. This was largely due to the excessive amount of activity (~20%) eluted into the spent substrate. Long term immobilized enzyme storage showed an activity loss of 50% after the first week, with an additional 20% of activity lost over the next 4 weeks.

After obtaining similar results, Chakrabarti (1988) suggested that 2 subsets of immobilized enzymes could be bound to the bone: a labile group which is more susceptible to denaturation and release, and a more tightly immobilized group which may prove more resilient to long term storage and reuse. Calcium, which is present in high levels in bone, is also a glucose isomerase inhibitor (Findlay *et al.*, 1986; Antrim *et al.*, 1985). Thus its presence may also contribute to long term glucose isomerase deactivation.

Although long term stability with storage is poorer for glucose isomerase than the other enzymes in this study, other studies with glucose isomerase showed similar results. Lee *et al.* (1976) maintained a half life of 15 days, and Chakrabarti (1988) had a half life of only 150 hours.

Glucose isomerase is a metalloenzyme, requiring both cobalt (Co^{2+}) and magnesium (Mg^{2+}) for maximal activity (Verhoff *et al.*, 1985). These two cofactors function in a synergistic manner, their binding at separate sites creating conformational changes in the active site required for glucose isomerization (Jensen and Rugh, 1985; Bucke, 1983). However, since cobalt is a toxic metal it must be removed following isomerization (Jensen and Rugh 1985; Verhoff *et al.*, 1985). More important, the mere presence of cobalt in a process designed to create a food product would probably make government approval and therefore eventual industrial use unlikely (Chakrabarti, 1988).

Thus, since adequate isomerase activity was observed with Mg^{2+} as the sole metal cofactor, cobalt was not added.

Mass transfer limitations for immobilized glucose isomerase were virtually non-existent. Both substrate and product are small molecules (mw=180.2), and therefore should not be subjected to great diffusional limitations upon immobilization, compared to larger molecular weight substrates such as starch and glycogen ($\sim 1 \times 10^8$) (Banks, 1975). Indeed, mixing the reaction tubes had minimal effect on the kinetic values for both free and immobilized glucose isomerase (Table 3-5). This implies that the need for mixing to reduce the formation of both substrate and product concentration gradients at the bone surface was not necessary.

Results from Arrhenius and Lineweaver-Burk plots for free and immobilized glucose isomerase also indicated no diffusion limitation. Furthermore, the bone powdering experiment, designed to increase surface area and therefore alleviate any problems of substrate approach and product escape, showed that none existed (Table 3-6).

The decrease in V_{max} observed for fructose as a substrate corresponded to the amount of enzyme immobilized. However, the 3 fold increase in K_m upon immobilization indicated that the enzyme had become a much less effective catalyst. Increases in K_m values often occurs upon immobilization. Chakrabarti (1988), showed a 1.6 fold increase in K_m following isomerase immobilization. Such increases are often due to immobilization in an unfavourable configuration, or due to steric hindrance from overcrowding of the immobilized enzyme (Kennedy, 1985). Indeed, the 1.5 fold increase in K_m for free glucose isomerase in the presence of 10% PEG, may indicate enzyme activity restriction due to overcrowding. The 2.4 fold decrease in the specific activity upon immobilization also implies a drop

in catalytic activity due to immobilized enzyme deactivation or overcrowding on the bone surface.

It is difficult to say whether overcrowding was a problem, for the amount of enzyme used in the standard experiments (9 U), represents a fraction of what could be usefully immobilized (Figure 3-1).

Since calcium is a naturally occurring component in starch syrup and in bone (Al-Tai, 1987; Findlay *et al.*, 1986), the immobilization of glucose isomerase onto bone suffers an additional setback. No experiments were designed to specifically address the inhibitory effect of Ca^{2+} in bone, if such a problem does exist. Increasing the Mg^{2+} concentration would be a simple solution, for magnesium can prevent the inhibitory effect of calcium if present in 10-20 fold surplus (Al-Tai, 1987).

Despite these stability and elution problems, glucose isomerase bound to bone still has practical application. Because it remained active at moderate pH and temperatures, successful co-immobilization of glucose isomerase with amyloglucosidase and pullulanase for the production of high fructose corn syrups from starch is feasible.

CHAPTER 4
STUDIES OF FREE AND CO-IMMOBILIZED
AMYLOGLucosidase, Pullulanase and
Glucose Isomerase

INTRODUCTION

Presently, the process of starch hydrolysis and isomerization of the resulting glucose syrup is conducted in 2 separate stages using very different reaction conditions (particularly pH values). The preliminary step which involves the saccharification of starch to glucose syrup using amylases, is conducted in the range pH 4-5, followed by isomerization performed at pH 7-9 (Verhoff *et al.*, 1985). A one step conversion of starch to a glucose-fructose syrup accomplished at a single pH would be ideal.

Presented here is the co-immobilization of amyloglucosidase, pullulanase and glucose isomerase onto Biobone™. Emphasis was placed on determining specific temperature and pH values for obtaining optimal glucose and fructose production.

MATERIALS AND METHODS

The experimental protocol for free and co-immobilized amyloglucosidase, pullulanase and glucose isomerase was followed exactly as that found for amyloglucosidase (Chapter 1), except for the following changes:

Enzyme Co-immobilization

The standard amount of amyloglucosidase, pullulanase and glucose isomerase used in all experiments was 0.7, 10 and 22.3 units, respectively. Acetate buffer (pH 4.5, 100 mM) was added to pre-washed bone, along with the standard amounts of amyloglucosidase and pullulanase. Following mixing for 30 minutes, excess buffer was poured off and phosphate buffer (pH 9, 50 mM) was added along with the standard amount of glucose isomerase.

Following agitation for 30 minutes, excess buffer was poured off, followed by successive washes with pH 6.5 phosphate buffer (4 x 1 ml).

Amyloglucosidase, Pullulanase and Glucose Isomerase Assay

The standard experimental conditions, unless stated otherwise, consisted of a 60 minute reaction time at high temperature (55°C), using phosphate buffer (pH 6.5, 50 mM), containing 2 mM bisulfite and 1.5 mM Mg^{2+} . Glycogen (16% w/v) was used as the substrate. To stop amyloglucosidase and pullulanase activity, 0.1 ml of the reaction mixture was mixed with 0.1 ml 0.5N NaOH.

The glucose concentrations of these samples were assayed enzymatically using the hexokinase/glucose-6-phosphate dehydrogenase coupled enzyme assay described by Lowry and Passonneau (1972). This assay involves measuring the reduction of NAD^+ at 340 nm. The reactions involved are as follows:

Hexokinase (0.28 U) was added to a cuvette containing a 1 ml mixture of buffer (100 mM Tris, pH 8.0), NAD^+ (0.50 mM), $MgSO_4$ (5.0 mM), ATP (0.50 mM), glucose-6-phosphate dehydrogenase (G6PDH, 0.10 U) and the test sample 0.1 ml (Lowry and Passonneau, 1972).

Blanks (containing no added sample) and glucose standards were run with each set of samples. An initial O.D. reading was taken before hexokinase addition. A final O.D. reading was taken after the coupled

enzyme reaction had run to completion (approximately 20 minutes). The amount of glucose produced was calculated using the equation:

$$\mu\text{moles glucose} = \frac{\Delta \text{O.D.} \times \text{volume}}{6.22}$$

6.22

where $\Delta \text{O.D.}$ = change in O.D. at 340 nm (corrected for O.D. changes of blanks).

volume = total volume of assay mixture in cuvette (1.11 ml).

6.22 = μ molar extinction coefficient of NADH at 340 nm

The concentration of glucose present in the samples could be calculated by multiplying the amount of glucose produced by the respective dilution factors. This value could then be converted to a reaction rate through division by the time period used.

Fructose concentrations of these samples were determined by the addition of phosphoglucose isomerase (PGI, 0.07 U per assay) after the hexokinase reaction ran to completion. The reaction catalyzed by phosphoglucose isomerase is as follows:

Fructose-6-phosphate is present due to the action of hexokinase on fructose. A final O.D. reading is taken after the reaction has reached completion (about 20 minutes). The amount of fructose can then be calculated in the same manner as for glucose.

Samples that had changes in absorbance values outside the linear range (about 0.8) were diluted with excess Tris buffer and re-assayed.

Optimum pH

For the determination of optimum pH, phosphate buffer (50 mM), containing 2 mM bisulfite and 1.5 mM Mg^{2+} , was adjusted to the required pH. Reactions were run at 55°C for 1 hour.

RESULTS

The distribution of the sum of the activities of co-immobilized amyloglucosidase, pullulanase and glucose isomerase (measured as the total amount of glucose and fructose produced) was $14.4 \pm 1.9\%$ bound, with $44.2 \pm 5.1\%$ being eluted into the washes (Table 4-1). Individual activities were impossible to determine due to the interconversion of glucose and fructose produced. The specific activity decreased substantially upon immobilization, from 13.4 ± 1.8 to 2.9 ± 0.6 U/mg, respectively.

