This is to certify that the

dissertation entitled

Enzymatic studies on the production of high fructose glucose syrup: Immobilization and stability studies of glucose isomerase.

presented by

Ramadan Ahmed Abd El-Ghani Habiba

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Food Science

Major professor

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ENZYMATIC STUDIES ON THE PRODUCTION OF HIGH FRUCTOSE GLUCOSE SYRUP: IMMOBILIZATION AND STABILITY STUDIES OF GLUCOSE ISOMERASE

By

Ramadan Ahmed Abd El-Ghani Habiba

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ABSTRACT

ENZYMATIC STUDIES ON THE PRODUCTION OF HIGH FRUCTOSE GLUCOSE SYRUP: IMMOBILIZATION AND STABILITY STUDIES OF GLUCOSE ISOMERASE

By

Ramadan A. Habiba

A crude glucose isomerase from *Flavobacterium arborescens* was covalently immobilized on Titanium(IV)-coated controlled pore glass (CPG) beads using several different techniques. The CPG beads were first coated with $TiCl_4$, then derivatized with gamma aminopropyltriethoxysilane or diamino compounds. Finally the derivatized beads were activated using either glutaraldehyde or ethyldimethylaminopropyl carbodiimide (EDC) and the enzyme was bound to the activated beads. In addition, the enzyme was directly immobilized to the titanium-coated CPG beads which gave inactive immobilized preparation. However, the rest of bound enzyme preparations were active and conditions of their immobilization were optimized. The type of derivatization did not affect the percent of protein (enzyme) binding. However, the later and the percent of activity retained significantly varied according to the type of activator used.

The optimum activity conditions of the bound enzyme preparations and the free (soluble) enzyme were determined. The bound enzyme required less amount of Mg^{2+} and Co^{2+}, exhibited wider pH and temperature activity ranges, and gave higher specific activity than those of the soluble enzyme. The K_m and V_m of glucose for the bound enzyme preparations were higher than those of the soluble enzyme.

Ramadan A. Habiba

Thermal, pH, and storage stabilities were studied for the bound and free enzyme. The soluble enzyme was stabilized upon immobilization as indicated by the following evidence: 1) the bound enzyme had a wider pH activity range, pH stability range, activity temperature profile, and a better storage stability than the soluble enzyme 2) the bound enzyme was more thermostable than the soluble enzyme at temperatures of 70 and 80°C. 3) the bound enzyme showed better (48 hour half life) long-term stability at 60°C than the soluble enzyme (12 hour half life) 4) regardless of the lower thermal stability of the soluble enzyme compared to a commercial enzyme (Spezyme GI, M600) and the carrier dissolution releasing the enzyme from the column bed, a half life of up to 22 days was obtained in a continuous isomerization process.

Diffusional limitation study on the bound enzyme preparations indicated no significant external or internal diffusion resistance. Thus the operational stability was not disguised by such limitations.

بسم الله الرحمن الرحيم

In the name of Allah the most merciful and the most beneficient

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INTRODUCTION

The increasing demand for sucrose and the wide fluctuation of its price have enhanced the search for alternative sweeteners. The discovery of glucose isomerase by Marshall and Kooi in 1957 paved the way for the production of a new natural sweetener called high-fructose corn syrup (HFCS). This product became successful due to the efforts of Japanese researchers in the1960's and the development of technology in the 1970's by American companies. Such development has enabled certain nations such as the United States, Europe, Japan, and some third world countries to cut imports and encourage home agriculture (Barker and Petch, 1985).

The most important development is the utilization of enzyme immobilization technology which permits the economic production of HFCS. However, the large scale of commercial utilization of immobilized enzymes is severely hindered by enzyme deactivation and loss of enzyme activity due to thermal denaturation, pH effects, shear effects, pore blockage, inactivators in feed stream, microbial contamination, attrition by weak bonding or support erosion, and other factors (Park et al., 1981).

Stability is one of the most critical biotechnological characteristics of an enzyme. Relatively little is known about mechanisms of enzyme inactivation and the stabilization of enzymes remain one of the most challenging areas of biotechnology. The importance of this area will increase as more enzymes are used commercially (Klibanov, 1983).

The objective of this research was to study the possibilities of improving the stability of glucose isomerase by investigating:

(a) the optimum conditions for the activity of soluble glucose isomerase

(b) the pH, thermal, and storage stabilities of soluble glucose isomerase

(c) the immobilization of the soluble glucose isomerase using different immobilization techniques

(d) the optimum conditions for the activity of the immobilized glucose isomerase

(c) the effect of the immobilization techniques used on the pH, thermal, storage, and operational stabilities of the immobilized enzyme

(d) the internal and external diffusion limitations of the immobilized enzyme preparations, if any.

LITERATURE REVIEW

I.INTRODUCTION

A. Commercial Importance

Fluctuation in the world sugar market and price support systems has encouraged the food and beverage manufacturers in countries which import a substantial portion of their sugar supply to seek a more stable and cheaper source of sweeteners. Corn syrups have been used in many applications, but their limited sweetness and physical characteristics were major obstacles toward expanding the use of these syrups. The discovery of glucose isomerase (GI), commercialization of immobilized glucose isomerase (IGI) and of fructose enrichment techniques permit the economic production of syrup similar to invert sugar, but containing various levels of fructose which is about twice as sweet as dextrose. The sweetness can thus be tailored to suit the particular application. High Fructose Corn Syrups (HFCS) containing 42% and 55% fructose have received wide acceptance in the sweetener market, particularly, for soft drinks. HFCS containing 90% fructose and fructose powder are also available, but in limited amounts for dietetic purposes (Blabchard and Geiger, 1984). Certainly, the enzymic isomerization of glucose to fructose is, at present time, the largest process using immobilized enzymes in the world (Marconi, 1980 and Swaisgood, 1985). The U.S. imports about half of its sugar consumption and the U.S. sweetener market surveys have shown a dramatic increase in the consumption of fructose containing sweeteners. This trend is projected to continue with fructose based sweeteners displacing the traditional

caloric sweeteners due to the availability of reliable technology, change in lifestyle and economic incentives (Teague and Arnold, 1983).

B. Process outline

The overall process for producing HFCS has been described by MacAllister (1980), and Blanchard and Geiger (1984). A typical process sequence based on that work and on the recommendations of Novo is given in Figure (1). The system is basically simple. However, the sensitivities of individual enzymes and exchange resins require close control of specific pH, temperature, and dry substance requirements at various stages of the process. Where practicable, the process stream is kept at pH about 4.0, to minmize color formation and sugar destruction, and at a temperature of about 60^{o}C to avoid microbial problems.

The starch slurry is liquified and saccharified to the maximum practicable dextrose content. Then, the product syrup is filtered, evaporated to an intermediate density and purified extensively with carbon and ion exchange. Calcium ions which adversly affect the life of the IGI must be effectively eliminated. The resulting syrup is treated with magnesium salts, and other activators or protectors, and the pH is adjusted to the pH optimum of the IGI applied, usually about 8.0, prior to passage over the immobilized enzyme bed. The flow rate is regulated in relation to enzyme activity so as to produce a syrup with 42% fructose. Then, the pH of the isomerized syrup is adjusted to about 4.0 to minimize color formation. The residual activators and traces of impurities that may have formed are removed by further carbon and ion exchange treatment prior to final evaporation of the high fructose-containing syrup (HFCS-42).

For fructose enrichment, the separation system (mostly elution chromatography) used requires a syrup feed at 60% dry substance. The fractionation results in a "raffinate", largely dextrose, which is returned to earlier stage in the process, and a product at 90% fructose which is

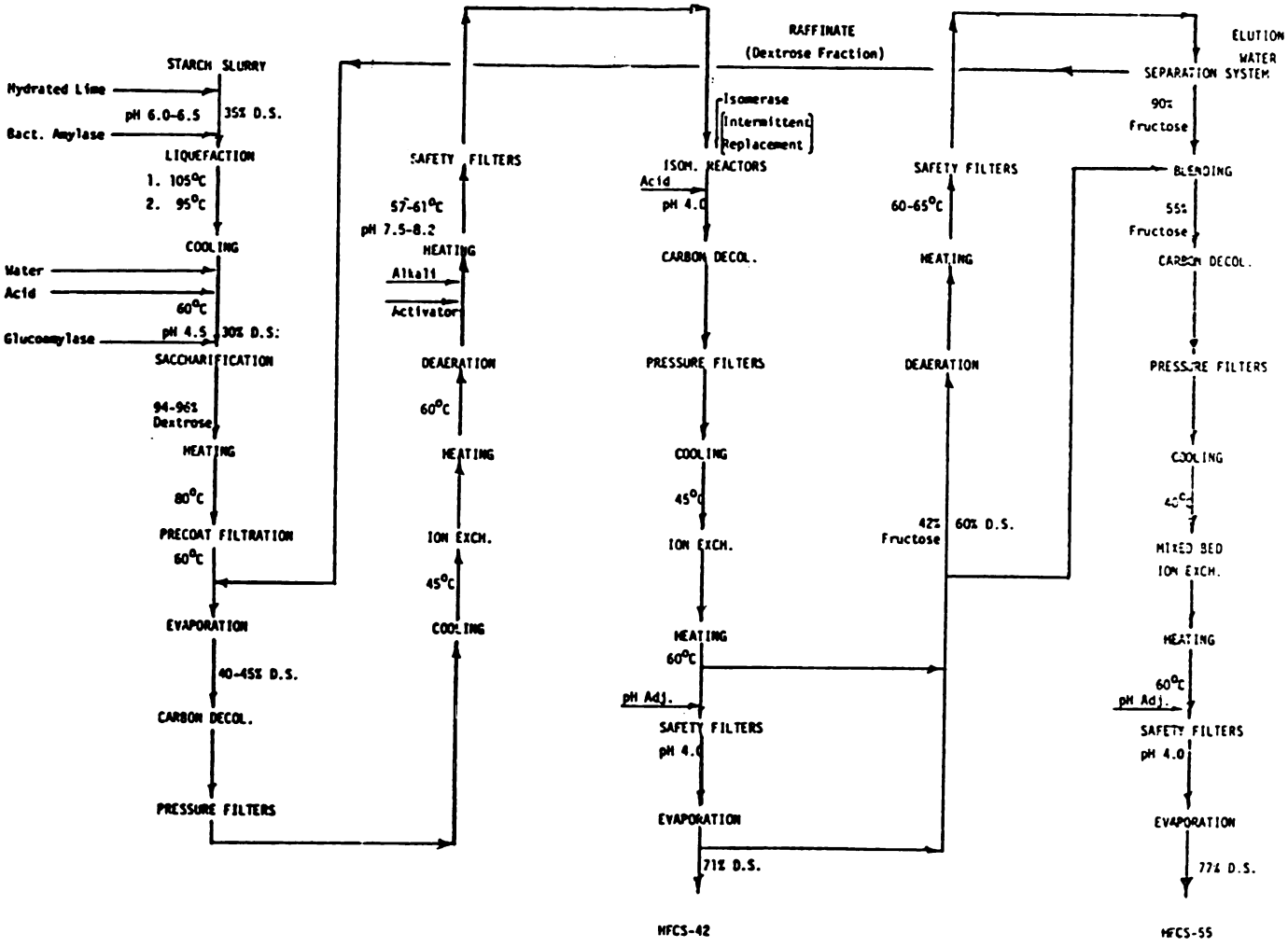


Figure 1. A typical process sequence for the production of HFCS

blended with 42% fructose syrup to form a 55% high fructose corn syrup (HFCS-55). Further purification may be required before the final evaporation of the HFCS-55.

II. ENZYME CONVERSION OF STARCH

Although enzymes have long been used to hydrolyze starch, the technology for using specific enzymes to make high-dextrose syrups of the type suitable for making today's 42% fructose and higher fructose-containing syrups was not commercialized until late 1950's. At that time the acid-enzyme and enzyme-enzyme treatment of starch was developed to obtain a refined 92-96% dextrose liquor to make crystalline dextrose (Antrim et al., 1979) .

Alpha amylase, more correctly known as alpha (1-4) glucan-4-glucanohydrase EC 3.2.1.1 , is the first enzyme in the hydrolysis of starch . This enzyme catalyzes the hydrolysis of alpha 1-4 bonds within the starch molecule . It is an endo amylase, i.e., it attacks bonds randomly within the starch molecule producing dextrins having a chain length of about 8 glucose units. The enzyme has a pH optimum at 6-7 and requires Ca^{++} for both activity and stability. The temperature is extremely important during this stage of hydrolysis. The temperature must be maintained sufficiently high to gelatinize the starch and prevent retrogradation of the starch from occurring. On the other hand, it must be kept low enough to prevent inactivation of the enzyme(Blanchard and Geiger, 1984). The discovery of alpha amylase which is stable at $95^{o}C$ has largely eliminated the problems in this area (Rosendal et al., 1979, and Slott and Madsen, 1975). The application of immobilized alpha amylase is hindered by its preferential hydrolysis of the amylose fraction of the starch and low molecular weight dextrins. While this produces some interesting dextrins and starch fractions, the immobilized enzyme does not produce the uniform molecular weight distribution desired for starch hydrolysis(Blanchard and Geiger, 1984). In practice, 30% starch

suspensions are thinned with either acid or bacterial alpha amylase in the presence of Ca^{++} at pH 7 to a dextrose equivalent (DE) of 15-20 (Schwimmer,1981).

The second step in the hydrolysis of starch to glucose is saccharification of the dextrins, produced by the action of alpha amylase, to glucose. This is accomplished using the enzyme glucoamylase (also known as amyloglucosidase). Glucoamylase (alpha 1-4 glucan glucohydrolase EC 3.2.1.3) catalyzes the hydrolysis of starch or dextrin to D-glucose. The enzyme has a broad pH optimum between 3.5-5.0 (Reilly, 1979). As an exo-enzyme, glucoamylase hydrolyzes the alpha 1-4 bonds from the non-reducing end of the the starch molecule. The presence of alpha 1-6 bonds, such as those which form the branch points in amylopectin, presents an obstacle to further hydrolysis. Glucoamylase can hydrolyze alpha 1-6 bonds, but at a rate only 1/30 that of the alpha 1-4 bonds(Pazur and Ando, 1960).

The glucoamylase treatment, at pH 4.0-4.5 and at a temperature of about $60^{o}C$ to avoid microbial contamination, is continued until at least 95% of the theoretical amount of glucose is formed. Unfortunately, one of the problems encountered with acid hydrolysis, namely repolymerization of dextrose to form maltose and isomaltose, also persists in the enzyme process. This problem is due to the troublesome enzyme, transglucosidase (alpha 1-4 glucosyltransferase EC 2.4.1.24) that often accompanies amyloglucosidase production from *Aspergillus niger*, the preferred industrial enzyme source for glucose feedstock preparation from starch dextrins(Bensen et al.,1982). To eliminate this problem, pullulanase(pullalan 6-glucanohydrolase EC 3.2.1.41) has been used. The enzyme hydrolyzes the alpha 1-6 bonds in amylopectin and dextrins. These bonds must be preceded by at least one and preferably more glucose units linked by an alpha 1-4 bond to be attacked by pullulanase. The use of pullulanase offers two advantages: first, it can lower the amount of glucoamylase needed for saccharification; second, its use can result in higher dextrose levels(Blanchard and Geiger, 1984).

III. ALKALINE ISOMERIZATION

During the time that work was underway to develop an enzymatic isomerization process in the 1960s, chemical isomerization of glucose under alkaline conditions was also evaluated (Tsao, et al. 1969, Parrish, 1970, and Katz and Ehrenthal,1972). The alkaline isomerization method encountered problems which could not be economically resolved. It was difficult to attain more than 40% fructose without forming non-dextrose and non-fructose degradation products. This reduces product sweetness and contributes to color development and off-flavor that could not be easily removed (Antrim et al. 1979). This is due to the lack of selectivity of the alkaline isomerization which allows the production of non-metabolizable materials such as psicose and objectionable, colored, materials. Consequently, chemical isomerization of glucose has not been employed commercially(Bucke, 1979).

IV. GLUCOSE ISOMERASE

A. Introduction

Glucose isomerase (D-glucose ketol-isomerase EC 5.3.1.5) has been used as a name describing different enzymes having glucose isomerase activity. At least four different types of glucose isomerase enzyme exist in microorganisms(Antrim et al. 1979, and Barker and Petch 1985).

The first report of an enzymic catalyzed interconversion between D-glucose and D-fructose was by Marshall and Kooi in 1957. They showed that sonic extracts and washed lyphilized cells of xylose-grown *Pseudomonas hydrophila* do in fact convert D-glucose to D-

fructose in the presence of arsenate. This enzyme had pH and temperature optima of 8.5 and 42-43^{o}C, respectively. It's affinity for glucose was lower than that of xylose (Km=0.5 and 0.003 M, respectively). Yamanaka (1963) observed the same activity with a xylose isomerase preparation made from D-xylose-grown cells of *Lactobacillus brevis*, but the addition of arsenate was not required.

A second glucose isomerizing activity from *Escherichia intermedia* which had no activity toward xylose and did not require xylose as an inducer was reported by Natake and Yoshimura (1963). This enzyme required arsenate and was confirmed by Natake (1968) to be glucose phosphate isomerase (D-glucose-6-phosphate ketol-isomerase EC 5.3.1.9).

A third glucose isomerase activity was described by Takasaki and Tanabe (1962, 1963), who isolated from *Bacillus megaterium* A1. This enzyme was NAD-linked and was named glucose isomerase (D-glucose ketol-isomerase EC 5.31.18). The enzyme was specific for glucose with pH and temperature optima of 7.8 and 35^{o}C, respectively.

A fourth type of glucose isomerase activity from *Paracolobacterium aerogenoides* was described by Takasaki and Tanabe (1964). It was similar to the previous (third type) one, but able to isomerize both glucose and mannose to fructose. It's pH and temperature optima were 7.5 and 40^{o}C.

The only enzyme that is of commercial importance is xylose isomerase because, in general, all xylose isomerases are heat-stable and do not require any regenerating cofactors such as NAD^{+} or ATP. The absence of regenerating cofactors makes the reaction a simple one and the high temperature stability allows the control of microbial contamination. Hence, offering a potentially feasible commercial operation (Antrim et al. 1979).

B. Enzyme Source

D-xylose isomerase is widely distributed. It is produced in most microorganisms capable of growing on xylose sources. Chen (1980) reviewed the glucose (xylose) isomerase producing microorganisms. Among these, *Pseudomonas hydrophila, Aerobacter cloacae, A. aerogenes, Escherichia freundii and E. intermedia* produced the enzyme which required arsenate for its activity. Hence, these sources were not commercially exploited. The heterolactic acid bacteria, *Lactobacillus brevis* produced the highest yield of the enzyme. Although, this enzyme appeared to have attractive properties for economic development, in particular it's low pH optimum, it is less stable at high temperature than it's competitors. Consequently it has not been developed commercially. Streptomyces species have been the most extensively studied and used as a source of the enzyme. At least 27 species of this genus have been reported to have the capability of producing GI. Among these, *S. albus* YT-4 and YT-5 were efficient producers of the enzyme. *S. wedmorensis* ATCC-21175 was used commercially in 1974. *S.olivaceus* NRRL B-3588 is used to produce the enzyme by Miles Laboratories,Inc. A mutant of this organism, NRRL B-3916 is used in an industrial scale by Miles-Cargill Inc. *S. glaucescenes* ETC 22794 was distinguished by it's ability to produce comparatively large amount of extracellular GI. Corn Products used a mutant of *S. olivochromogenes* ATCC-21114 in the production of High Fructose Corn Syrup. Some actinomycetes are potent producers of GI. Of these, *Actinoplanes missouriensis* produces the enzyme commercially used by Anheuser-Bush Inc., *Micromonospora* and *Norcardia* also produce GI as demonstrated by Standard Brands Inc. . *Bacillus coagulans* HN-68 is used commercially by Novo Industries, Inc. and *B. stearothermophilus* has been shown to produce a thermostable GI. Other organisms that have industrial importance include *Arthrobacter sp.* NRRL B-3726, 3727, 3728, and *A. levanicum.*

C. Enzyme Production

Most of the organisms require xylose to induce GI production. However, pure xylose is too expensive to be used in a commercial fermentation process(Chen, 1980). Therefore, many attempts were made to select organisms which do not require xylose for GI production. Takasaki (1966) isolated *Streptomyces albus* YT-4, YT-5, and YT-6 which could produce GI on xylan or xylan-containing materials such as wheat bran, corn hulls, and corn cobs. *Actinoplanes missouriensis* produced GI when supplied with beet molasses and corn steep liquor (Shieh, 1976 and 1977). *Arthrobacter sp.* NRRL B-3726, 3727, and 3728 were capable of producing the enzyme in the presence of glucose instead of xylose (Lee et al. 1972).

Various organic nitrogen sources including corn steep liquor, peptone, polypeptone, tryptone, meat extract, yeast extract, casein, and soy flour have been used for GI production by *Lactobacillus, Actinomycetes, Arthrobacter,* and *Bacillus.* Among these nitrogen sources, corn steep liquor appears to be the most efficient and is used most commonly. Inorganic nitrogen sources such as NH_4Cl, $(NH4)_2HPO4$, and $(NH4)_2SO4$ could be utilized by some of the enzyme sources (Chen,1980).

The pH of the culture medium used in GI production is in the neutral range (6.8-7.2) and the temperature range depends on the type of the organism being used. Most of GI producers are mesophiles. However, a few belong to the thermophiles such as *Bacillus coagulans* and *B. stearothermophilus.* Organisms usually require around 24 h to reach their maximum yield of GI (Chen, 1980).

In general, there is no set formula for the modern commercial production of GI. Each organism, and even each strain, needs its own special conditions for maximum enzyme

production. The enzyme is generally produced commercially by scaling up a submerged aerated fermentation in several stages (Antrim et al., 1979). The fermentation stages are described by Dworschack and Lamm (1972) as: (a) slant development, (b) culture development-two substages, and (c) final fermentation stage.

The most common method used in laboratories for the extraction of GI is sonification (Tsumura and Sato, 1965, and Strandberg and Smiley, 1971) Abrasive grinding is also employed for enzyme extraction (Natake and Yoshimura, 1964). However, mechanical disruption is time consuming and expensive and is not suited to large scale production. GI is easily liberated by autolysis using cationic detergents such as cetylpyridinium chloride, dimethylbenzylalkylammonium chloride or octadecyltrimethylammonium chloride. Lysozyme, toluene or combinations of them are also used (Chen 1980). Complete solubilization of *Streptomyces phaechromogenes* glucose isomerase by suspension of the cells in water at $5^{o}C$ for two weeks was observed by Korus and Olson (1977).

Most, but not all glucose isomerases(GI's) are intracellular enzymes (Schwimmer, 1981). Both extra- and entracellular GI's are produced by some microorganisms (Chen et al. 1979 and Mikhailov et al. 1986).

D. Molecular properties

Purified GI's from most of microorganisms studied have quite similar molecular weights ranging from 16500 to 191000 daltons (Antrim et al. 1979). However lower values, 52000, 80000, and 117000 are reported (Suekane et al. 1978 and Gong et al. 1980).

Molecules of GI isolated from various sources differ from each other in structure. For example, GI from *Bacillus stearothermophilus* consists of one polypeptide chain (Suekane et al. 1978). However, GI from *Bacillus coagulans* Strain HN-68 is reported to dissociate with sodium dodecylsulfate into three or four subunits with equal molecular weights,49000 (Danno, 1973). Moreover, the quaternary structure of GI from *Lactobacillus xylosus* (Yamanaka and Takahara 1977), *Streptomyces albus* (Huge-Angeletti, 1975), *S. griseofuscus* S-41 (Kasumi et al. 1981), and *Flavobacterium arborescens* (Boguslawski, 1983) exhibited a tetramer structure with four identical subunits, while GI's from *Actinomyces olivocinereus* Strain 154 (Rezchikov et al. 1980) has a quaternary structure consisting of four non-identical subunits. Dimer structures of the enzyme molecules were reported by Suekane et al. (1978) for GI from *Streptomyces olivochromogenes* and by Gong et al. 1980 for GI from *Actinomyces missouriensis*.

Some of the physicochemical properties of purified GI were studied. Sedimentation coefficient ($S_{20,w}$) varies from 6.98S for *Streptomyces olivaceus* Strain 13 GI (Mikhailov et al., 1986) to 10.2S for *Bacillus coagulans* Strain HN-68 enzyme (Danno, 1970). Values for partial specific volume (ml/g) and diffusion coefficient ($D_{20,w}$) were similar (0.690-0.736 and $4 \cdot 10^{-7}$ - $6.82 \cdot 10^{-7}$, respectively) as reported by Takasaki et al. (1969), Danno (1970), Suekane et al. (1978) and Kasumi et al. (1981). Stoks' radius of the enzyme studied ranged from 47 Å for GI from *Streptomyces griseofuscus*, S-41 (Kasumi et al. 1981) to 51 and 53Å for GI from *Streptomyces olivochromogenes* and *Bacillus stearothermophilus*, respectively (Suekane et al. 1978). The extinction coefficient ($E_{1cm}^{1\%}$) of the *Bacillus coagulans* Strain-68 enzyme is reported to be 10.6 while that of *Streptomyces griseofuscus* S-41 is 11.4. The isoelectric point (p^I) varied from 4.0 (Kasumi et al., 1981) to 4.95 (Mikhailov et al., 1986).