Table 4-2 indicates that both bisulfite and magnesium no significant inhibitory effects on the activities of free and immobilized amyloglucosidase and pullulanase.

Figure 4-1 shows the effect of increasing concentrations of glucose isomerase on to set amounts of immobilized amyloglucosidase and pullulanase. Optimum fructose production occurred at an isomerase concentration of 22.3 units.

Figure 4-2(a,b,c) shows the effect of varying pH on the relative production of glucose and fructose. While the total hexose sugar yield decreased with increasing pH, the percentage of fructose increased for free,

Table 4-1
Amount of Amyloglucosidase, Pullulanase and
GLucose Isomerase Activity Retained
During Bone Immobilization Following Ten Washes

	Activity, %	Specific Activity U/mg Protein
Free	100 $\pm$ 2	13.4 $\pm$ 1.8
Immobilized	14.4 $\pm$ 1.9	2.9 $\pm$ 0.6
Wash	44.2 $\pm$ 5.9	—
Lost	41.4	—

Data are means $\pm$ SEM, n=3. Activities are expressed relative to an equal amount of the free enzymes (100%). The amount of protein in free enzyme and bound to 100mg bone was determined by the Biorad protein assay. Activities were measured as the total amount of glucose and fructose produced.

Table 4-2
Effect of Bisulfite and Magnesium on Free and Immobilized
Amyloglucosidase and Pullulanase Activity

	Activity (nmoles glucoseproduced/ml/min)	
	Free	Immobilized
Control	355 $\pm$ 11 (100%)	76.7 $\pm$ 9.8 (100%)
2 mM Bisulfite	368 $\pm$ 6 (103%)	84.1 $\pm$ 4.0 (110%)
1.5 mM Mg ²⁺	367 $\pm$ 23 (103%)	65.1 $\pm$ 5.8 (85%)

Data are means $\pm$ SEM, n=3. Relative activities are given in brackets. Control values (using standard conditions) were set to 100% for comparison. Reactions were run at 55°C for 30 minutes.

Figure 4-1.

EFFECT OF INCREASING CONCENTRATIONS OF GLUCOSE ISOMERASE WITH SET AMOUNTS OF AMYLOGLUCOSIDASE AND PULLULANASE, ON THE FINAL OUTPUT OF GLUCOSE AND FRUCTOSE

Increasing concentrations of glucose isomerase was added to set amounts of amyloglucosidase and pullulanase, for immobilization onto bone. Following a 30 minute binding period, bone was assayed for immobilized activity.

Figure 4-2 a,b,c.

EFFECT OF pH ON THE RELATIVE SUGAR PRODUCTION BY
FREE AND CO-IMMOBILIZED AMYLOGLUCOSIDASE,
PULLULANASE AND GLUCOSE ISOMERASE

and immobilized enzymes up to pH 7.0. The greatest fructose yield was 20% over the pH range of 6.5-7.0 for the immobilized system. However, over the same pH range, the total hexose sugar yield dropped from 80% to 12%, relative to immobilized activity at pH 5.5. Therefore, pH 6.5 was considered optimal for the immobilized system. For the free enzyme system, the maximum fructose yield was 30% over the pH range 7.5-8.0. However, since only 29-37% of the maximal output of total hexoses were being produced in this pH range, a compromise was made between the absolute amounts of hexose sugars produced and product ratios. Therefore, pH 6.5-6.8 was considered optimum, for although fructose constituted only 8-15% of the total sugar output, the total hexose sugar yield was maintained at 72-86%. With respect to a slight bias towards optimizing the immobilized enzyme system, all subsequent experiments were done in pH 6.5 buffer.

The effect of temperature on glucose and fructose production is shown in Table 4-3. The immobilized system showed a constant decrease in hexose output at temperatures higher than 55⁰C. The free enzyme system demonstrated maximal glucose and fructose production at 55⁰C. Thus, subsequent experiments were performed at 55⁰C.

The time course of glucose and fructose production by the free and immobilized enzyme systems is shown in Figure 4-3. Good rates of glucose production were maintained for both immobilized and free enzymes. Long term fructose production was very slow for both immobilized and free systems, representing a fraction of the total hexose output.

Figure 4-4 outlines the effects of increasing the reaction mixture pH from 6.0 to 7.2 on the relative amounts of glucose and fructose produced. Product ratio shifted 89:11 to 75:25 of glucose to fructose, for the immobilized

Table 4-3
Effect of Temperature on the Activity of
Free and Immobilized Amyloglucosidase, Pullulanase and
Glucose Isomerase

Temperature °C	Activity (nmoles glucose produced/ml/ min)			
	Free		Immobilized	
	Glucose	Fructose	Glucose	Fructose
45	416 + 20	42.4 + 5.5	57.8 + 6.9	5.9 + 0.1
55	488 + 25	40.9 + 1.6	66.3 + 9.7	7.1 + 0.4
60	317 + 42	46.4 + 6.0	11.0 + 1.6	3.1 + 0.1
67	214 + 9	51.9 + 4.7	11.0 + 1.2	4.7 + 0.5
75	124 + 15	19.6 + 2.0	7.1 + 0.7	1.4 + 0.2
82	134 + 6	15.7 + 1.2	7.1 + 0.9	1.3 + 0.3

Data are means + SEM, n=3. Reactions were run for 1 hour using glycogen as substrate.

Figure 4-3.

TIME COURSE OF GLUCOSE AND FRUCTOSE PRODUCTION
BY FREE AND CO-IMMOBILIZED AMYLOGLUCOSIDASE,
PULLULANASE AND GLUCOSE ISOMERASE AT 55°C

Figure 4-4.

EFFECT OF pH INCREASE ON FRUCTOSE OUTPUT DURING SACCHARIFICATION FOR FREE AND CO-IMMOBILIZED AMYLOGUCOSIDASE, PULLULANASE AND GLUCOSE ISOMERASE

Amyloglucosidase, pullulanase and glucose isomerase were co-immobilized under standard conditions. Following the addition of substrate, both free and immobilized systems were run at pH 6.0 for 45 minutes. Following this, the pH was increased to 7.2 for an additional 45 minutes.

system. For the free enzymes, the ratio changed from 98:2 to 88:12 glucose:fructose. Therefore, reactor pH could be favourably manipulated to increase fructose output.

DISCUSSION

Previous experiments establishing the pH and temperature parameters for co-immobilized amyloglucosidase and pullulanase, and for glucose isomerase alone (Chapter 2, 3) indicated that the three enzymes could be successfully co-immobilized onto bone. A further inducement was that both amyloglucosidase and pullulanase activities were not adversely affected by the presence of bisulfite or magnesium. Bisulfite (2 mM) and Mg^{2+} (1.5 mM) ensures maximum glucose isomerase stability and activity (Antrim *et al.*, 1985).

Amyloglucosidase/pullulanase and glucose isomerase showed optimum binding at different pH extremes (pH 4.5 and 9.0 respectively). Therefore, they were immobilized at their pH optimum to ensure maximum enzyme binding. Under these conditions, only $14.4 \pm 1.9\%$ of total activity was successfully immobilized. The 4.6 fold drop in specific activity, coupled with the relatively large amount of activity lost upon immobilization, indicated less than optimal binding had occurred. Immobilization likely resulted in conformations less favourable to catalysis.

The specific pH used when all 3 enzymes were co-immobilized strongly influenced the yield and ratio of hexose sugars produced. Lowering the pH increases the glucose yield by increasing the activity of amyloglucosidase and pullulanase. At the same time, fructose production decreases as a result of glucose isomerase inhibition. Therefore, pH can be altered so as to suit the

product ratio goals of HFCS producers. The neutral pH value deemed optimal for total hexose production (pH 6.5), was also ideal for the production of high quality sweeteners. The quality of syrup is improved by lowering the isomerization pH as a low pH reduces the formation of colour and by-products (Jensen and Rugh 1985).

It would have been ideal to run the reactions at temperatures higher than 55°C to enhance fructose output, particularly since glucose isomerase functions well at high temperatures (Chapter 3). However high temperature reactions appeared to be limited by the hydrolysing enzymes, particularly with the bound system (Table 4-3). Low fructose production may have also been the result of a catalytically restricted glucose isomerase, with this effect accentuated at high temperatures. Indeed, the time course of glycogen hydrolysis and glucose isomerization showed minimal fructose produced over time. Also, glucose isomerase had previously shown signs of steric hindrance when immobilized (Chapter 3). It is probable the co-immobilization enzymes suffer from steric hindrance as well.

Glucose isomerase, as with most enzymes, is susceptible to thermal deactivation. In general, at every given temperature, irreversible thermoinactivation of an enzyme results, usually by a combination of covalent and conformational processes (Ahern and Klivanov, 1987). Industrial HFCS production therefore optimizes production capacity, economy and syrup quality largely through maintaining low temperature reactions (55-57°C), despite the fact that the equilibrium shifts in favour of fructose production at higher temperatures (Jensen and Rugh 1985).

One of the key goals of the companies involved in the production of HFCS is to increase the degree of conversion of glucose to fructose without resorting to complicated separation procedures (Jensen and Rugh 1985).