The amino acid composition of the purified GI's was determined by several investigators (Danno, 1970, Hogue-Angeletti,1975, Suekane et al., 1978, Rezchikov et al., 1980, Kasumi et al., 1981, Antrim and Auterinen, 1986, and Kwon et al., 1987). These studies revealed that all GI's

examined have the same kind of amino acids, except half-cystine which was absent in most of the GI's , but was present in GI from *Streptomyces albus* which has four residues per molecule (Huge-Angeletti, 1975) and GI from *Streptomyces griseofuscus* S-41 which contains six residues per molecule. Moreover, all GI's are characterized by having high acidic/basic amino acid ratio. i.e., the content of dicarboxylic acids (aspartic and glutamic) exceeds that of basic amino acids (lysine, arginine, and histidine).

The computed values for the contents of secondary structure of GI from *Streptomyces griseofuscus*, S-41 were as follows: alpha-helix, 40%; ß-form, 36%; and random coil, 24% (Kasumi et al., 1981)

E. Catalytic properties

The enzymes classified as D-xylose isomerase catalyze the interconversion of D-xylose to D-xylulose. Furthermore, all known xylose isomerases are thought to catalyze the conversion of D-glucose to D-fructose. In general all isomerases require the presence of some metal ion such as Co^{2+} Mn^{2+} or Cr^{2+} for their catalytic activity (Antrim et al., 1979).

Most of the GI's studied have a fairly high optimum temperature around $80^{o}C$. The enzyme from *Streptomyces phaeochromogenes* showed higher optimum temperature ($90^{o}C$) in the presence of Co^{2+} than the $80^{o}C$, which was optimumwithout Co^{2+} (Chen, 1980). In an extreme case, Takasaki (1974) through the use of thermophiles, isolated GI's that could react at a temperature as high as $100^{o}C$. The arsenate-requiring GI's from *Aerobacter aerogenes* Strain HN-56, and *Escherichia intermedia* Strain HN-500 have significantly lower temperature optima (40-$50^{o}C$) than others (Natake and Yoshimura, 1963).

The pH optimum of GI is generally greater than pH 7 (Takasaki et al., 1969, Strandberg and Smiley, 1971, and Scallet et al., 1974) with a very broad peak. However, GI's with lower pH optima are also known such as the *Lactobacillus brevis* enzyme, pH 6.5 (Yamanaka, 1968), and the *Bacillus coagulans* , Strain HN-68, pH 7.0 (Danno, 1970). At the other extreme, the enzyme from *Streptomyces phaeochromogenes* has been reported to have a pH optimum of 9.0-9.5 (Tsumura and Sato, 1965a). Interestingly, Tsumura and Sato (1967) were able to modify the pH optimum of *S. phaeochromogenes* enzyme by the addition of 10^{-3}M Co^{2+} in the fermentation medium.

GI's from different sources showed great variations with regard to their specificity. Some of GI's are specific for D-glucose and D-xylose. Others, in addition to glucose and xylose, isomerize D-ribose or 2-deoxyglucose. Some less specific GI's are known to isomerize L-arabinose, L-rhamnose, D-allose, and D-galactose. Moreover, this specificity is not even genus-specific (Chen, 1980). Indicators have shown steric correlation among these carbohydrates, with the exception of L-rhamnose and 2-deoxyglucose, since these sugars have a C1 conformation and the hydroxyl group on carbon 2 is in the equatorial position. When the hydroxyl groups of carbon 3 and 4 are in the equatorial position, as in glucose and xylose, maximum isomerization is obtained (Sanchez and Smiley, 1975). It is generally assumed that D-xylose is the natural substrate for this class of enzymes because the enzyme has a higher affinity for xylose than its other substrates. However this rule cannot be generalized for all isomerases as GI's from *Streptomyces albus* NRRL 5778 and *Actinoplanes missourriensis* have higher affinity toward D-glucose than D-xylose (Antrim et al., 1979).

In general, GI requires a divalent cation such as Mn^{2+}, Co^{2+}, or Mg^{2+} for its catalytic activity. However, the same ions do not affect every isomerase in the same manner. In fact, an ion

may be necessary for the activity of an isomerase from one source but may have no effect on an isomerase from another source. Both Mg^{2+} and Co^{2+} were required for the full activity of GI's from *A. missouriensis*, *B.coagulans* Strain HN-68, *Aerobacter cloacae*, *S.albus*, and other Streptomyces species (Antrim et al., 1979). For the enzyme of *S. flavogriseus*, Mg^{2+} was more effective than Co^{2+}, but the reverse was true for GI of *B. coagulans*. Like the GI from *L. brevis*, the enzyme from *B.coagulans* required Co^{2+} to isomerize glucose and ribose but Mn^{2+} to isomerize xylose. It was concluded that the active form of the enzyme was the one which contained Co^{2+} or Mn^{2+} and proposed that binding of Co^{2+} and Mn^{2+} caused suitable conformation changes in the catalytic site of the enzyme for glucose and xylose isomerization, respectively (Danno, 1971).

An important feature for the enzyme of *Arthrobacter Sp.* was that it did not require Co^{2+} for activity or stability (Lee et al., 1972). It has been reported that Mg^{2+} is required for activity, while Co^{2+} is required for enzyme heat stability (Aschengren, 1975).

F. Enzyme inhibitors

The catalytic activity of GI is generally competitively inhibited by Ag^{+}, Hg^{2+}, Cu^{2+}, and Ca^{2+} (Danno et al., 1967, Takasaki et al., 1969, Scallett et al., 1974, Ascengren, 1975, and Kasumi et al., 1981). Other inhibitors are the sugar alcohols, xylitol, arabitol, sorbitol, and mannitol (Danno,1970, Scallett et al., 1974, Yamanaka and Takahara, 1977, and Kasumi et al., 1981). Tris (hydroxy methyl) aminomethane has been shown to be a competitive inhibitor of GI from *B. coagulans* HN-68 with a K_I of 0.30 - 0.75mM, which is smaller than the Km value of the substrate D-xylose (Danno, 1970). The same inhibitory effect was reported by Kasumi et al., (1981) for GI from *S. griseofuscus*, S-41. Para-chloromercuribenzoate, monoiodoacetic acid, sodium azide, potassium cyanide, sodium fluoride, and 2-mercaptoethanol had no inhibitory effect on the activity of GI, while EDTA caused a significant loss of activity (Kasumi et al., 1981).

For some but not all GI's, inhibition by Ca^{2+} conflicts with the requirement of this metal for the stability and functioning of most bacterial amylases used in starch thinning operations at the beginning of the process leading to the production of HFCS (Schwimmer, 1981). Most GI's are sensitive to oxygen, but the GI from a species of Streptomyces genus is not (Tsumura and Sato, 1965).

G. Enzyme kinetics and mechanisms

It has been generally assumed that the reaction catalyzed by D-xylose isomerase follows the simple reversible Michaelis-Menten mechanism (Takasaki, 1967, Havewala and Pitcher, 1974, and Lloyd and Khaleeluddin, 1976). A realistic kinetic model which attempts to describe the interconversion of D-glucose to D-fructose must also consider the complex mutarotation of the reactants and the stereospecificity of the isomerase to the alph-D-glucose anomer (Schary and Rose, 1971 and Mckay and Tavlarides, 1976).

Through the use of tritium-labeled substrate, Rose et al. (1969) suggested that the enzyme from *L. brevis* acted via a mechanism involving a *cis* -enediol substrate intermediate, and the required metal ion (Mn2+) acted as an electrophilic moity that polarizes the carbonyl group of the aldose sugar during the ces-enediol substrate intermediate step. Young et al. (1975) further examined the interaction of Mn2+ with GI from *L.brevis* and *Streptomyces sp.* using magnetic resonance studies. The authors found that carbon 1, carbon 2, and carbon 5 protons of the enzyme-bound substrate (alpha-D-xylose) are equidistant, 9.1 $\pm$ 0.7 Å, from the bound Mn2+. This distance is too great for direct coordination. Moreover, small ligands such as water molecules or a large portion of the protein intervene between the bound metal and the bound substrate in the active ternary complex. Thus, the results suggested that the coordination of the metal ion in

activating the enzyme was structural rather than catalytic. Similar conclusions were reached by Danno (1971) for the *B. coagulans enzyme.*

The kinetics Km values for GI greatly varied according to the source of the isomerase studied. The reported Km for glucose ranged from 86 mM for GI from *Streptomyces albus* NRRL B-5778 to 920 mM for the *Lactobacillus brevis* enzyme. The same wide variations were reported for the Km values for xylose. In this case the km values were lower than that of glucose and ranged from 5 to 93 mM for *Lactobacillus and Streptomyces* enzymes, respectively (Sanchez, and Smiley, and Yamanaka, 1968).

The equlibrium constant (Keq) of the reversible interconversion of glucose to fructose reaction varied with temperature. The fructose concenteration at equilibrium increased from 46.5% at 30^{o}C to 54.7% at 85^{o}C (Lloyd and Khaleeluddin, 1976). However, results differ from one study to another. The fructose concenteration at equilibrium at 60 ^{o}C was reported to be 49.9, 50.4, 50.7 and 53.4% by Lloyd and Khaleeluddin (1976), Havewala and Pitcher (1974), Jorgensen, et al. (1988), and Takasaki (1967), respectively. It is worth mentioning that the Takasaki studies were made using dilute sugar solution while the Lloyd and Khaleelulddin (1976) and Havewala and Pitcher (1974) studies were carried out using relatively concentrated sugar solutions. Hence, the apparent differences of the above equilibrium results might arise from variation of activity coefficients in dilute and concentrated sugar solutions, or from chelation effects (Hamilton et al., 1974).

V. ENZYME IMMOBILIZATION

A. Introduction

Enzyme immobilization is used to fix the enzyme so it can be retained. Another, but broader definftion is that an immobilized biocatalyst consists of enzymes, cells, or organells which are in a state that permit their reuse (The Working Party on Immobilized Biocatalysts-The European Federation of Biotechnology, 1983).

The advantages of enzyme iommobilization include recovery of the immobilized enzymes, closer control of the reaction, and less expense in terms of overall processing costs (Weetall, 1985). In addition to the previous advatages, IGI permits the isomerization process to operate continuously which in turn reduces the amount of enzyme used, contact reaction time, and by-product build up. This affects enzyme activity, stability and productivity, as well as cost of product purification.(Carasik and Carroll, 1983)

The penetration of immobilized enzyme systems into industrial operations is restrained by several consequential factors. These limitations include: a) the comparatively low cost of soluble enzymes for many industrial processes, coupled with traditional attitudes that are slow to change, b) the capital costs involved in introducing new equipment to existing process plants, c) the nature and cost of the immobilizing support and the immobilization process, including losses of activity at this stage, d) cleanup costs which are usually related to the stability of the immobilized enzyme. and e) in food operations, matrix compatablity with the substrate and material being processed must not be overlooked to avoid food adulteration (Godfrey, 1983 and Swaisgood, 1985).

B. Methods of immobilization

Methods of enzyme immobilization have been extensively reviewed (Mosbach, 1976 and 1987, Goldstein and Manecke, 1976, Bucholz, 1979, Pitcher, 1980, Trevan, 1980, Swaisgood, 1985, and Scott, 1987). More than 100 immobilization techniques have been elaborated. These techniques can be divided into the following five groups: (1).Covalent attachment of enzymes to solid supporrts. Porous glass and ceramics, stainless steel, sand, charcoal, cellulose, synthetic polymers, and metalic oxides have been utilized. (2) Adsorption of enzymes on solid supports such as ion exchangers. (3) Entrapment of enzymes in polymeric, organic and biological gels. (4) Cross-linking of enzymes with bifunctional reagent. Among the most popular cross-linkers are glutaraldhyde, dimethyladipimidate, dimethyl suberimidate, and aliphatic diamines. (5) Encapsulation of enzymes so that the enzymes are enveloped within various forms of membranes that are impermeable for enzymes and other macromolecules, but permeable for low molecular wieght substrates and products (Klibanov, 1983). However, Swaisgood (1985) in his review pointed out that enzymes retained by microencapsulation or with a semipermeable memberane are, in effect, soluble enzymes; hence these methods do not permit potential structural stabilization by matrix-enzyme interaction.

C. Glucose isomerase immobilization

It is the GI immobilization technology that permits the production of HFCS which is by far the most successful application of immobilized enzymes over the world.

GI immobilization processes have been reviewed by Hamilton et al. (1974), Bucke (1977), Antrim et al. (1979), Chen (1980), MacAllister (1980), Verhoff et al. (19..) and Jensen and Rugh

(1987). In general GI immobilization is outlined as follows: a) after the maximum concentration of the enzyme is reached in the fermentation process, the cell mass is harvested simbly by filteration. Then, the cell mass can be processed in several alternative ways to make IGI. b) the cells may be stored, usually at low temperature, or heat-treated until subsequent processes. The untreated cell mass may contain lytic enzymes which would tend to solubilize the isomerase when exposed for a long period of time. c) The cell mass with or without heat treatment, to stabilize the enzyme against solubilization, can be dried. Hot air drying is very effective if the hot air has a wet bulb temperature well below that which corresponds to the onset of rapid heat denaturation of the enzyme. Formation of large masses of the dry cell complex should be avoided. Mixing filter aid of various kind and/or crosslinking agents with the wet cell mass before drying is sometimes effective in developing dry immobilized enzymes which are of rigid and open structure readily contacted throughout its mass by the solution of reactants. d) GI can be obtained from the aquous suspension of cells containing GI by sonication or treatment with surfactants of the quaternary ammonium salt type. The insoluble cell material is then removed by filtration or centrifugation and the solution containing GI is contacted with an insoluble carrier, to which the enzyme is attracted and held. The insoluble carrier-glucose isomerase combination can then be removed from the suspension by filteration, washing, drying and adjustment to particle size distribution as needed for a given reactor configuration and reactor flow pattern. The degree of GI purity and the enzyme/carrier ratio contribute to the efficiency of the IGI when the other parameters are constant. e) cells containing GI or solution of GI can be suspended or dissolved in solution of monomers which can be polymerized to entrap the cell enzyme complex or the soluble enzyme within a polymer gel matrix. This material can be further processed to form a dry solid structure which allows penetration of the solution and flow of reactants and products to and from the reactive site of the enzyme, but prevents the enzyme-cell complex from leaking out into the reactant solution.

Generally, in making IGI it is desirable to attain a high specific activity because that tends to decrease the net cost of the carrier per unit substrate processed (other things being equal) and

for a given reactor volume, tends to decrease the residence time in the reactor. The latter is especially important because the condition of isomerization, high pH and temperature tend to cause formation of undesirable color, taste, acids, and psicose, so the less time under those conditions the better.

The methods of immobilizing GI which have been described in the literature can be classified as:

1. Glucose isomerase immobilized within bacterial cells

2. Cell-free glucose isomerase immobilized by sorption onto an insoluble carrier

3. Cell-free glucose isomerase immobilized by covalent bond formation between the enzyme and the insoluble carrier.

4. Entrapment of glucose isomerase, with or without bacterial cells within an insoluble matrix which is permeable to glucose and fructose, but from which the enzyme can not be extracted into the reaction solution phase.

1.Glucose isomerase immobilized within bacterial cells

Whole cell immobilizations are rather simple and cheap techniques. However, generally low potency enzymes are produced due to extraneous cellular material. Leaching of entrained impurities from the cellular material during use contribute to high purification costs, and problems regarding structural integrity and mass transfer limitations often arise(Antrim et al., 1979, and MacAllister, 1980).

The earliest commercially used enzyme immobilization technique was the heat fixation of Streptomyces species reported by Takasaki et al. (1969). After 24 hr incubation at 30^{o}C, the

broth was heated at 65^{o}C for 15 minutes and the heated cells were recovered by filteration. This product was used on a small industrial scale by Clinton Corn Processing Company from 1967 until about 1970. This method was also applied to cells of *S. phaeochromogenes* and *S. flavogriseus* (Chen,1979, Ellaiah, 1987, and Ryu et al. 1977). Spray dried cells of *Actinoplanes missouriensis* were used by Anheuser-Busch Inc. in an industrial process(Scallet et al. 1974). The effect of heat treatment was ascribed to selective inactivation of lytic enzymes which would otherwise tend to break up the cell wall structure, liberating the enzyme. In addition, Van Kuelen et al. (1981) reported that permeability of the cell membrane of Arthrobacter species was increased by heating. Increasing the permeability of the cell would tend to reduce the diffusional resistance, and therefore increase the efficiency of the intracellular bound enzyme.

Modification of the basic heat treatment of cells involved treating the aqueous cell suspension prior to heating with various inorganic salts at concentration usually below 0.1M (Lamm,1974), synthetic polyelectrolytes (Lee and Long, 1974, and Bungard et al. 1979), and citrate solutions (Tsumura and Kasumi, 1977)

GI was fixed within the cells, which produced it, by adding glutaraldhyde to the suspension of cells which could then be recovered by filteration, washed, and used in continuous flow reactors to catalyze the isomerization reaction (Zienty, 1973). In another process, the suspension of GI containing cells of *Bacillus coagulans* NRRL B 5636, was homogenized, and then treated wih glutaraldhyde to immobilize the enzyme within the solid phase formed. This material was then broken up mechanically, diluted with one volume of water, and flocculated with a cationic flocculant to give a clear water phase. The mixture was filtered and the filter cake was ventilated to remove some of the free water. The filter cake was granulated on 1-mm screen and air dried at 50^{o}C. The dried product is ground and sieved to 100-350 um particles, as needed for continuous fixed bed catalyst isomerization reactors (Amotz et al., 1976). This product is produced by Novo Industries under the trade name of Sweetzyme® and used commercially for

the production of HFCS. The Novo enzyme went through several developing stages which paralleled the shifting from conventional batch reactors to the more economical continuous column reactors (Carasik and Carroll, 1983). Recently, SweetzymeT® was introduced by simply using another microorganism, *Streptomyces murinus.* The new immobilizate claimed to be able to produce 10 ton syrup dry substance per kg enzyme under optimal industrial conditions, which makes it more productive than previous Sweetzymes (Jorgensen et al., 1988). Similarly, Miles Laboratories Inc. introduced a whole-cell immobilized enzyme known as Takasweet®FM (Lantero,1982). However, the microorganism and the crosslinking system were different. Cells of *Flavobacterium arborescens* were used and the crosslinking system consisted of polyethyleneimine, chitosan, and glutaraldhyde. In another study (Hu, 1986), a multifunctional crosslinking agent, epoxypolyamine (EPA), was used to produce an immobilized whole cell containing GI. Cells of *Actinoplanes missouriensis* ATCC 14538 were cross linked with EPA and the crosslinked precipitate was dried at 60^{o}C and broken or shaped into 2-3 mm particles.

Entrapment of cells in gels or fibers has been widely employed in many organisms containing GI. Cells of several Streptomyces species including *S. griseus, S..olivaceus, S. phaeochromogenes,* and *S. spp. S*-41-10 were reported to be entraped in polyacrylamide gels (PAG) and used in a continuous or batch process (Ohwaki, 1975 and Lai, 1977).

Several succesful techniques were evolved as combinations of the basic methods. Cells of *Streptomyces phaeochromogenes* were immobilized by first heating the cells, then stirring them with a slurry of hide collagen, adjusting the pH to 12, and casting the mixture on a surface. The resulting preparation was tanned with glutaraldhyde or formaldhyde (Vieth et al., 1973). A commercially utilized whole cell IGI known as Maxazyme® GT-IMMOB was prepared at Gist-Brocades N.V. (Hupkes and Tilburg, 1976). *Actinoplanes missouriensis* NRRL B-3342 mycelium fixed enzyme,heated at 72^{o}C pH 8.6, was mixed with gelatin solution (final gelatin concentration was about 8%) and heated above 40^{o}C. The gelatin enzyme mixture was transferred into a cold

water immiscible solvent (e.g. butyl acetate, butanol, or amyl alcohol) and the suspension was stirred. The coagulated spherical enzyme containing gelatin particles was collected, washed with ethanol, suspended in cold water , and crosslinked with a solution of 2.5% glutaraldhyde. Finally the excess of glutaraldhyde and other impurities were washed out with tap water and the immobilized enzyme particles were dried after washing with alcohol or acetone, stored in either a sodium chloride solution, or in a 0.3% formaldhyde solution. Similar IGI preparation, using the same organism, was described by Linko et al. (1977). However, the entrapment in this case was in cellulose fiber. This IGI preparation exhibited good operationl stability, with 45 days half life (the time at which the enzyme lost half of its original activity) in the presence of Co2+, in a continuous isomerization process.

Immobilized cells of *S. phaeochromogenes* with a half-life of 60 days were prepared by heating the cells at 70-80^{o}C, lyophilizing, mixing with gelatin or sodium caseinate , and then steeping in acetone containing glutaraldhyde to gelatinize (Natkajima, 1978). Another similar technique, but involving a new aggregation reagent, chitosan, was prepared by Kasumi et al. (1979). A cell suspension of *streptomyces spp.* (from Godo Shusei Co. LTD.) was mixed with an equal volume of 0.1% chitosan solution, added dropewise with vigorous agitation, and the mixture was allowed to stand for 30 minutes. Then, the aggregated cells were collected and dispersed in another equal volume of 0.1% chitosan solution. After 1 hr, cell flocks were harvested by filteration. Ten percent gelatin (to dry cell weight) and water were added so that the moisture content of the whole mixture was 75%. After kneading well in a mortar, the mixture was forced through a press with 1 mm holes and cut into 1.0-1.5 mm lengths when half dried. Cells granules obtained were air dried at room temperature overnight, then immersed in 0.25% glutaraldhyde solution (20 ml : 1g dry cells) for 40 minutes at room temperature with occasional shaking. Finally, the granules were washed throughly with water and dried in the room. This product showed a considrable durability (37-40 days half life) in a continuos isomerization process, and was much superior to the free enzyme in heat and pH stability and resistance to

inhibitory effects of several metal ions. However, the immobilized enzyme was thought to be influenced by diffusion as shown from results of kinetic and activation energy studies.

A much more stable (80 days half life) whole cell GI preparation was reported by Young et al. (1980). Cells of *Streptomyces species* K-14 (KFCC 3501) were mixed with aqueous gelatin solution and cellulosic materials, such as DEAE-cellulose or wood cellulose powder. The mixed enzyme paste was then cast into the desired shape, usually a pellet form, and treated with 5% aquous glutaraldhyde solution, pH 4.0, at room temperature for 1 minute for crosslinking. The gel formed was washed throughly with distilled water and dried overnight at room temperature. Finally, the dried IGI was ground and sieved to obtain the desired size.

Recently, gelatin entrapped cells of *Lactobacillus brevis* 74 were reported (Ananichev et al., 1986). The method involved heat treatment, crosslinking with glutaraldhyde, stabilization with tannin, and incorporation into gelatin. This IGI preparation was used in a continuous column reactor for obtaining a glucose-fructose syrup under laboratory conditions. The half life of the preparation was 43 days at 60^{o}C. However, the half life was calculated by extrapolation of the data and no detailed information was presented regarding the isomerization conditions.

Calcium alginate entraped IGI was prepared by Ellaiah (1987). The wet cell mass was suspended in 2% (W/V) sodium alginate solution to give a 50% slurry. The slurry was then extruded into a stirred 0.1 M calcium chloride solution, to form beads of 2-3 mm diameter. The beads were centrifuged and washed with sterile phosphate buffer, pH 7.0. Bead size varied according to the concentration of alginate or the diameter of syringe needle used in extrusion.

Whole cell IGI was prepared by mixing cells containing GI with a photopolymerizable resin and then irradiating the mixture with U.V. light or gamma rays (Fukui, et al., 1977). A glass forming monomer was used to produce IGI using *Streptomyces phaeochromogenes* cells and applying

the radiation-induced polymrization method (Kumakura et al., 1979). Hydrophilic monomers of 2-hydroxyethyl methacrylate and 2-hydroxyethyl acrylate were used. The mixture of cells, monomers, and salt solutions was poured into a test tube and quickly shaken. This resulted in a separated phase of fine monomer particles due to the salting out effect. Immediately, the test tube was cooled at - 78^{o}C, then gamma ray irradiation (total dose 10^{6} R) from a ^{60}Co source was carried out for 1 hr at low temperatures. The diameter of particles was varied by changing the irradiation temperature or the concenteration of the monomer and salt solutions. The magnitude of the enzymatic activity increased with decreasing particle diameter (1000-2000u).

2. Cell-free glucose isomerase immobilized by sorption onto an insoluble carrier.

Adsorption is not only simple, but also the oldest technique to immobilize enzymes, dating back to 1916 when Nelson and Griffin used both charcoal and aluminum hydroxide to adsorb invertase. Since that time, a wide range of organic and inorganic substances have been utilized as supports for enzyme adsorption (Pitcher, 1980). Generally, conditions of low ionic strength and a pH at which no enzyme elution occurs are required for use of these type of immobilized enzymes (Antrim et al., 1979).