Producers of HFCS concentrate fructose by chromatographically separating it from fructose (Jensen and Rugh 1985). Attempts made to increase fructose yield involved adjusting the pH to optimize glucose output, followed by pH adjustment to optimize fructose output (Table 4-4). Such pH manipulation resulted in 5.8 and 2.2 fold increase in fructose levels for free and immobilized enzymes. More importantly, maximum fructose output was increased by this method, compared to the results obtained for the earlier pH studies (Figure 4-2). Iso-syrups consisting of 42% fructose are more easily achieved by starting with as high a glucose level as economy allows (Antrim, 1985).

Isolation or creation of more thermo-stable glucose isomerases would make the HFCS production more cost effective. Isolation or creation of a glucose isomerase capable of significant operation at pH values below 7 would also be of great value (Cheetham, 1985). In addition to reducing colour formation, such an isomerase would greatly assist the development of the "one-step" starch to fructose reactor that has been presented here.

This work is not the first to outline a "one-step" reactor for use in biomass conversion. Storey and Chakrabarti (1990c), co-immobilized cellulase, β -glucosidase and glucose isomerase for the purposes of converting cellulose into fructose. However, the work presented here is more industrially relevant, due to the characteristics of Biobone™. Manufacturers of HFCS require simple, cheap, efficient and durable immobilization systems. The nature of the support material becomes important as ideally the spent enzyme may be thrown away or even included in the animal feed fractions which are inevitably produced as by-products of corn wet-milling (Burke, 1983). Biobone™ is therefore the ideal support matrix due to its food grade quality and non-existent cost.

A final advantage of the co-immobilized enzyme system outlined here is that the mixture of amyloglucosidase, pullulanase and glucose isomerase may allow for a greater degree starch and glycogen conversion to take place. This is due to a possible reduction or elimination of feedback inhibition or reversion reaction if glucose is removed.

CHAPTER 5
STUDIES OF AMYLOGLucosidase IMMOBILIZED ONTO
CHEMICALLY DERIVATIZED BIOBONE™

INTRODUCTION

Amyloglucosidase in free form is used commercially for the production of glucose from starch (Fogarty, 1983). This enzyme has been immobilized to a variety of matrix carriers in the hopes of producing a system that would be industrially suitable (Kennedy, 1985). Some immobilization procedures have resulted in activity retentions reaching values as high as 45% (Lee *et al.*, 1976). However, my previous work had shown that amyloglucosidase immobilized onto BioboneTM results in only 14% activity retention (Chapter 1).

Since chicken bone is food grade, non-toxic, abundant and inexpensive, it is an ideal support matrix for immobilization (Findlay *et al.*, 1986). Thus, it seemed worthwhile to investigate methods to improve amyloglucosidase binding to bone, thereby making its industrial application more attractive.

The techniques employed here are chemical methods of incorporating reactive groups onto the bone surface, thus making it more suitable for enzyme binding. Bone derivatization methods were introduced which would encourage enzyme immobilization through covalent attachment and ionic interactions. Temperature, pH and kinetic properties, as well as substrate comparisons were done to determine whether any improvements to enzyme binding, activity and stability were achieved.

MATERIALS AND METHODS

Preparation of Derivatized Biobone™

Carbonyl Coupling (GLUT-bone)

This procedure couples glutaraldehyde groups to the bone surface. To 100 mg bone was added 1 ml of a 1% (v/v) solution of glutaraldehyde in distilled water. The reactants were allowed to shake for 2 hours. Following this the bone was washed repeatedly with distilled water (Figure 5-1) (Weetall, 1973).

Alkylamine Coupling with Carbonyl Derivative (SG-bone)

This procedure first couples silane groups to the matrix, followed by glutaraldehyde groups. Derivatized bone was prepared as described by Weetall (1973).

To 1 g of Biobone™ was added 18 ml of distilled water plus 2 ml of 3-aminopropyltriethoxysilane. After adjusting the pH to between pH 3 and 4 with 6 N HCl, the reactants were placed in a 75°C water bath for 2 hours. Following this the bone was filtered on a Buchner funnel and wash repeatedly with distilled water. It was then dried in a 115°C oven overnight (Weetall, 1973) (Figure 5-2a).

To 1 g of silanized bone was added 25 ml of a 2.5% solution of glutaraldehyde in 0.05 M Na₂HPO₄ buffer adjusted to pH 7.0. The reactants were shaken for 2 hours. The bone was then washed repeatedly with distilled water on a Buchner funnel (Weetall, 1973) (Figure 5-2b).

Figure 5-1.

Immobilization of amyloglucosidase by glutaraldehyde derivatized bone (GLUT-bone). (Weetall, 1973).

Figure 5-2a.

Preparation of a derivatized bone by silanization (Weetall, 1973).

Figure 5-2b.

Immobilization of amyloglucosidase by silan-glutaraldehyde derivatized bone (SG-bone) (Weetall, 1973).

Alkylamine Coupling with Carbodiimide Derivative (CD-bone)

This technique, which introduces an aldehyde group onto the already silanized bone, was performed as described by Weetall (1973).

To 1 g of silanized bone was added 50 ml of 0.03 M H_3PO_4 adjusted to pH 4.0. To this was added 150 mg of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide, metho-p-toluene sulfonate. The reactants were allowed to shake overnight at 4⁰C. The bone was then filtered on a Buchner funnel and then washed repeatedly with distilled water. (Weetall, 1973) (Figure 5-2c).

Diethylaminoethyl Derivative (DEAE-bone)

DEAE modified bone was prepared by the method described by Yamazaki (1984), but with a few modifications. 20 g of bone was added to a solution 20 g of NaOH in 80 ml of H_2O (precooled), and left to soak for 30 minutes at 25⁰C. A solution of 20 g of solid 2-diethylaminoethyl chloride in 20 ml of water was then added to the soaking bone. The mixture was then incubated at 50⁰C for 2 hours. After cooling to room temperature the bone was washed with water (5 x 100 ml), followed by hot ethanol (3 x 100 ml), 0.2N HCl (2 x 50 ml), water (3 x 100 ml) and 0.5 N NaOH (3 x 50 ml) (Yamazaki, 1984)(Figure 5-3).

Amyloglucosidase Experiments

The experimental protocol for free and immobilized amyloglucosidase was followed exactly as that found in Chapter 1.

Figure 5-2c.

Immobilization of amyloglucosidase by silan-carbodiimide derivatized bone (CD-bone). An amide linked ligand is prepared with the alkylamine derivative and a carbodiimide. R'' and R' represent groups on the carbodiimide, ie., cyclohexyl groups (Weetall, 1973).

Figure 5-3.

Immobilization of amyloglucosidase by diethylaminooctyl derivatized bone (DEAE-bone) (Yamazaki, 1984).

RESULTS

Bone Derivatization

Only DEAE prepared bone resulted in less than 100% of usable bone recovered (16.9%). DEAE-bone was bleached white and was very brittle. SG-bone was red in colour and like Glut-bone was more solid and less prone to flaking than the underivatized bone.

Immobilization of Amyloglucosidase

The results of enzyme coupling to bone using the various techniques are presented in Table 5-1. Under standard immobilization conditions, the average amount of activity immobilized to the control (plain) bone was $13.6 \pm 0.04\%$, with approximately 57.8 U+ 6.6% being eluted in the washes.

SG-bone, CD-bone, DEAE-bone derivatization methods showed greater amounts of activity bound, immobilizing $18.7 \pm 1.4\%$, $22.5 \pm 1.7\%$ and $18.5 \pm 1.7\%$ of added activity, respectively. Glut-bone, immobilizing $17.7 \pm 2.2\%$, showed no significant increase. DEAE-bone and SG-bone had the lowest activity "lost" upon immobilization, as well as the highest specific activity values (60.0 ± 16.8 and 71.6 ± 18.6 U/mg respectively) (Table 5-2). In contrast, CD-bone had the highest amount of lost activity and the lowest specific activity (26.9 ± 5.4 U/mg).

Table 5-3 shows the effect of binding time among the 5 types of bone. All but GLUT-bone showed a decrease in the amount of activity bound after 16 hours. Thus, a 30 minute binding time was used in all instances. Since free amyloglucosidase also lost activity over time, the continuous shaking was believed to have a detrimental effect on enzyme activity.

Table 5-1
Amount of Amyloglucosidase Activity Retained
During Bone Immobilization Following Ten Washes

Immobilization Method	Bone	Activity, % Wash	Lost
Adsorption (control)	13.6 $\pm$ 0.4	57.8 $\pm$ 6.6	28.6
Covalent Attachment:			
Glut-bone	17.7 $\pm$ 2.2	61.2 $\pm$ 5.1	31.1
SG-bone	18.7 $\pm$ 1.4	64.8 $\pm$ 2.5	16.5
CD-bone	22.5 $\pm$ 1.7	41.6 $\pm$ 3.4	35.9
Ionic Attachment:			
DEAE-bone	18.5 $\pm$ 1.7	78.1 $\pm$ 1.1	3.4

Data are means $\pm$ SEM, n=3. Activities are expressed relative to an equal amount of the free enzyme (100%).