Cell-free GI's have been adsorbed on anion exchange celluloses (Sipos, 1973, Thompson et al., 1974) and anion exchange sephadex (Tsumura and Ishikawa, 1967). DEAE-cellulose immobilized GI was prepared (Sipos, 1973) at Clinton Corn Products and replaced the heat fixed cells in Clinton's filter press reactor (Schnyder, 1974). This adsorbed purified GI represented a major improvement. The enzyme loading was high, the syrup contamination with the solubilized cell material was avoided, the support material (DEAE-cellulose) was not expensive and it's cost could be reduced by reuse. The recovery of fermentor activity in

the immobilized form was good (up to 90%), and the use of this product minimized pressure drop problems when used in shallow bed reactors. However, the isomerase product was still a very soft gel and could not be used in deep bed reactors which are considered by the industry to be simpler in design and operation. A granular form of the enzyme was therefore developed (Antrim and Auterinin, 1986) to allow use both in existing deep bed reactors and smaller second generation reactors. The developed IGI was prepared using highly purified GI from *Streptomyces rubiginus* ATCC 21175, which was also the previous IGI enzyme source. The purified GI electrostatically adsorbed onto a granular DEAE-cellulose -polystyrene-TiO2 composite. This provided a stable enzyme with a half-life in the neighborhood of 70 days, and exremely high productive capabilities with greater than 9 metric tons of 42% fructose syrup per Kg of immobilized enzyme. However, the system is still susceptible to accidental changes in process conditions.

DEAE-celluloses were also utilized for the immobilization of partially purified GI from *S. flavogriseus* (Suekane et al., 1978), *B. coagulans* (Huitron and Limon-Lason, 1978), *Actinomyces olivocinereus* (Ananichev et al., 1980), and *Actinoplanes missouriensis* (Chen et al., 1981). The Actinoplanes product retained over 70% of the original activity and its half-life was around 1000 hr at $60^{o}C$. Moreover, the product was less sensitive to pH changes and Co^{2+} was not required for enzyme stability.

Derivatives of 3-aminobenzene-boric acid of CM-cellulose and DEAE-cellulose were used to adsorb GI and were utilized in continuous isomerization processes (Kanbayashi and Hasegaw, 1977). Moreover,GI's have been immobilized by adsdorption on other ionic materials such as films of collagen or zein (Vieth et al., 1974), coordinated to polymeric materials activated by salts of Ti, Zr, Fe (Emery et al., 1974), adsorbed onto Chitin (Stanley et al., 1976), or glutaraldhyde-crosslinked chitin (Chen et al., 1983). The chitin-immobilized isomerases exhibited a good rigidity

which resulted in a low pressure drop when the immobilized product was used in a continuous isomerization process.

GI's have been adsorbed on various inorganic materials such as basic $MgCO_3$ (Heady and Jacaway, 1974), porous ceramic bodies, porous alumina (Messing, 1974 and 1975), and blends of MgO-Al2O3 (Eaton and Messing, 1976).

Miles Kali-Chemi offered, under the trade name Optisweet 22, a purified soluble GI adsorbed to fine SiO2 beads (Weidenbach et al., 1983). The principle of the method is the following: purified isomerase from *Streptomyces rubiginosus* is adsorbed to spherical silicate particles, 100-200 um, and crosslinked with glutaraldhyde. This product, like the Clinton's enzyme, is of high potency and required an intensive syrup purification. Moreover, the column height is limited to 0.4 meter.

Immobilized GI adsorbed on porous silochrome under vacuum and additionally treated with a tannin solution, to form associates that were more strongly adsorbed in the carrier pores, and with glutaraldhyde to cross-link the protein molecules was prepared (Ananichev et al., 1984). Also, an adsorbed enzyme on phenol-formaldhyde resin was reported (Yokote et al., 1974 and Kimura et al., 1978).

3.

3. Cell-free glucose isomerase entrapped in an insoluble matrix

The first immobilized cell-free GI was prepared by entrapping the enzyme from *Streptomyces phaeochromogenes* NRRL B-3559 in polyacrylamide gel. The polymerized gel containing GI was passed through a stainless steel sieve (24 1-mm holes/cm^2) and washed with buffer. The composition of the gell did not affect the amount of isomerase entrapped nor did it affect the enzyme activity. About 40% of the enzyme added to the system was entrapped and one third of this was assayed as GI. The PAG-entrapped enzyme was sensitive and inactivated in the reaction mixture, which limited its use (Strandberg and Smiley, 1971).

Simple entrappment of cell-free isomerase enzyme extract from various sources have been flocculated in the presence of filter aid and synthetic polyionic floccuating agents (Nystrom, 1976). More complex procedures have been utilized, such as extruding partially purified GI from Streptomyces species with cellulose triacetate in methylene chloride. About 45% of the enzyme offered was recovered in an active form in the resulting fibers. The immobilizeed enzyme was used for the isomerization of industrial glucose solutions and exhibited good stability (70 days at 65^{o}C half-life) in a radial reactor. However, diffusional limitations and high enzyme concentration inside the microcavities of the fiber decreased the efficiency of the entrapped enzyme.

Both entrapment and covalent binding techniques were utilized to immobilize GI from Streptomyces species (Kasumi et al., 1977). The purified enzyme was entrapped in chitosan in the presence of water-soluble carbodiimide. Chitosan, a deacylated polysaccharide prepared from chitin, in the dissolved state is a strong flocculant because of its polycationic caracteristics. The chitosan-bound enzyme is easily solidified by adjustment of the pH from acidic to alkaline. Water-soluble carbodiimide catalyzes the formation of covalent bonds between the amino groups of chitosan, derived from glucoseamine upon hydrolysis of chitosan, and carboxyl groups of the

enzyme. The activity yield of chitosan-bound GI was found to be only 32% which is fairly low. The isomerization power of the immobilized enzyme , in a continuous operation , went down to 60% of the original value after 5 days. However, no significant drop of activity was noticed from day 5 up to 20 days of operation.

4. Cell-free glucose isomerase immobilized by covalent bond formation between the enzyme and the insoluble carrier.

Probably the most frequently reported methods of enzyme immobilization in the literature are the various types of covalent attachment of enzymes to support materials. However, covalent bonding has not found as extensive use in the food processing industry as one might expect from the numerous articles describing different chemical coupling schemes. This may be due to the health hazard criteria for most of the coupling reagents as food processing aids. Other chemical compounds, or at least the resulting derivatives, are probably quite safe but, the testing program to establish this fact, in light of the regulatory situation, may be prohibitively expensive. Finally, the many covalent bonding techniques are complicated and expensive. Undoubtedly, the greatest advantage of covalent coupling is the durability of the derivative. In addition, various pH, ionic strength, and temperature will not normally cause the enzyme elution problem that can plague certain adsorbed enzyme systems (Pitcher, 1980 and Swaisgood, 1985). Another distinct advantage of binding soluble enzyme to an insoluble support is that the support itself has good structural integrity. Therefore, by careful selection of the support matrix to immobilize enzymes, little emphasis has to be placed on improving particle integrity (Verhoff et al., 19).

Isomerases have been reported to be covalently coupled to silicate derivatives. Strandberg and Smiley (1972) prepared a glass-bound GI by covalent binding a partially purified GI from *Streptomyces phaeochromogenes* NRRL B-3559 to diazotized porous glass beads in

0.05M sodium borate buffer pH 8.5 at $4^{o}C$ for 4 days. About 41-47% of the bound enzyme activity was expressed and the highest activity was with approximately 200 units/g glass. Stability of the glass-bound enzyme was tested in continuous column operation and the half life was about 12-14 days at $60^{o}C$. The loss of activity was attributed to dissolution of the exposed surface of the porous glass. This dissolution tended to increase as pH of the substrate and surface area of the glass increase.

Lee et al. (1976) slightly improved the previous method by using ZrO_2-coated glass beads. The binding method involved silanization of the coated glass beads followed by glutaraldhyde coupling of the isomerase. The enzyme-bound preparation retained 56% of the free enzyme activity with an enzyme loading of 48mg/g of glass. The stability of this bound enzyme at $60^{o}C$ in a continuous isomerization process was tested and the half life was 16 days in the presence of Co^{2+}.

Ananchiv et al. (1980a) immobilized purified GI from *Actinomyces olivocinereus* on cellulose, CM-cellulose, spheron, aminated silochrome, porous glass beads, and nylon activated with cyanuric chloride, carbodiimide, $TiCl_4$, and glutaraldhyde reagents. Aminated porous glass and silochrome proved to be the best carriers, binding about 70% of the added amount of protein. The aminated silochrome bound enzyme preparation was used in a continuous column isomerization process (Ananchiv et al., 1980b). During 32 days of continuous operation, the activity of the preparation decreased to 84% of the original level. The isomerization conditions were 2.0M glucose in 0.1 phosphate-citrate buffer pH7.5 , 50mM Mg^{2+} at $60^{o}C$ and flow rate of 40 ml/hr. No cobalt was added.

The enzyme from the previous source was immobilized by the same binding method, but utilizing macroporous silica carriers, silica gels and silochromes of different pore sizes (Voroshilova et al., 1984). The immobilized enzyme with pore sizes of 50-80 nm containing about 60 mg of

bound protein per 1 g carrier had the maximum activity. The immobilized enzyme showed greater pH and thermal stabilities than the soluble enzyme.

A novel microporous PVC-silica support was developed by Amerace Corporation and used for the immobilization of GI from *Bacillus coagulans* NRL-5666 (Goldberg et al., 1979). The support is noncompressible under normal conditions with a porosity of 70-80% and a pore size of 0.2-2.0 um range. It is extremely hydrophilic, has a negative charge, and a surface area of 80 m^2/g. The microporous support has received FDA approval for direct food contact. The immobilization method involved activation of the PVC-silica sheet by polyethyleneimine (PEI) of approximately 40000 molecular weight, then, rinsing away the excess PEI and cutting the sheet into 47mm diameter discs having a thickness of about 0.5 mm. The discs were mounted in reactors comprised of 47 mm Millipore Swinnex filter housings or were molded into a stacked disc configuration. The system was filled with 2.5% glutaraldhyde at pH 9.5 for 1 hr. Excess glutaraldhyde was removed from the reactor by flushing with pH 7.5 buffer solution containing 2g/l $MgSO_4.7H_2O$ and 2g/l $NaHCO_3$. After maintaining the pH at 7.5, the enzyme solution was recycled through the reactor for 1 hr. Excess enzyme was removed by flushing the reactor with the same buffer and collecting the effluent. The entire procedure was carried out at room temperature. In the above sequence, the glutaraldhyde cross-linked PEI rendering it totally insoluble and acted as a leash for the enzyme pendent group with which it was subsequently reacted. Recovery yields of 90-100% activity of the immobilized enzyme were achieved, with about 50% of the soluble enzyme recovered after immobilization. This bound enzyme was 78% more efficient than that prepared on CPG beads and the half-life was 920 hr in an Amerace flow-through reactor. Conditions of isomerization were 40% dextrose solution (w/w) containing 2g/l $MgSO_4.7H_2O$, 1g/l $NaHCO_3$, and 1 g/l $NaHSO_3$ with a pH of 7.5 and an operating temperature of 60^oC.

An anion exchange resin of phenol-formaldhyde, Duolite A7, was successfully used for the covalent immobilization of partially purified GI from *Streptomyces phaeochromogenes* , a product of Nagase Sangyo CO Ltd. (Kimura et al., 1978). The Duolite A7 was activated by the bifunctional crosslinking agent, triazinyl chloride to form 2,4-Dichloro-s-triazinyl Duolite A7 which was continuously stirred with GI solution in 0.05M sodium bicarbonate buffer, pH 8.0, containing o.o5M $MgSO_4$ and 1mM $CoCl_2$ for 16 hr at 5^{o}C. The amount of protein bound was 40.3 mg/ml resin with 36.6% retention of activity. The immobilized GI was used for continuous isomerization of 40% glucose solution at pH 8.2 and at 60^{o}C in the column reactor. The half life was 40 days, which is somewhat better than that obtained with the same enzyme adsorbed on the same resin.

UOP,Inc. has been granted several patents on immobilizing GI extracted from *Actinoplanes missouriensis* on derivatized inorganic support. Rohrbach (1981) described a method in which alumina particles (60-80 mesh) were coated with polyethyleneimine prior to treating with glutaraldhyde. The enzyme was then added to the alumina-polyethyleneimine glutaraldhyde complex. Similarly, Rohbach and Lester (1981) immobilized GI to alumina particles that were first treated with polystyrene, then, with nitric acid, and finally reduced to obtain an amino-polystyrene-alumina complex. This complex was then treated with glutaraldhyde prior to adding the enzyme.