Table 5-2
Specific Activities of Free and Immobilized
Amyloglucosidase

	Specific Activity U/mg Protein
Free	75.3 $\pm$ 0.8
Immobilization Method:	
Adsorption (control)	43.5 $\pm$ 9.6
Glut-bone	48.1 $\pm$ 11.0
SG-bone	71.6 $\pm$ 18.6
CD-bone	26.9 $\pm$ 5.4
DEAE-bone	60.0 $\pm$ 16.8

Data are means, $\pm$ SEM, n=3. The amount of protein in free enzyme and bound to 100 mg bone was determined by the BioRad protein assay. Units are expressed as nmole/ml/min.

Table 5-3
Effect of Binding Time on the Amount of
Immobilized Amyloglucosidase Activity

	Binding Time hours	Activity nmoles/min/ml
Free	0.5	271 + 18
	2	240 + 22
	16	224 + 7
Immobilization Method: Adsorption (control)	0.5	38.7 + 0.5
	2	37.6 + 1.9
	16	31.7 + 0.4
Glut-bone	0.5	55.4 + 4.8
	2	45.0 + 5.7
	16	48.9 + 4.7
SG-bone	0.5	40.9 + 3.4
	2	33.3 + 2.2
	16	25.5 + 2.1
CD-bone	0.5	57.6 + 1.1
	2	45.8 + 2.5
	16	42.1 + 5.4
DEAE-bone	0.5	47.1 + 2.9
	2	34.6 + 2.0
	16	34.5 + 1.6

Data are + SEM, n=3.

Temperature Studies

Figure 5-4 compares the activities of free and immobilized amyloglucosidase when reactions are carried out at high temperatures. Free, GLUT-, and CD-bone immobilized enzyme maintained fairly constant activity at high temperature. However, the control, SG- and DEAE-bone linked enzyme showed some activity loss with an increase in temperature.

pH Studies

Figure 5-5 compares the effect of pH on the activities of free and immobilized amyloglucosidase. Like the control bone, SG-, CD- and GLUT-bone immobilized enzymes maintained high activities over the pH range 3.0 to 7.0, with GLUT-bone showing an optimum at a pH below 4.5. DEAE-bone behaved like the free enzyme, showing a distinct pH optimum at 4.5 and low activity at the pH extremes.

Substrate Comparison

Figure 5-6 shows amyloglucosidase activity with various substrates. Amyloglucosidase immobilized to the derivatized bones showed 30-40% greater activity against glycogen, and 10-40% better activity against potato starch, than the control bone. All the enzymes utilized Lintner starch to a similar extent. Overall, SG-bone and CD-bone were the most efficient systems.

Figure 5-4.

EFFECT OF HIGH TEMPERATURE ON THE ACTIVITIES
OF FREE AND IMMOBILIZED AMYLOGLUCOSIDASE

Figure 5-5.

EFFECT OF pH ON THE ACTIVITIES OF FREE AND IMMOBILIZED AMYLOGLUCOSIDASE

Figure 5-6a,b,c.

EFFECT OF DIFFERENT METHODS OF AMYLOGLUCOSIDASE
IMMOBILIZATION ON GLYCOGEN AND STARCH HYDROLYSIS

Kinetic Properties

Table 5-4 outlines the kinetic properties for free and immobilized amyloglucosidase. The K_m value had increased by 3 fold for the control immobilized enzyme, compared to the free. The K_m values for GLUT-, SG-, CD- and DEAE-bone showed an additional 1.9, 5.3, 2.4 and 3.4 fold increase respectively, compared to the control bone. Thus, amyloglucosidase immobilization to the derivatized bones resulted in poorer catalysts.

The 6.5 fold decrease in the V_{max} value upon immobilization (with respect to the control bone) was largely a reflection of the initial amount of activity immobilized. Compared to the control bone, the V_{max} values for CD- and SG-bone were similar, but the values for GLUT- and DEAE-bone were 1.6 and 1.5 fold higher, respectively.

DISCUSSION

Bone was previously shown to be a good support material for enzyme immobilization because of its mechanical strength, porosity and relatively large number of potentially reactive surface hydroxyl groups (Findlay *et al.*, 1986). The primary goal of this section of the study was to introduce intermediate groups of higher reactivity than hydroxyls, particularly amino groups and carbonyl groups, in the hope that enzyme immobilization would be enhanced by the increased number of attachment groups on the bone surface now available for binding.

The choice of bone derivatization techniques seemed logical in view of the commercial use of resins and cellulose that are prepared in a similar manner (Weetall, 1973). Furthermore, methods utilizing these cross-linking agents have previously been successful in binding enzymes, including

Table 5-4
Kinetic Parameters of Free and Immobilized
Amyloglucosidase

	K_m (mg/ml)	V_{max} (nmole/ml/min)
Free	2.92 $\pm$ 0.4	210 $\pm$ 11
Adsorbed (control)	8.38 $\pm$ 1.92	32.3 $\pm$ 0.4
Glut-bone	15.6 $\pm$ 2.3	51.8 $\pm$ 2.1
SG-bone	44.2 $\pm$ 4.8	38.5 $\pm$ 3.1
CD-bone	19.8 $\pm$ 0.3	33.7 $\pm$ 0.7
DEAE-bone	28.3 $\pm$ 2.5	50.7 $\pm$ 1.0

Data are means $\pm$ SEM, n=3. 15% (w/v) glycogen was used as substrate.

amyloglucosidase. Yamazaki *et al.* (1984) immobilized invertase to DEAE coated cotton flannel. Chen *et al.* (1977) immobilized amyloglucosidase onto cellulose beads previously treated with glutaraldehyde and 3-aminopropyltriethoxysilane. Findlay *et al.* (1986) also had varied success with a number of enzymes immobilized to bone using the methods of direct adsorption, Glut-bone, SG-bone and CD-bone. Although Findlay was the first to use bone and derivatized bone for enzyme immobilization, the present work represents the first attempts to immobilize amyloglucosidase to derivatized bone.

The success with the covalent coupling methods implies that the enzyme amino groups favour binding to a carbonyl-type binding surface (ie. SG-bone) over the largely hydroxyl surface of plain bone. Since the glutaraldehyde modified bone did not retain significantly greater activity than the control, it was thought that an initial silanization step would further increase the amount of enzyme bound. This preliminary step would supply glutaraldehyde with the necessary amino groups needed for the cross-linking reaction, which are not abundant in bone (Findlay *et al.*, 1986). This procedure proved to be successful, with SG-bone showing a 1.4 fold increase in the amount of activity compared to the control bone (Table 5-1).

A comparison of substrate use by the different types of bone immobilized amyloglucosidase revealed greater amounts of glycogen and potato starch hydrolysis over the control bone. The slight differences in temperature and pH optima further attests to the uniqueness of the derivatized bone surfaces in terms of surface charge of the microenvironment and enzyme stability when bound.

Based on the substantial increases in the K_m values for the enzyme linked by the various coupling techniques, it appears that immobilization

may have had a drastic effect on the enzyme conformation. It was surprising also that the amyloglucosidase immobilized to those bones having the highest specific activities (DEAE-bone and SG-bone), also showed the greatest increase in their K_m values. One would have thought that the attached spacer groups would have decreased any steric hindrance as well as limitations due to substrate diffusion. However, active site modification due to multiple point attachments of the enzyme onto the highly reactive bone surface may have caused this decrease in substrate affinity (Zaborsky 1973). Thus, it appears that enzyme immobilization by adsorption is the most "gentle" method, causing the least number of alterations to catalytic activity.

Although the V_{max} values for the newer immobilization methods did increase by 4-38% compared to direct adsorption, the highest amount of immobilized enzyme activity still amounted to only 23%. Such low yields may be still indicative of a relatively small number of functional sites available for binding.

Methods to test whether the these bone derivatizations actually took place were not done. However, the observations of physical bone changes as well as the experimental data do indicate that some favourable changes did take place.

This section of the report presents a preliminary investigation into the potential uses of some enzyme attachment methods for enhanced binding to BioboneTM. Overall, the results are encouraging and warrant further study.

Other modifications of bone that could enhance binding are also possible. Initially treating bone with collagenase might liberate free amino groups and thus make the bone surface more attractive for enzyme binding (Findlay *et al.*, 1986). Other derivatization methods do exist, and show promise in enhancing amyloglucosidase immobilization. Wilson *et al.* (1969)

employed the use of the bifunctional reagent 2-amino-4,6-dichloro-5-triazine to successfully attach amyloglucosidase to DEAE cellulose.

CHAPTER 6
STUDIES OF FREE AND IMMOBILIZED
 β -AMYLASE

INTRODUCTION

β -amylase (α -1,4-glucan maltohydrolase EC 3.2.1.2) hydrolyses α -1,4-glycosidic linkages in starch and glycogen in an exo-fashion by a multimolecular attack mechanism, from their non-reducing ends, releasing β -maltose units (Fogarty, 1983). It is unable to bypass α (1,6)glycosidic linkages in the amylopectin portions of starch and glycogen and does not attack maltotriose, resulting in incomplete degradation of these branched polymers. Therefore, the typical end products of β -amylase hydrolysis on liquified starch is 55% maltose, 15% maltotriose and 25-30% β -limit dextrins (Finnsugar Co. Ltd.).

β -amylase is an industrially important enzyme, occurring widely in higher plants and microorganisms (Kennedy *et al.*, 1987). Maltose and high maltose syrups have demonstrated a variety of commercial uses in confectionary, baking and brewing industries for its low viscosity in solution, low hygroscopicity, resistance to crystallization, low sweetness, heat stability and lack of colour formation (Fogarty, 1983). In light of the commercial importance of β -amylase, a variety of immobilization experiments have already been done (Fogarty, 1983).

The immobilization of barley β -amylase onto granular chicken bone was recently studied in the hopes of obtaining an active and stable preparation. The pH, temperature and kinetic parameters were determined. Also, due to its poor stability when immobilized, β -amylase was subjected to a variety of stabilizing agents in the hopes of creating a stable preparation.

MATERIALS AND METHODS

Immobilization of β -amylase

The standard amount of β -amylase used in all experiments, unless stated otherwise was 1.5 U (Finnsugar Chem. Co.). Sugars in the commercial enzyme preparation were first removed by passage through G-25 Sephadex gel equilibrated with 100 mM phosphate buffer (pH 6.0). The standard immobilization procedure included the addition of enzyme to a tube containing 100 mg of bone and .5ml phosphate buffer, pH 6.0, followed by agitation for 30 minutes at 23°C. Excess solution was poured off, followed by successive washes with buffer (3 x 1.0 ml).

β -amylase Assay

β -amylase activity was determined by measuring the maltose liberated from dialysed glycogen and corn starch. Standard reactions were carried out at 45°C in 100 mM phosphate buffer (pH 6.0), 12% (w/v) glycogen and 20% (w/v) corn starch. To initiate a reaction, 0.5 ml of substrate was added to a tube containing the enzyme followed by a 30 minute incubation and mixing period at 45°C. To stop the reaction, 0.1 ml of the reaction mixture was added to 0.1 ml of 0.5 N NaOH. The amount of reducing sugar liberated was estimated with the Dinitrosalicylate (DNS) reagent.

After 0.6 ml of DNS reagent was added to a total sample volume of 0.4 ml, samples were heated in a water bath for 5 minutes. The amount of reducing sugar produced, measured at 575 nm, was proportional to the change in colour intensity from orange to brown. The amount of maltose produced was estimated using a maltose standard curve (Miller, 1959).

β -amylase Experiments

The experimental protocol for studying free and immobilized β -amylase was virtually identical to that described for amyloglucosidase (Chapter 1), except for the following changes:

Determination of Optimum Binding Conditions

Phosphate buffer of different pH (4.0, 5.0, 5.5, 6.0, 6.5) and concentrations (100 mM, 50 mM), were used for the determination of optimum binding pH.

Optimum Temperature and Temperature Stability Studies

In addition to the standard temperature experiments (Chapter 1), free and immobilized enzyme were also heated at a designated temperature for 20 minutes, followed by a cooling to room temperature. After substrate addition, the reaction mixture underwent a 30 minute mixing at 23°C.

Kinetic Parameters

The kinetic parameters for free and immobilized β -amylase were determined using dialysed oyster liver glycogen type II and whole corn starch as substrates. Maltose production after 10 minutes was determined using 3-150 mg/ml glycogen at 45°C and 4-230 mg/ml of starch at 23°C. Kinetic parameters in the presence of 10% polyethylene glycol (PEG) with glycogen as substrate were also determined. K_m and V_{max} values were calculated using Lineweaver-Burk or Hill plots.

Effects of Potential Disrupting Agents

For the studies to determine disrupter effects, all reagents were made up in phosphate buffer, pH 6.0.

Storage stability

β -amylase was immobilized and stored overnight at 4⁰C in phosphate buffer (50mM, pH 6.0) containing one of the following: glycogen 70 mg/ml, KCl 50 mM, CaCl₂ 50 mM, TritonX-100 (1%), glycerol (10%, 20%, 50% v/v), bovine serum albumin (BSA) (15% w/v), mercaptoethanol (10 mM) or dithiothrietol (0.1 M). β -amylase bound to the following derivatized bone was also tested for overnight stability: diethylaminoethyl (DEAE), glutaraldehyde (GLUT), silanized-glutaraldehyde (SG), carbodiimide (CD), and to CD-bone in the presence of 1.0 M sorbitol or sucrose. Assays for the production of maltose were done at 23⁰C for 30 minutes in the presence of the stabilizers. These were compared with respective controls stored for T=0 time. Results were obtained following specific blank subtraction.

β -amylase was also immobilized and stored at 4⁰C in phosphate buffer containing .04% sodium azide and one of the following: sorbitol or sucrose (.05, .10, .25, .50 and 1.0 M) and glycogen (.0035, .007, .014, .028, .056, .07 mg/ml). Assays for the production of maltose were done at room temperature after the first 5 days and then weekly, for a period of 5 weeks, to determine long term storage stability.

Methods Employed for Mass Transfer Studies

Kinetic data for glycogen using 1.5, 6 and 15 units were plotted on Lineweaver-Burk plots. The resulting slopes were compared.

RESULTS

Amount of Enzyme Bound

The results from Table 6-1 and Figure 6-1 indicate that the best binding conditions were pH 4-5, 50 mM buffer for 30 minutes. It was apparent that activity decreases steadily with time the enzyme is left to adsorb to the bone.

Figure 6-2 shows the effect of incubation with varying amounts of β -amylase (.45-52 U) on the final activity immobilized. Although only 1.5 U of enzyme was used in the standard experiments, as much as 24 times more activity could be immobilized per 100 mg bone, with maximum loading reached at about 30-42 units added per incubation. Under the standard immobilization conditions, the average amount of activity immobilized to bone following 10 washes was $21.2 \pm 0.4\%$ with about $59.0 \pm 3.0\%$ being eluted in the washes (Table 6-2). The 20% lost activity could be attributed to enzyme deactivation upon immobilization. These calculated values were made relative to an equal amount of added free enzyme. The specific activity of free and immobilized β -amylase was 18.4 ± 2.1 and 8.1 ± 2.0 U/mg protein respectively.

Optimum pH

Figure 6-3 shows the effect of pH on the activities of free and immobilized β -amylase. Both reached maximum activity at pH 6.0, with the free enzyme demonstrating a slightly broader optimum range.

Figure 6-1.

EFFECT OF BINDING TIME ON THE AMOUNT OF
IMMOBILIZED β -AMYLASE ACTIVITY

Table 6-1
Determination of Optimum Conditions for Immobilizing
 α -amylase onto Bone By Variations in pH and Ionic Strength

Activity, μ moles maltose produced/ml/min

	Ionic Strength mM	
	50 mM	100 mM
4.0	0.34 $\pm$ 0.01	0.25 $\pm$ 0.01
5.0	0.35 $\pm$ 0.02	0.28 $\pm$ 0.02
5.5	0.28 $\pm$ 0.02	0.27 $\pm$ 0.02
6.0	0.17 $\pm$ 0.01	0.22 $\pm$ 0.01
7.0	0.15 $\pm$ 0.01	0.17 $\pm$ 0.01

Data are means $\pm$ SEM, n=3. To achieve immobilization, the standard amount of enzyme (1.5 U), was added to each with a 30 minute incubation, followed by 4 washes. Reactions were then run at pH 6.0, 23°C for 30 minutes following binding.

Table 6-2
Amount of α -amylase Activity Retained
During Bone Immobilization Following 10 Washes

	Activity, %	Specific Activity U/mg Protein
Free	100 $\pm$ 5	18.4 $\pm$ 2.1
Immobilized	21.2 $\pm$ 0.4	9.1 $\pm$ 2.0
Wash	59.0 $\pm$ 3.0	—
Lost	19.8	—

Data are means $\pm$ SEM, n=3. Activities are expressed relative to an equal amount of the free enzyme. The amount of protein in free enzyme and bound to 100 mg bone was determined by the BioRad protein assay.

Figure 6-2.

EFFECT OF THE ADDITION OF INCREASING CONCENTRATIONS
OF β -AMYLASE ON THE AMOUNT OF ACTIVITY IMMOBILIZED

Figure 6-3.

EFFECT OF pH ON THE ACTIVITY OF FREE AND IMMOBILIZED β -AMYLASE

Optimum Temperature and Temperature Stability Studies

Table 6-3 displays the effects of temperature on the activities of free and immobilized β -amylase. Both show relatively poor activity at temperatures greater than 67°C. However, free β -amylase retained approximately 90% of $V_{\max}$ over a wide but moderate temperature range (23-55°C) with a slight peak at 47°C, whereas the bound enzyme peaked at an optimum of 42°C. Thus, all standard experiments were performed at the mean optimum of 45°C.