The transition metal-link method was used to immobilize partially purified GI from *Lactobacillus brevis* on microcrystalline cellulose (Kent and Emery, 1974). Microcrystalline cellulose designated as Segmacell Type 19 was mixed with titanium chloride (15% w/v) in 15% hydrochloric acid. The suspension was dried at 45^{o}C and washed with 0.02M, pH 7 Tris buffer. Coupling was effected by stirring the washed powder and the enzyme preparation at 4^{o}C for 18 hr. Then excess enzymes were washed by Tris buffer and 0.5M sodium chloride in the same

buffer. The enzyme product was capable of functioning satisfacforily, in the presence of Mn^{2+} and Co^{2+}, in aqueous glucose syrups of up to 30% w/v. The operating pH could be as low as pH 5 (optimum pH 6). However, the immobilized enzyme had a very low density.

Korus and Olson (1976) immobiled GI by encapsulating the cell-free enzyme in a hollow fiber reactor. The anisotropic hollow fiber memberanes used in that study were non-cellulosic inert polymeric fibers which would be superior to cellulosic membranes with regard to thermal and chemical resistance, and resistance to microbial attack. The fibers were soaked with enzyme solution so that enzyme could diffuse into the porous sponge region of the fibers. Substrate solution was then pumped through the inner lumen of the fibers. GI was inactivated by fresh P10 hollow fibers , polysulfone polymers with nominal molecular weight cut-off of 10,000, and it was necessary to precondition the fibers by soaking in 2% bovine serum albumin. The half life in a continuous operation at $53^{o}C$, using 1M fructose solution at a flow rate of 0.4ml/min, was 3 days. GI was more stable in X50 hollow fiber ,copolymers of poly vinylchloride and polyacrylonitrile with a nominal molecular weight cut-off of 50,000, than in P10 fibers and stability improved with time, perhaps because of a thermal inactivation of proteases. The hollow fiber enzyme has the advantage of large enzyme loading and ease of use, cleaning and reloading with enzyme.

VI.CHANGES OF ENZYME PROPERTIES UPON IMMOBILIZATION

Once an enzyme is attached to an insoluble carrier, its physicochemical charateristics must be compared with those in the native state. Parameters such as pH profile, thermal profile, kinetics, and even substrate specificity may be altered upon immobilization (Trevan, 1980, Godfrey, 1983, and Weetall, 1985).

A. Changed pH optima

Most carriers are charged to some extent. These charges will change and modify pH optima of enzymes immobilized in such carriers. The mechanism of this pH change has been elegantly explianed by Goldstein et al., (1964) and Goldstein and Katchalski (1968). Highly negatively charged carriers create a lower pH at the boundry between the carrier and the bulk solution, so that the enzyme is in a more acidic environment than most of the solution. The opposite occurs with positively charged supports. Even with uncharged supports, the overall changes on the enzyme may be sufficient to change the apparent pH optima (Weetall, 1985).

B. Altered temperature optima

Although in a few cases reduced thermal stability has been noted, most industrial immobilized enzymes show an increased thermal tolerance. In some cases immobilization increases thermal stability by increasing the molecules rigidity (Weetall, 1974), thus preventing changes in the molecules tertiary structure upon heating. Other factors which tend to heighten the stability of immobilized enzymes are prevention of autolysis, protection against the effect of

microorganims, and inaccessibility of the enzymes to the effect of inhibitors (Kostner and Kreen, 1974).

C. Changes in apparent kinetics

Enzyme activity in the process of attachment almost always decreases. The basic factors which cause the diminution of immobilized enzyme activity are diffusion resistance, shielding of the enzyme's active center, and chemical and conformational modification of the enzymatic protein macromolecules. Full inactivation of the enzyme can be expected in the case of a covalent bond with active center amino acid residues. Blocking the active center by the enzyme substrate or its inhibitor is applied to protect the enzyme active center from the attack of bonding groups. It should be noted that covalent attachment, sometimes proceeds with full preservation of enzymatic activity. In some instances, the enzyme is even activated during the process of attachment (Kostner and Kreen, 1974).

Generally, the apparent Km of an immobilized enzyme is greater than that of the soluble form. This increase is usually related to various parameters, including charge of the substrate and carrier diffusion effects, and even mode of operation (Weetall, 1985). Where the enzymatic reaction is subject to product inhibition, it is commonly observed to be more inhibited when operated with immobilized enzymes (Godfrey, 1983). The support material might exert constraints directly on the enzyme molecules (Trevan, 1980).

Significant decreases in enzyme activity can be caused by denaturation. However, the elution of the enzyme from the support with time also can contribute significantly to operational half-life. Even covalently coupled enzymes can be eluted by bond scisson (Lasch and Koelach, 1978). Inorganic carriers were compared (Weetall, 1985) with several organic supports including resins, celluloses, and cross-linked polymers. In all cases, some enzyme leakage occurred.

VII. ISOMERIZATION

A. Feedstock preparation

The preparation of dextrose liquor for isomerization is reviewed by Antrim et al. (1979) and dealt with extensively by Blanchard and Gieger (1984). The saccharified starch, which typically contains 94-96% dextrose is normally refined by, filteration, carbon treatment, and ion exchange resin treatment. Filteration is very important to remove any fines or suspended materails from the feed stream. Carbon treatment mainly removes colored materials and colored precursors, such as methyl furfural. A mixture of strong acid (sulfonate form) and weak base (mixture of primary, secondary, and tertiary amines) resin is used to demineralize the dextrose syrup, particularly to remove calcium ions which were added during the liquefaction process. The refined dextrose liquor is then reconcentrated or blended to a dry solids level of 40-50% which is pumped to a makeup tank where various salts are added. Levels of magnesium ions ranging from 0.5 to 5.0 mM have been described for a number of commercail isomerization processes. Magnesium ions are required for optimal catalytic activity of almost all commercially used isomerases. Controlling Ca^{2+}/Mg^{2+} ratio within certain limits (1/10 to 1/20) has been recommended (Poulsen and Zittan, 1977). Addition of other ions, such as bisulfite or iron has been described (Cotter, 1971 and Fujita et al., 1977) for enhancing the activity or stability of glucose isomerase. The pH of the dextrose is then adjusted to 7.0-8.5, depending upon the reactor type and choice of isomerizing enzyme used. Batch isomerization would usually require a lower pH operating range to prevent formation of color and undesirable isomerization by products (Schnyder, 1974) due to the relatively long contact time btween enzyme and substrate.

B. Process development

The development of isomerization techniques has been well reviewed by Antrim et al. (1979), Hemmingsen (1979), Carasik and Carrol (1983), and Blanchard and Gieger (1984). The initial soluble enzyme was too costly for commercial application. Early types of immobilized enzymes were used in batch reactors, but the long residence time required a relatively low pH and the use of cobalt as an activator. Later, the continuous isomerization was introduced. Back-mixed continuous reactors were inherently inefficient and tended to degrade the enzyme particles. Fluidized bed reactors involved too much back-mixing and channeling. Down-flow fixed beds compressed the enzyme and led to unacceptable pressure drops. Shallow beds have been used by Clinton Corn Products to suit their soft adsorbed glucose isomerase. This system may suffer channeling if not closely controlled.

The introduction of stronger enzyme particles permitted the general adoption of the downflow fixed-bed system which was described by Novo in 1977 and proved to be the most efficient and simple to run.

C. Current practice

The design criteria given by Oestergaard and Knudsen (1976) provides the basis of most systems in use in the USA. Satisfactory feed purification is normally achieved by filteration, carbon treatment, demineralization by ion exchange, and deaeration. Depending on the form of immobilized enzyme, hydration by soaking the enzyme in cool syrup with magnesium activators prior to being put into use may be required.

Fixed bed columns are usually operated downflow except for a short time period of upflow to settle the bed after charging. Columns can be arranged singly, in parallel or up to four columns in series. The series configuration of the columns provides further protection against enzyme poisoning since the fresh enzyme is inserted at the downstream end of the train. Thus any inhibiting reaction is concentrated on the most exhausted enzyme. However, in this case, pressure drop can be a serious problem if the enzyme particles are not strong enough.

Downflow fixed beds are inherently resistant to channeling and have "plug flow" characteristics. The residence time is less than four hours which permits the use of feed liquor at the optimum pH of "7.5-8.2" without serious degradation of sugar and the use of cobalt is unnecessary. A small amount of magnesium is added and the temperature is normally adjusted at 60^{o}C. As the isomerization process proceeds the enzyme activity decays exponentially (i.e. the rate of decay is proportional to the amount of activity present) and it can be represented by half life. Park et al. (1981) outlined three control requirements of an enzyme reactor to maintain the quality of the product. These involved (1) reducing the flow rate, (2) increasing temperature, or (3) adding fresh enzyme to the system to compensate for enzyme loss. Usually, the flow rate is reduced until the total flow through the system reaches the minimum that is acceptable. Then, the most exhausted column is discharged and refilled with fresh enzyme, and inserted in the downstream position of a series configuration system. In general, the enzyme will be discarded after three half lives.

Recently, Clinton Corn Products introduced a new "on-column loading" system which permits a gradual addition of enzyme to the column during the isomerization process (Antrim et al., 1989). As soon as possible, the isomerized syrup is adjusted to pH 4.0 in order to avoid color formation and sugar degradation. The productivity of commercially used IGI ranges from 1000 to 9000 lb 42% HFCS/lb enzyme.

D. Pressure drop

Pressure drop across a reactor is measured as the difference in the influent and effluent pressure. The compressibility and pressure drop for different makes of enzyme were studied by Norsker et al. (1979) and Hupkes (1978). Pressure drop may range from 1 to 10 psig or even 20 psig.

Pressure drop is directly related to viscosity of the substrate, physical nature of the immobilized enzyme particles, particle size distribution, and presence of fines. Generally a compromise in particle size is reached whereby pressure drop, caused by smal particle size, and diffusional resistance are minimized. Serious pressure drop problems can also be caused by substrate impurities such as carbon fines, protein floc, and precipitated salts. Swelling of IGI particles can increase pressure drop unless an adequate enzyme pretreatment is performed. Once the IGI and the reaction condition have been defined, pressure drop will be controlled by reactor design, mode of operation, number of reactors, and reactor configuration. Pressure drop is a function of bed height and increases with the number of reactors in a series configuration (Venkatasubramanian and Harrow, 1979).

To ensure good flow distribution with parallel operation, a minimum height to diameter ratio of 3 is required. Pressure drop problem may arise if this ratio exceeds 3 and the possiblity of chanelling problem will increase with lower ratio. However, height to diameter ratio may be reduced if series configuration is applied (Blanchard and Geiger, 1984). Generally, this ratio depends on the pressure drop criteria and affects the performance of the IGI reactor system under a given set of operating conditions (Kim et al. 1977).

E.

F.

E. Diffusional limitations

Immobilized catalysts are subjected to external and internal diffusion limitations. External diffusion, reflecting substrate transport from the bulk solution to the catalyst surface, is a function of the stirring or linear flow rate and is relatively easy to minimize with most reactor types. Most catalysts exhibit some degree of internal diffusion limitation, which reflects the diffusion of substrate within the matrix of the enzyme particle. Enzyme loading and particle size are the major factors influencing internal diffusion inhibition and results in an increase in the apparent Michaelis constant (Lilly and Dunnill, 1976 and Swaisgood, 1985). IGI is no exception and the effect of diffusion limitations on reactor efficiency has been studied. Boersma et al. (1979) concluded that under normal operating conditions, the external mass transfer rate does not influence the overall reaction rate. However, the effect of internal mass transfer resistance on the overall reaction rate can be accounted for by the so called porous sphere model. Verhoff and Goldstein (1982) found that although increased diffusion resistance lowers the apparent decay rate of the enzyme, it also decreases the productivity of the enzyme. Thus, the enzyme pellet should be made as small as practical.

F. Enzyme activity decay

Enzymes are subject to irriversible loss of catalytic activity by a variety of causes, most prominently by heat denaturation. Extremes of pH and other reaction parameters can also cause deactivation independently or by interacting with thermal denaturation. With immobilized enzymes the deactivation is further complicated by additional factors such as poisoning, physical and chemical changes(Lee et al., 1976). However, enzymes can be stabilized by either covalent multipoint attachment of the enzyme to a matrix, or by embedding the enzyme in a stabilizing

protective microenvironment (Mosbach, 1976). Martinek et al. (1980) added that it is not known whether the stability of an enzyme is dependent upon its conformation or microenvironment. Moreover, if this dependency exists it is likely that no general mechanism of denaturation of proteins exists, except that inactivation of enzymes under the action of heating or denaturating agents entails significant conformational changes, i.e. unfolding in protein molecules. The thermostability of some enzymes can be increased by rigidifying (fixing) the native conformation of the protein globule by applying intramolecular crosslinkages, multipoint covalent attachment, multipoint non-covalent interaction of enzymes with support, and by mechanical entrapment into "tight" pores of an inert support.