In an effort to determine more specifically the temperature stability of β -amylase, the free and immobilized enzymes were first heated to the designated temperature than returned to room temperature to be assayed. These results, shown in Figure 6-4 indicate that free β -amylase experienced a more drastic loss of enzyme activity with increasing temperature than when immobilized.

Effect of Disrupting Agents on β -amylase Activity

The effects of the disrupting agents on the firmness of binding and enzyme activity of free and immobilized β -amylase are presented in Table 6-4. The immobilized enzyme had better catalytic function and showed enhanced stability against these denaturants compared to control values, than did the free. In particular, high activity of the immobilized enzyme was retained at pH 3 whereas the free enzyme was completely inactivated at low pH. The immobilized enzyme also showed much higher activity in the presence of ethanol than did the free. Aside from the ethanol (20%), relatively little enzyme activity was present in the pretreatment washes, implying relatively firm enzyme binding had occurred.

Table 6-3
Effect of Temperature on the Activity
of Free and Immobilized β -amylase

Temperature	Activity, μmol maltose produced/ml/min			
	Free		Immobilized	
0	1.31 $\pm$ 0.05	(45%)	0.22 $\pm$ 0.01	(45%)
12	1.90 $\pm$ 0.20	(66%)	0.24 $\pm$ 0.01	(48%)
23	2.53 $\pm$ 0.16	(87%)	0.32 $\pm$ 0.01	(66%)
37	2.67 $\pm$ 0.13	(92%)	0.40 $\pm$ 0.002	(82%)
42	2.73 $\pm$ 0.07	(94%)	0.49 $\pm$ 0.02	(100%)
47	2.90 $\pm$ 0.08	(100%)	0.41 $\pm$ 0.03	(87%)
55	2.68 $\pm$ 0.18	(92%)	0.32 $\pm$ 0.02	(66%)
67	1.67 $\pm$ 0.36	(57%)	0.18 $\pm$ 0.01	(36%)
75	0.53 $\pm$ 0.01	(18%)	0.03 $\pm$ 0.002	(6%)

Data are means $\pm$ SEM, $n=3$. The values in brackets are the relative activities (compared to 42°C and 47°C for bound and free β -amylase respectively) observed at the various temperatures. After the enzyme and substrate solutions were preheated to the desired temperature, they were combined and left to react for 30 minutes.

Figure 6-4.

EFFECT OF TEMPERATURE ON THE STABILITY OF
FREE AND IMMOBILIZED β -AMYLASE

Table 6-4
Effect of Disrupting Agents on Free and
Immobilized α -amylase

Treatment	Activity, μmol maltose produced/ml/min		
	Free	Immobilized	Wash
control	2.2 $\pm$.2 (100%)	0.3 $\pm$.02 (100%)	0.07 $\pm$.01 (3%)
pH 3	---	0.2 $\pm$.02 (77%)	---
Triton(1%)	1.6 $\pm$.1 (71%)	0.4 $\pm$.02 (119%)	0.08 $\pm$.01 (5%)
EtOH (30%)	0.3 $\pm$.01 (14%)	0.3 $\pm$.04 (95%)	0.06 $\pm$.01 (20%)
KCl 1.0 M	1.7 $\pm$.1 (77%)	0.4 $\pm$.02 (128%)	0.07 $\pm$.03 (4%)
KCl 0.5 M	1.9 $\pm$.1 (87%)	0.5 $\pm$.03 (143%)	0.11 $\pm$.01 (6%)
KCl 0.2 M	2.2 $\pm$.1 (97%)	0.4 $\pm$.02 (124%)	0.12 $\pm$.01 (6%)

Data are the means, $\pm$ SEM, $n=3$. Relative activities are given in brackets. Control values (using standard conditions) were set up to 100% for comparison with the other samples. Activity in the washes is relative to free values under each of the conditions. Reactions were conducted at 45°C for 30 minutes.

Kinetic Properties

Table 6-5 outlines the kinetic properties of free and immobilized β -amylase. K_m values for glycogen and starch both decreased upon immobilization, to levels 38 and 16% of the values for free. V_{max} values also dropped 8 and 1.5 fold compared to the free enzyme values for both glycogen and corn starch, respectively. Both of these V_{max} decreases largely reflect the percentage of enzyme that was immobilized to bone. Furthermore, glycogen experiments done in the presence of 10% PEG had no real effect on the K_m or V_{max} values (data not shown).

Substrate Comparison

Table 6-6 shows β -amylase activity with both crude and dialysed glycogen and soluble and whole corn starch. The conversion rate of glycogen to maltose by both free and immobilized β -amylase was greater than that for starch. Both bound and free enzyme hydrolysed crude glycogen more efficiently than the dialysed. Furthermore, the immobilized enzyme utilizes corn starch much better than the free relative to their abilities to hydrolyse glycogen. Also, enzyme attack is largely limited to the insoluble portions of starch.

Analysis of the reducing sugars produced from the hydrolysis of Lintner and potato starches could not be done with the DNS reagent, due to the excessively large background absorbance produced from each (data not shown).

Long and Short Term β -amylase Storage and Stability

The results from Table 6-7 indicate that immobilized β -amylase loses 50% activity overnight when assayed at 23°C, but 77% when assayed at

Table 6-5
Kinetic Parameters for Free and Immobilized β -amylase

Substrate	K_m mg/ml	V_{max}^a
Glycogen		
Free	28.2 ± 0.9	3.18 ± 0.40
Immobilized	10.7 ± 0.4	0.39 ± 0.03
Corn Starch		
Free	58.0 ± 0.1	0.16 ± 0.02
Immobilized	49.0 ± 0.1	0.11 ± 0.01

Data are means $\pm$ SEM, n=4.

^a μ mol maltose/ml/min

Reactions with glycogen were carried out at 45°C, whereas reactions with corn starch were done at 23°C.

Table 6-6
**Comparison of Crude and Dialysed Glycogen,
and Soluble and Whole, Corn Starch as Substrates
for Free and Immobilized β -amylase**

Substrate	Activity, μ mol maltose produced/ml/min	
	Free	Immobilized
Glycogen		
Dialysed	2.53 ± 0.16 (100%)	0.32 ± 0.01 (100%)
Crude	3.30 ± 0.09 (130%)	0.37 ± 0.07 (116%)
Corn Starch		
Whole	0.18 ± 0.02 (100%)	0.13 ± 0.03 (100%)
Soluble	0.02 ± 0.01 (10%)	0.01 ± 0.001 (8%)

Data are the means $\pm$ SEM, n=3. The relative activities are given in brackets. Control values (dialysed glycogen) were set to 100%. All reactions were carried out at 23°C for 30 minutes.

Table 6-7
Effects of Stabilization Treatments on the Retention of
Immobilized β -amylase Activity Following Overnight Storage

Treatment	Temp. °C	Storage Time Hours	Activity mole maltose/ml/min	
Free	45	0	4.31 + 0.12	(100%)
		24	4.60 + 0.53	(100%)
	23	0	3.41 + 0.09	(100%)
		24	3.40 + 0.06	(100%)
Immobilized Control	45	0	0.31 + 0.02	(100%)
		24	0.07 + 0.01	(23%)
	23	0	0.20 + 0.01	(100%)
		24	0.10 + 0.01	(51%)
Glycogen (7%w/v)	23	24	* 0.20 + 0.01	(98%)
KCl (0.5 M)	23	24	0.14 + 0.01	(68%)
CaCl ₂ (0.5 M)	23	24	0.11 + 0.01	(53%)
TritonX (1%)	23	24	0.10 + 0.01	(52%)
Glycerol (10%)	23	24	0.10 + 0	(48%)
(20%)	23	24	0.09 + 0.03	(45%)
(50%)	23	24	0.08 + 0.02	(38%)
BSA (15%)	23	24	0.13 + 0.01	(67%)
Mercaptoethanol (10 mM)	23	0	0.26 + 0.01	(100%)
		24	0.19 + 0.02	(72%)
Dithiothreitol (.1 μ M)	23	0	0.17 + 0.02	(100%)
		24	0.11 + 0.02	(65%)
DEAE-Bone	23	0	0.15 + 0.01	(100%)
		24	0.09 + 0.01	(62%)
GLUT-Bone	23	0	0.20 + 0.02	(100%)
		24	0.14 + 0.01	(67%)
SG-Bone	23	0	0.08 + 0.003	(100%)
		24	0.03 + 0.001	(31%)
CD-Bone	23	0	0.35 + 0.01	(100%)
		24	0.28 + 0.02	(80%)
CD-Bone 1M Sorbitol	23	0	* 0.06 + 0.004	(100%)
		24	0.06 + 0.001	(100%)
1M Sucrose	23	0	* 0.07 + 0.003	(100%)
		24	0.07 + 0.001	(100%)

Data are the means + SEM, n=3. The relative activities are given brackets. Individual control values (where applicable) were set to 100%. Asterisks indicate conditions where 100% activity was maintained. * umoles maltose produced/ml/min

45°C. This is in comparison to the free enzyme, which maintains 100% activity overnight when assayed at both temperatures.

A variety of additions were made to the enzyme preparation to see if these would improve the overnight storage stability; of these only storage in the presence of glycogen maintained full enzyme activity.

The stability of β -amylase bound to derivatized bone was also tested. Although DEAE- and SG-bone showed lower activity retentions, GLUT-bone showed similar values, and CD-bone showed higher activity retentions compared to the control bone, at 0 storage time and 23°C. β -amylase immobilized onto the derivatized bones did not demonstrate overnight stability. However, β -amylase bound to CD-bone in the presence of 1M sorbitol and sucrose maintained 100% activity following overnight storage.

The effect of different concentrations of additives -those that demonstrated successful overnight stability- in maintaining stability over 5 days of storage was also tested. Stability improved markedly with increasing levels of glycogen, sorbitol or sucrose with 100% activity retained at additive concentrations of 0.07 mg/ml, 1 M and 1 M, respectively (Figure 6-5a,b). Free enzyme without stabilizers by comparison, lost only 5% activity over the same time. Approximately 8% of activity was present in the washes for sucrose and sorbitol at all concentrations, for both 0 and 5 day storage times. The 0 and 5 day controls lost only ~ 4% by comparison (data not shown).

These storage studies were further extended to 2 weeks. At this time the glycogen storage studies could not be analysed. After several washes (before assaying), the presence of residual maltose resulted in readings that were greater than the 0 storage time controls, even after repeated washings. The 1 M sucrose and sorbitol samples maintained approximately 72% activity after 5 weeks, resulting in an operational half life of approximately 65 days.

Figure 6-5a.

β -amylase was immobilized under standard conditions and then stored for 5 days in the presence of the given amounts of glycogen. Following this, glycogen of the same concentration was added to the respective bones, for a 30 minute incubation period. Control bones (ie. T=0 days), were prepared in a similar manner.

Figure 6-5b.

EFFECT OF INCREASING SORBITOL AND SUCROSE
CONCENTRATION ON IMMOBILIZED β -AMYLASE
STABILITY AFTER 5 DAYS

β -amylase was immobilized and then stored for 5 days in the presence of the given amounts of sucrose or sorbitol. Following this, the immobilized enzymes were assayed under standard conditions, in the presence of the same amounts of sucrose or sorbitol. Control bones (ie. T=0 days), were prepared in a similar manner.

Free enzyme, in the absence of stabilizers lost 16.5% of its activity, after 2 weeks.

To test the effects of these storage stabilizers on enzyme activity, β -amylase activity was measured in the presence of varying concentrations of sucrose and sorbitol. High concentrations of sucrose and sorbitol resulted in substantial activity losses, when compared to reactions run without these solutes present (Table 6-8). However, approximately 86% relative activity was restored after excess solute containing these agents was removed by successive washings.

β -amylase Continuous Use and Stability

Figure 6-6 shows the effects temperature and 1M sucrose on enzyme recycling. Continuous use at 45⁰C resulted in a loss of nearly 90% in activity after 1 cycle. Continuous reuse at 23⁰C resulted in a more gradual decline for both plain and CD-bone immobilized β -amylase, whereas the presence of 1M sucrose gave some improvement of activity retention. Furthermore, residual activity (approximately 10% relative to free enzyme amounts) was present in the excess substrate of the first cycle, for each of the cycling experiments.

Time Course of Glycogen Degradation and β -amylase Stability

Figure 6-7 shows the time course of maltose production for free, immobilized and CD-bone immobilized β -amylase in the presence of 1 M sucrose. The CD-bone immobilized enzyme with 1 M sucrose showed a gradual but relatively linear rate of maltose production over the entire 24 hour period, which indicated a constant activity with time. Free enzyme and enzyme simply bound to bone show 2 levels of linear activity with slope changes occurring at 1 and 4 hours, respectively. The change in the rate of

Table 6-8
Effects of Increasing Concentrations of Sorbitol and Sucrose
in Reaction Mixture on the Activity of Immobilized α -amylase

Concentration (M)	Activity%	
	Sorbitol	Sucrose
0	100	100
.01	100	100
.05	100	100
.10	100	100
.25	90	90
.50	66	90
1.0	32	32

Data are means $\pm$ SEM, n=3. The values are expressed with respect to control conditions. All reactions were carried out at 23°C for 30 minutes after T=0 storage time.

Figure 6-6.

EFFECT OF CONTINUOUS USE ON IMMOBILIZED β -AMYLASE ACTIVITY

β -amylase was immobilized under the given conditions. After each 30 minute reaction period, bone was thoroughly washed with buffer, and then fresh substrate was added. Spent substrate was left to incubate for an additional 30 minutes for the determination of eluted activity. This cycle was repeated 5 times.

Figure 6-7.

TIME COURSE OF GLYCOGEN DEGRADATION BY FREE
AND IMMOBILIZED β -AMYLASE AT 23°C

substrate use for these 2 enzyme systems could indicate either a slow loss of enzyme activity, or a decrease in the availability of substrate sites (ie. increase in number of $\alpha(1,6)$ branch point sites).

Presence of Bone Washes and β -amylase Stability

Tests were done to determine whether immobilized β -amylase instability may be due to something given off by the bone itself. A comparison of free β -amylase activity in buffer to free activity in the presence of bone washes shows that free β -amylase activity over 24 hours is essentially unaffected by the presence of bone wash (Table 6-9).

Diffusion Limitation of the Immobilized System

Lineweaver-Burk plots for immobilized β -amylase at 1.5, 6 and 15 units do not result in increasing slopes, which is usually indicative of internal diffusional resistance (Hon *et al.*, 1979).

Arrhenius plots for immobilized β -amylase showed slopes that were similar to the free enzyme over the temperature range 0-47°C, indicating similar activation energies. However, at high temperatures (55-75°C), the immobilized enzyme showed a much steeper negative slope (data not shown).

Increasing immobilized enzyme surface area (by first freezing the bone immobilized enzyme in liquid nitrogen and then grinding into a powder) resulted in a 1.5 fold increase in activity (Table 6-10).

Table 6-9
Effects of Bone Wash on the Activity of Free β -amylase

Enzyme Condition	Activity, μ moles maltose produced/ml/min
control	2.5 $\pm$ 0.1
bone wash 1 hr pretreatment	2.1 $\pm$ 0.3
24 hr pretreatment	2.4 $\pm$ 0.1

Data are means $\pm$ SEM, n=3. The effects of bone wash on free β -amylase activity over 24 hours was compared to free activity in buffer only. Reactions were conducted at 23°C for 30 minutes

Table 6-10
Effects of Increasing Surface Area on the Apparent
Activity of Immobilized α -amylase

Treatment	Activity, μ moles maltose produced/ml/min
Control-bone	0.21 $\pm$ 0.01 (100%)
Powdered-bone	0.32 $\pm$ 0.04 (152%)

Data are means $\pm$ SEM, n=3. Relative activities are given in brackets. Control values were set to 100% for comparison. Immobilized enzymes (including controls) were frozen in liquid nitrogen then left to return to room temperature. Immediately following freezing the immobilized enzyme was ground into powder with a mortar and pestle. All reactions were carried out at 23°C for 30 minutes with glycogen as substrate.

DISCUSSION

Large quantities of high maltose syrups (>80%) are not produced in Europe or the United States, but are in high demand in Japan (Fogarty, 1983). High maltose syrups are made by a process where corn syrup, produced from corn starch by either acid or α -amylase hydrolysis, is further hydrolyzed in combination with fungal amylase mixtures to give high yields of maltose (Bohnenkamp *et al.*, 1980). β -amylase, acting similarly to the fungal amylases (but producing no glucose) could be substituted for them were it not for their high production costs (Bohnenkamp *et al.*, 1980). However, a successful immobilized β -amylase could be a possible alternative.

The focus of the work done with β -amylase was to optimize operational conditions and also increase its functional stability to make the immobilized system more feasible, especially from a commercial point of view. Immobilization allows for the continued reuse of enzyme, but is only worthwhile provided the preparation is stable and the product remains essentially enzyme free (Caldwell, *et al.*, 1976).

Preliminary experiments for the immobilization of β -amylase onto bone were promising. After determining optimum binding conditions (pH 5.0, 50mM buffer, 30 minute binding period), the amount of activity immobilized relative to the free enzyme $21.2 \pm 0.4\%$, was comparable to those for other methods. Although Roy *et al.* (1987), achieved only 3-7% bound activity when β -amylase was immobilized onto a cation exchange resin, Vretblad *et al.* (1973), immobilized 6-35% of added activity onto epichlorohydrin linked Sepharose and Martensson (1974), immobilized 23% with acrylimide acrylic acid.

The amount of enzyme used in standard experiments (1.5 U), represented a fraction of the amount of enzyme that could be used to achieve maximum enzyme loads (42 U) (Figure 6-2). Thus, since the maximum binding capacity was not reached at this point, the immobilized enzyme is likely not suffering from steric hindrance.