The large-scale commercial utilization of immobilized enzymes is, however, severely hindered by the enzyme deactivation and loss of enzyme activity due to thermal denaturation, pH effects, pore blockage, inactivators in feed stream, microbial contamination, attrition by weak bonding or support erosion, as well as other factors (Park et al., 1981).

The activity of GI, as with other enzymes, declines with time. At some time, the reactor must be stopped and recharged with fresh enzyme. The enzyme activity decay decreases the productivity and increases the isomerization costs. Adler et al. (1979) reported that the stability of free and immobilized cellulose acetate fiber-entrapped preparations of GI, with respect to proteolysis, were improved by treating the soluble GI, prior to immobilization, by recycling through hollow fiber, heat treatment, or by the addition of protease inhibitors.

Stabilization of GI activity was to some extent accomplished by immobilization. However, caution should be exercised when evaluating the stability of such preparations because it is possible for diffusional resistances or microenvironmental effects to cause an apparent increase in immobilized enzyme stability over native enzyme stability when actually there may be no real stability increase at all (Hamilton et al., 1974). The effect of temperature, pH, oxygen, enzyme

activators, and other factors on glucose isomerase stability were elegantly reviewed by Antrim et al. (1979). More details can be found in the changes on enzyme character upon immobilization, diffusional limitations, and half-life sections in this review.

G. Half-life

The half-life of an enzyme is a measure of the stability of such enzyme. As the activity decay of GI, the half-life of the enzyme depends on many factors which makes it difficult and some times impossible to compare the half-life of different IGI products.

As far as type of carrier is concerned, GI from different sources was immobilized on silica carriers. Cell-free GI bound to diazotized porous glass beads, packed in fixed bed column, and operated at 60^{o}C with buffered 0.5M glucose liquor containing 50 mM magnesium ions gave 12-14 day half-life (Strandberg and Smiley, 1972), while GI bound through immine glutaraldhyde linkage on zerconia-coated controlled porous glass (CPG) beads gave 16-20.2 days half-life when continuously used to isomerize purified glucose solution containing 5-10 mM magnesium ions and 1 mM cobalt ions (Havewala and Pitcher, 1974 and Lee et al., 1976). Recently, UOP,Inc. and Miles Kali Chemie introduced a high stable IGI. Ketmax GI-100 of UOP,Inc., which is prepared by covalently binding GI to ceramic alumina treated with polyethyleneimine and activated with glutaraldhyde, gave 33 days halfe-life. Even better half-life (50 days) was claimed by the Miles Kali Chemie for its product, Optisweet 22, which is produced by the adsorption of GI onto silica particles and crosslinked with glutaraldhyde. Ketomax GI-100 is expensive and the Optisweet 22 suffers the drawbacks of being adsorbed, it also needs specially designed reactors and requires an intensive syrup purity (Jensen and Rugh, 1987)

Purified GI from *Actinoplanes missouriensis* adsorbed on macoporous beads of Celite or Titania gave a very short half-life (2 days) when the substrate feed had no cobalt. However, the addition of Co^{2+} considerably increased the half-life, 20 and 46 days at 0.3 and 1.5 mM Co^{2+}, respectively (Rosevear et al. 1977). An improved IGI preparation was reported by Goldberg et al. (1979). Purified GI from *Bacillus coagulans* bound to a microporous polyvenylchloride (PVC)-silica filled plastic sheet packed in a flow through reactor and operated at 60°C, pH 7.5, 40% (w/w) dextrose substrate containing 1 g/l $NaHCO_3$, 1 g/l $NaHSO_3$, and 2 g/l $MgSO_4.7H_2O$ gave 38 days half-life.

Cell-free GI from *Streptomyces sp.* immobilized to carriers other than silica gave a wide range of half-lives (3-70 days) depending on the type of carrier and the operating conditions. The half-life of crude GI adsorbed on hollow fibers x50, used in a continuous isomerization at 55°C, was 3 to 4 days (Korus and Olson, 1976), while that of semipurified GI adsorbed on DEAE-cellulose gave about 8 days at 60°C (Thompson et al., 1974), and10-11 days at 50°C (Park and Toma, 1975). A 6 day half-life was reported (Kasumi et al., 1977) when pure GI was bound to chitosan. Recently, FinnSugar/Fermco improved the DEAE-cellulose IGI, Spezyme IGI, by adsorbing highly purified GI onto DEAE-cellulose-polystyrene-Titania which gave a very stable preparation (50-70 days) when operated at 55-57°C (Antrim et al., 1986). This product is the only one that has gained a foothold in the market (Jensen and Rugh, 1987).

Whole-cell GI from *Streptomyces sp.* exhibited a very wide stability. The heat-fixed cells had a 4 days half-life at 70°C (Ryu and Chung, 1977), which was slightly increased to 6.5 days when crosslinked with glutaraldhyde at Miles. Not surprising, the half-life of this product increased to 17 and 41 days at 65°C and 60°C, respectively in the presence of Co^{2+} (Lantero, 1977). However, a very stable product which does not need Co^{2+} as stabilizer in the feed stream was reported by Park et al. (1980). The cells were heat-fixed, entrapped in gelatin, flocculated with DEAE-cellulose, and crosslinked with glutaraldhyde. Even more stable IGI has been commercially

produced by Novo Industries. The cells are heat-fixed, flocculated with polyethyleneimine (PEI), and crosslinked with glutaraldhyde (Jorgensen et al., 1988). The Japanese (Nagase/Denki company) produce a product called Sweetase by binding the cells to ion-exchange resins and crosslink it with glutaraldhyde. This product half-life is about 54 days in continuous isomerization (Jensen and Rugh, 1987).

Whole-cell IGI products were produced from sources other than *Streptomyces sp.* *Bacillus coagulans* cells containing GI was heat-fixed and crosslinked with glutaraldhyde at Novo Industries. This product exhibited a 20 day half-life at 65^{o}C without Co^{2+} requirement (Poulson and Zittan, 1976). However, the same preparation gave only a four day half-life at 56.5^{o}C as reported by Park et al. (1981). AN *Actinoplanes missouriensis* cell preparation is reported (Linko et al., 1977) to have a half-life of 45 days in the presence of Co^{2+}. Gist-Brocades used the same source to produce Maxazyme, which exhibited 62 days half-life without Co^{2+} addition (Hupkes and Tilburg, 1976). About the same stability or slightly higher (62-75 days) was exhibited by IGI (Takasweet FM®) prepared from *Flavobacterium arborescens* at Miles Laboratories,Inc.(Lantero, 1982). Recently, a gelatin entrapped *Lactobacillus brevis* 74 cell preparation was reported to give a 43 day half-life at 60^{o}C , however the half-life was calculated by extrapolation of the data (Ananichev et al., 1986).

VIII. FRUCTOSE ENRICHMENT

The development of fructose enrichment techniques has overcome the inherent limitation of isomerization equilibrium and permitted the production of syrup with up to 95% fructose (Blanchard, 1984). Chromatographic separation is successfully used for the production of 90% fructose syrup from the conventional 42% fructose syrup. The 90% fructose syrup is then blended to produce 55% fructose syrup which has a large demand in the market, particularly for

carbonated beverages. This separation technique can be applied in either batch or continuous system.

The batch separation technique involves the use of adsorbent that has a particular affinity for fructose. The dry substance of the 42% fructose syrup is adjusted and then syrup is passed over such adsorbent, followed by a portion of water. Since dextrose is not retained by the adsorbent, it discharges from the column first, followed with some overlap by the fructose, producing a super-rich fructose syrup. The remaining material can be recycled in variuos ways (Lauer et al., 1974).

The continuous adsorption chromatographic technique was developed for petrochemical application in 1957 (Broughton and Gerhold, 1961) and adapted for dextrose-fructose separation by UOP, Inc. (Bieser and DeRosset, 1977 and Teague and Arnold, 1983) using zeolite adsorbent. In Japan, separation processes were developed by Troy Industries, Inc. (Odawara et al., 1979) and Mitsbishi Chemical Industries Ltd. (Ishikawa, 1980). A number of continuous adsorption processes utilize "simulated moving bed". Control and sequenceing are rather complex, but once a balance is established, operation is quite simple. The continuous system uses substantailly less water, and requires less stock of adsorbent than equivalent batch systems (Hirota, 1980).

The 55% fructose syrup could be manufactured by processes other than chromatographic separation, such as high temperature isomerization, crystallization, complexing, and isomerizing in an aqueous ethanol. Sproull et al. (1976) reported that at 80^{o}C the equilibrium constant is shifted so as to permit the production of 55% fructose syrup using 40% dextrose liquor. Unfortunately no isomerase enzyme currently available can be used continuously at this high temperature (Lloyd and Horwath, 1983). Dextrose readily crystallizes in small amounts from 42% fructose syrup. Conditions can be arranged at about 80% dry substances and cooling at

30^{o}C, so as to leave the mother liquor with 55% fructose. Addition of sodium or calcium salts may be included. The difficulty of separating the dextrose crystals and the increased proportion of higher sugars, which do not crystallize are major problems. Takasaki (1972), Barker (1973) and his coworkers (1978) demonstrated that isomerizing in the presence of borate, phenyl borate or germanate will complex fructose as it is formed, removing it from the equilibrium and causing the isomerization to proceed further. Fructose contents of up to 80% can be achieved by this technique, but there are considrable problems in decomplexing the fructose and the type of complexing agents available have not been attractive for food processors. Visuri and Klibanov (1987) demonstrated that the syrup containing 55% fructose can be produced either directly by the isomerization in 85% aqueous ethanol or by diluting the normal 42% fructose syrup with ethanol and passing it through a small GI column to enhance the fructose contents to the desired level. Ethanol is then recovered by distillation and reused.

MATERIALS AND METHODS

MATERIALS

Crude D-Glucose (xylose) isomerase (EC 5.3.1.5) from *Flavobacterium arborescens* in a lyophilized powder form was kindly donnated by Miles Labaratories Inc., Elkhart, IN U.S.A. This enzyme was in the experimental stage and was not a commercial one.

Controlled pore glass (CPG) beads, 20-80 mesh size, of different pore diameters, 266-727 Å, were purchased from Sigma Chemical Company, ST.Louis, MO. Anhydrous dextrose (glucose) was purchased from J.T.Baker Chemical Co., Phllipsburg, N.J. Gamma aminopropyltriethoxy silane, Titanium chloride, 1-ethyl -3(-3-dimethylaminopropyl) carbodiimide (EDC), $MgSO_4.7H_2O$, $CoCl_2.6H_2O$, Diaminopropane, Diaminohexane, diaminooctane, diaminodecane, and hydroxyethylpiprazine ethane sulfonic acid (HEPES) buffer were purchased from Aldrich Chemical Company,Inc. Milwaukee, Wisconsin. All chemicals were of chemical grade.

A glass column was constructed using the following design (Figure 2):

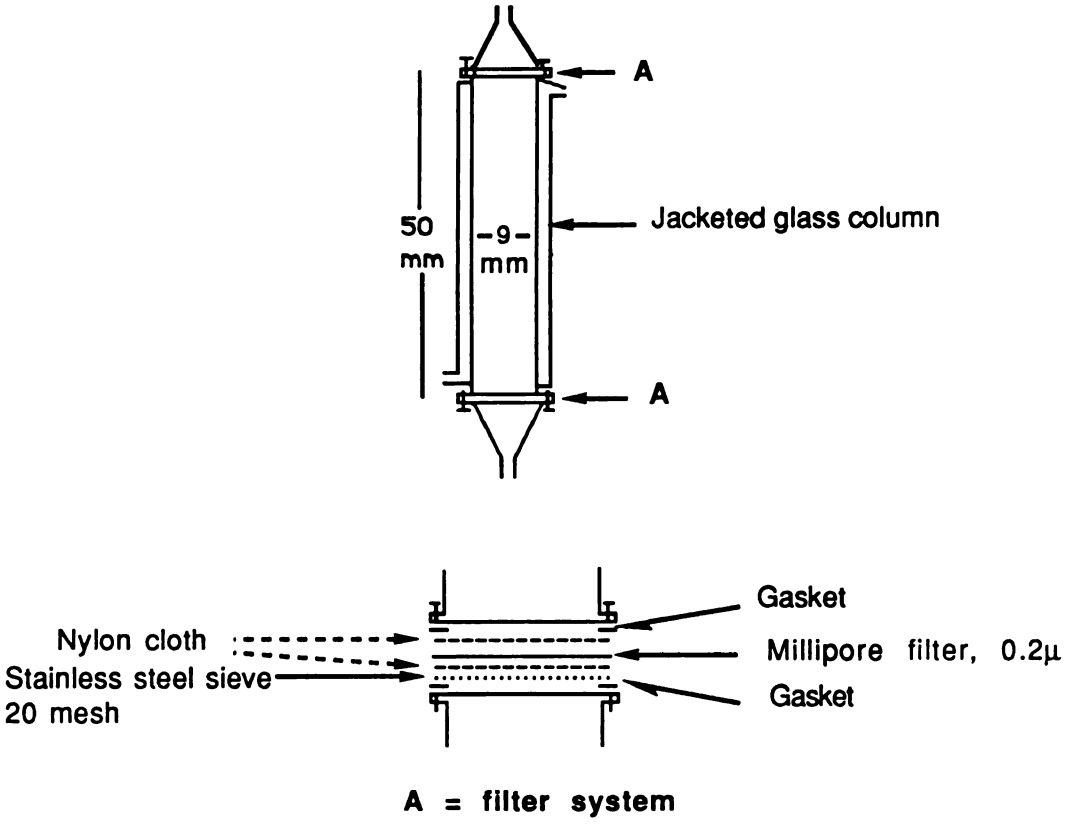


Figure 2. Digram of the column used in the operational stability of bound glucose isomerase.