The 2.6 and 1.2 fold decrease in K_m values upon immobilization likely indicate that the affinity of β -amylase for glycogen and corn starch respectively, had increased. This was an excellent result but surprising because diffusional problems usually occur with immobilized enzymes using such highly viscous polysaccharide substrates like corn starch (Vretblad *et al.*, 1973). 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 (Zaborsky, 1973). Furthermore, other studies showed substantial increases in K_m values upon immobilization of β -amylase. Roy *et al.* (1987), observed a 6 fold increase, Maeda *et al.* (1978) obtained a 65 fold increase, and Ramesh *et al.* (1981) showed a 1.7 fold increase. β -amylase immobilized to bone may have altered the conformation of the active site, increasing the affinity of β -amylase for its substrate (Chakrabarti, 1988). Examples in which the K_m of an enzyme had decreased upon immobilization have been reported (Chakrabarti and Storey, 1988).

Table 6-6 indicates that activity, both bound and free are largely confined to the insoluble portion of corn starch. Thus, immobilization doesn't limit the approach of high molecular weight substrates.

Corn starch was the only starch with background readings small enough to not impose a hindrance on the reducing sugar analysis with the DNS reagent. Although the major components of starch occur in similar

proportions from all sources (69-77% amylopectin, 23-31% amylose), the differences in background interference may be due to the effects of the minor components of starch (Banks, 1975; Galliard, 1985). Such components (proteins, lipids, phosphates) present in even highly purified commercial starch account for the differences between these starches and play significant roles in determining both the rheological properties of starch gels and the behaviour of starch during syrup production (Galliard, 1985).

The results from Lineweaver-Burk and Arrhenius plots did not support the existence of diffusion limitation when β -amylase was immobilized onto bone. Also, mixing the reaction tubes had previously been shown to lessen the effects of diffusion limitation for both soluble (glycogen) and highly insoluble (starch) substrates (Chapter 1). The decrease in K_m upon immobilization further supports the fact that the bone did not restrict the approach of large molecular weight substrates (Table 6-5). However, β -amylase immobilized onto crushed bone did show enhanced activity compared to the control bone (Table 6-10).

The stability that immobilization imparts to β -amylase in the presence of the potential disrupting agents was readily apparent, with enhanced activity resulting in the presence of TritonX-100 and KCl (Table 6-4). The initial washing procedure was sufficient to eliminate any unbound enzyme. Except for ethanol treatments β -amylase remained firmly bound to bone.

The results from pH and temperature studies established the instability of β -amylase following immobilization onto bone. Although the pH optimum did not change upon immobilization, free β -amylase did show a broader pH optimum range (Figure 6-3). Both Martensson (1974), and Hon *et al.* (1979), showed narrow pH optima for the immobilization of barley β -amylase. Vretblad *et al.* (1973) however, showed a very broad optimum.

Similarly, the temperature profiles indicated a much narrower optimum range upon immobilization (Table 6-3), as well as a faster rate of temperature inactivation (Figure 6-4). In contrast, other immobilized β -amylase systems showed greater temperature optima and greater resistance to heat inactivation compared to the free (Maeda *et al.*, 1978; Ohba *et al.*, 1979; Roy *et al.*, 1987).

Efforts to improve bound enzyme stability were attempted after both enzyme recycling and overnight stability studies under standard conditions resulted in substantial activity losses (Figure 6-6, Table 6-7). Experiments repeated at room temperature, improved stability, did not maintain full activity. By contrast, the free enzyme maintained 100% activity at both 23 and 45°C (Table 6-7).

β -amylase is known to be sensitive to oxidation of some thiol groups necessary for activity (Martensson, 1974). Overnight stability studies in the presence of antioxidants (mercaptoethanol, dithiothreitol), did not maintain 100% activity, but did show some improvement over the control bone studies. Martensson (1974), however, did achieve a stabilizing effect using reduced glutathione. He also discovered the protective effects of (0.05%) BSA on immobilized β -amylase activity, concluding that stabilizing protein-protein interactions were enhanced due to increased protein concentrations. However, 15% BSA failed to stabilize β -amylase under our immobilization conditions (Table 6-7).

Overnight stability studies in the presence of those compounds which previously showed enhanced enzyme activity (TritonX-100, KCl), as well as other potential stabilizers (glycerol, CaCl_2), did not help (Table 6-7). The choice of CaCl_2 was arbitrary since no special ion requirement is known for β -amylase (Fogarty, 1983).

β -amylase was also immobilized to derivatized bones (refer to Chapter 5), in an attempt to enhance enzyme activity and stability through covalent binding and ionic interactions. All were unsuccessful in maintaining 100% activity following overnight storage. However, further studies were performed with CD-bone, for it showed a distinct increase in the amount of immobilized activity ($23.8 \pm 1.14\%$)(Table 6-7).

Retention of constant overnight immobilized activity was achieved only in the presence of substrate glycogen and CD-bone immobilized β -amylase in the presence of 1M sorbitol or sucrose. Sugars and polyols are known protein stabilizers, which function by preferentially hydrating protein surfaces and thereby enhancing protein associations (Arakawa *et al.*, 1982). The fact that high concentrations of these solutes are needed to maintain stabilization argues against any specific binding being the dominant force involved in the stabilization (Carpenter *et al.* 1988). The 3 fold drop in activity in the presence of 1M sucrose and sorbitol was surprising (Table 6-8). Hinton *et al.* (1969), attributed the decrease in enzyme activity in the presence of sucrose due to conformational changes. Indeed, repeated washings to remove the 1M sorbitol did restore 86% of β -amylase activity.

Long term enzyme activity was retained but only under high concentrations of these stabilizers (Figure 6-5a,b). However, enzyme recycling in the presence of 1M sucrose did little to maintain constant enzyme activity with continuous use (Figure 6-6). Also, with respect to industrial production, use of β -amylase in the presence of 1 M sucrose would not be feasible.

CD-bone immobilized β -amylase in the presence of 1 M sucrose increased operational stability, for long term substrate degradation remained

essentially linear over 24 hours (Figure 6-7). However, the total output was very poor, much less than with bone alone.

Enzyme instability that existed only upon immobilization raised the question of whether this was due to the presence of some inherent destabilizer in the bone itself. However, free enzyme activity in the presence and absence of bone wash clearly indicates that the instability is not due to anything given off by the bone (Table 6-9).

Some enzyme desorption occurred under normal operating conditions (enzyme recycling, long term storage) as well as by treatment with ethanol. However, the amounts of enzyme eluted were not sufficient to explain the activity loss encountered after overnight storage, enzyme recycling or high temperature treatments. Caldwell *et al.* (1976), encountered similar problems after immobilizing β -amylase onto hexyl-Sepharose. Continuous column use resulted in hourly leakage of 0.5% of column activity.

From these results Caldwell *et al.* (1976), concluded that an enzyme molecule, after initial immobilization attachment, undergoes certain conformational changes which ultimately leads to denaturation. Such changes could be due to a steady increase in the number of attachment points with a resulting increase in binding stability, but at the same time introduce a strain on the conformation with subsequent loss of activity. The possibility that increasing thermal motion may provoke multipoint attachment for the already weakly immobilized enzyme, may explain the very poor temperature stability demonstrated by bone-immobilized β -amylase (Figure 6-4), as well as enzyme recycling and overnight storage assays done at 23°C versus 45°C (Figure 6-6, Table 6-8). Such motion would increase with increasing temperature and would manifest itself as a loss of enzymatic activity (Caldwell *et al.*, 1976). This apparent temperature dependence of bone-

immobilized β -amylase activity does limit its use on an industrial scale. However, the decrease in K_m values upon immobilization indicated an increase in substrate affinity. In addition, CD-bone immobilized β -amylase with 1M sucrose did demonstrate high operational stability. Such properties are equally important for successful and economical industrial utilization. Microbial β -amylases however, may prove to be more industrially useful, owing to their easy production by fermentation, and temperature and pH optimum in the neighbourhood of 55°C and 6-8, respectively (Fogarty, 1983).

GENERAL DISCUSSION AND CONCLUSIONS

Biobone™ was believed to be the ideal support matrix for the studies undertaken. To reiterate, chicken bone is abundant and inexpensive; it is a natural product and therefore food approved, and; it is a strong, porous material (Findlay *et al.* 1986).

In all of the studies undertaken, Biobone™ demonstrated high enzyme loading capabilities, and did not degrade when subject to harsh experimental conditions (ie. high temperature). Biobone™ was shown to be very versatile, in that it could be effectively used in granular and powdered form.

The choice of starch degrading enzymes in this work was ideal, since all sugar syrups produced for commercial purposes (ie. glucose, fructose, maltose), are derived from starch sources. Therefore, using chicken bone as a support matrix for these enzymes is highly suited for use in the food industry.

Although no experiments were conducted concerning scaling up, to mimic industrial level reactors, it is felt that any of the enzymes studied could be used in either batch or column processes. Granular bone would be particularly well suited for column processes due to its good flow through properties (Findlay *et al.* 1986). Future work will also focus on immobilizing those enzymes involved in the breakdown of other forms of biopolymers that are usually discarded as waste (ie. lignin, hemicellulose, chitin).

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