METHODS

Enzyme assay:

A valid enzyme assay of glucose isomerase was developed by adding 5 µg/µl glucose isomerase solution (20 µl) in 0.05M phosphate buffer, pH 7.0 to 2 ml of 0.04M hydroxyethylpiperazine ethane sulfonic acid (HEPES) buffer, pH 7.7 at room temperature, containing 0.1M glucose, 0.1M $MgSO_4.7H_2O$, and 2.0mM $CoCl_2.6H_2O$. This mixture was allowed to react at 60°C for 30 minutes. At the end of the reaction time, the reaction was stopped with 3 ml 0.5M perchloric acid. The formed fructose was measured by the sulfuric acid-cysteine hydrochloride-tryptophan method of Dische and Borenfreund (1951) modified by Messineo and Musarra (1972). To a 0.1 ml sample containing 1-10 ug fructose, 2.8 ml 75% (v/v) sulfuric acid, and 0.1 ml 2.5% (w/v) cysteine hydrochloride were added. This was thoroughly mixed and incubated in a 45°C water bath for 10 minutes. One ml 0.1M HCl containing 100 ug/ml tryptophan was immediately added with shaking. Then,the mixture was allowed to stand at room temperature for 20 minutes. Absorbance was read at 518 nm (A_{518}). A blank was prepared by adding 20 µl 0.o4M HEPES buffer, pH 7.7 instead of the enzyme solution.

The immobilized glucose isomerase preparations were assayed as the soluble enzyme (as above) except that the amount of the enzyme was about 10 mg (wet basis), reaction mixture volume was 4.0 ml in 25 ml pyrex Erlenmeyer flasks with pyrex stoppers in a shaking water bath, and the reaction was stopped by sudden cooling in an ice bath.

Glucose isomerase activity is expressed as % relative activity (observed activity/maximum activity* 100), or micromoles of fructose formed per minute (units). Specific activity was calculated as units per mg protein.

Fructose standard curve:

The absorbance at 518 nm of different concentrations of fructose (1-10 ug) was measured as mentioned above in the enzyme assay section. All measurements were done in triplicate using a Bausch& Lomb Spectronic 70. HEPES buffer solution was used as blank.

Protein determination:

The protein content of the immobilized glucose isomerase (IGI) was measured by microKjeldahl method as described in the approved methods of the Association of American Cereal Chemists (AACC, 1983) with crystalline bovine serum albumin as standard and the 6.25 factor was used to convert the measured nitrogen to protein. However, the protein content in the output of the operational stability tests was measured by the dye binding method "Bio-Rad protein assay" according to Bradford (1976).

Optimum activity pH:

The effect of pH on the rate of glucose isomerase activity was studied with 0.04M HEPES buffer ranging between 6.0 and 9.0 for the soluble enzyme. However the pH range was extended (5.5-11.5) for the bound enzyme. 0.04 M acetate, HEPES, and glycine-NaOH buffers were used

for pH 5.5, 6.5-8.5, and 9.5-11.5, respectively. The pH of the reaction mixture was measured before the initiation of the reaction using Corning pH meter 610A.

Optimum activity temperature:

At the optimum pH of the enzyme, the optimum activity temperature was determined. The activity of the enzyme at various temperatures ranging from 50 to 90^{o}C were tested for soluble and immobilized glucose isomerase.

Mineral requirements:

Optimum activity concentration of Mg^{2+} and Co^{2+} were determined for both soluble and immobilized glucose isomerase.

A. Optimum activity concenteration of Mg^{2+}:

At the optimum activity pH, the Optimum activity concenteration of Mg^{2+} was measured using various levels of magnesium sulfate concentration ranging from 0 to 200 mM.

B. Optimum activity concenteration of Co^{2+}:

At the optimum activity pH, the optimum activity concentration of Co^{2+} was measured using various levels of cobalt chloride ($CoCl_2.6H_2O$) concentration ranging from 0 to 20 mM in case of soluble enzyme and from 0 to 5 mM for the immobilized enzyme.

Enzyme stability:

The pH, thermal, operational and storage stabilities of soluble and immobilized enzyme preparations were studied.

A. pH stability:

Residual activity of the enzyme was measured after incubating the enzyme in buffer solution at various pHs ranging from 3.5 to 11.5 at room temperature for 30 minutes. A 0.04 M acetate buffer was used for the pH range of 3.5-5.5, 0.04 M HEPES buffer was used for the pH range of 6.5-8.5, and 0.04 M glycine-NaOH buffer was used for the pH range of 9.5-11.5.

B. Thermal stability:

Glucose isomerase (free and bound) stability at various temperatures for 30 minutes and at 60^{o}C for up to 43 days was studied. In order to determine the enzyme stability over range of temperatures, the residual activity of free glucose isomerase was measured in 0.04M HEPES buffer solution, pH 7.7 or unbuffered solution, after the exposure to various temperatures ranging from 50 to 90^{o}C for 30 and 60 minutes. For the bound enzyme, the preincubation was in the HEPES buffer and the exposure time was 30minutes.

The enzyme stability at 60^{o}C (long-range stability) was determined by measuring residual activity of free glucose isomerase after preincubation in 0.04M HEPES buffer solution, pH 7.7, in 0.04M HEPES buffer containig 20 mM Mg^{2+}, 2mM Co^{2+}, 20 mM Mg^{2+} and 2mM Co^{2+}, and in 2.5 M glucose "in 0.04 M HEPES buffer pH 7.7" at 60^{o}C for various periods of time (up to 43

days). For the bound enzyme, the preincubation was only in 0.04M HEPES buffer, pH 7.7 since operational stability will be studied.

C. Operational stability

The stability of immobilized glucose isomerase preparations in a continuous isomerization process were also studied. The immobilized enzyme was packed in a 9 x 50 mm glass column. A 45% glucose solution containing 0.04 M HEPES buffer, pH 7.7, 20 mM Mg^{2+} and 10 mM $NaSO_3$ at 60°C was passed upward into the column which was immersed in a thermostatic controlled water bath at 60°C. The flow rate was about 20 ml per hour using a Buchler Instruments polystaltic pump. The enzyme bed was equilibrated with conditioning buffer containing 0.04 M HEPES buffer, pH 7.7, 20 mM Mg^{2+} and 10 mM Na_2SO_3 at 60°C for 30 minutes prior to substrate passage.

The activity of the immobilized enzyme in the continuous operation was monitor by measuring the fructose concentration or A_{518} in the column effluent with time. The time required for the % relative conversion (observed conversion/maximum conversion x 100) to reach half of its initial value was considered as the appearent half life. Protein content of the column effluent was measured, to determine any desorption of the enzyme from the column, by the Biorad method.

D. Storage stability

Residual activity of free and immobilized glucose isomerase stored, in 0.04 M HEPES buffer, pH 7.7 containing 0.06% sodium azide, at 4-6°C was measured at different entervals of time over a 9 week period.

ENZYME IMMOBILIZATION:

Glucose isomerase was covalently bound to Titanium(IV)-coated , derivatized and activated controlled porous glass (CPG) beads by four techniques. These techniques were:

A. Coupling Glucose isomerase to Titanium(IV)-coated CPG beads.

B. Coupling Glucose isomerase to Titanium(IV)-coated, silanized, and glutaraldhyde-activated CPG beads.

C. Coupling Glucose isomerase to Titanium(IV)-coated, aminated, glutaraldhyde-activated CPG beads

D. Coupling Glucose isomerase to Titanium(IV)-coated, silanized,EDC-activated CPG beads.

Carrier"CPG beads" derivatization:

CPG beads were coated, aminated, and silanized as preparation steps prior to immobilization.

A. CPG beads coating

CPG beads were coated with 5% titanium chloride solution according to the method of Lambrecht et al. (1987) previously described by Cabral et al. (1982). CPG beads were first cleaned and hydrated by an acid wash with 5% (v/v) nitric acid at 80-90^{o}C for 60 minutes, followed by rinsing with douple distilled water (d.d.H_2O) as mentioned by Weetal (1976). Then, the CPG

beads were steeped in 5% (w/v) titanium chloride in 15% hydrochloric acid for 6 hours at room temperature (about 23^{o}C). Finally, the beads were dried at 43^{o}C for 30 hours and washed with dry chloroform.

B. CPG beads amination

The dried Titanium(IV)-coated CPG beads were treated with 1% 1,6-hexanediamine (1,6 diaminohexane) in chloroform (5 ml/ g matrix) at 45^{o}C for 30 minutes. Then, the beads were washed successively with chloroform, methanol, and d.d.H_2O (Lambrecht et al., 1987). The aminated CPG beads could be dried and stored for later use. Diamines of 3-10 carbon atoms (1,3-diaminopropane - 1,10-diaminodecane) were used to get aminated CPG beads with various length spacer for the spacing effect experiment.

C. CPG beads silanization

The dried Titanium(IV)-coated CPG beads were silanized as described by Robinson et al. (1971). To 1g of clean coated CPG beads, 18 ml of d.d.H_2O were added plus 2 ml of 10% (v/v) gamma aminopropyltriethoxy silane in acetone. Then, the pH was adjusted between 3.0-4.0 with 6 N HCl and the reactants were placed in a 75^{o}C water bath for 2 hours followed by filteration and washing of the beads with d.d.H_2O. Finally, the beads were dried and stored for later use.

Determination of NH_2 groups on the derivatized CPG beads:

The funtional NH_2 groups on the silanized and aminated Titanium(IV)-coated CPG beads were determined according to Bucholz and Klein (1987). About 10 mg of the derivatized beads with 10^{-3} mmole NH_2 groups were subjected to hydrolysis for 60 minutes with 0.5 ml 1.0M NaOH solution at room temperature. The suspention was then neutralized with 0.5 ml 1.0M HCl solution followed by the addition of 1.0 ml borate buffer, pH 9.2 (4 g of sodium tetraborate in 100 ml d.d.H_2O) and 0.5 ml of 0.1% trinitrobenzene sulfonic acid solution. The mixture was incubated in a water bath at 45^oC for 80 minutes. To 0.5 ml of the incubated mixture, 0.5 ml 1.0M HCl and 0.5 ml d.d.H_2O were added and the absorbance at 405 nm was measured against a blank. A standard test was performed using 50 ul of aminopropyltriethoxy silane(0.1 $ml_{97\%}$ in 100 ml borate buffer, pH 9.2).

Carrier'derivatized CPG beads' Activation:

The derivatized CPG beads were activated either by glutaraldhyde or by 1-ethyl-3(-3-dimethylaminopropyl) carbodiimide "EDC" as follows:

A. Carrier activation with glutaraldhyde

A carbonyl derivative having an active aldhyde groups was prepared by treating the alkylamine derivative (Aminated or silanized CPG beads) with glutaraldhyde according to the method of Weetall (1976). To 1 g alkylamine carrier, 25 ml of 2.5% (v/v) glutaraldhyde solution in 0.05M phosphate buffer, pH 7 was added. Then, the reaction was allowed to continue for at least 1 hour at room temperature. Finally, the beads were exhaustively washed with d.d.H_2O to remove excess of glutaraldhyde.

B. Carrier activation with EDC

1-ethyl -3(-3-dimethylaminopropyl) carbodiimide (EDC) was used, simultaneously with the derivatized carrier and the enzyme, as a coupling reagent as described in the literature (Swaisgood et al., 1976, Kjellen and Neujahr, 1979, Janolino and Swaisgood, 1982, and Papisov et al., 1985). More details are described below.

Coupling glucose isomerase to the activated CPG beads:

The Titanium(IV)-coated, carbonyl, and silanized CPG beads were used to covalently bind glucose isomerase as follows:

A. Coupling Glucose isomerase to Titanium(IV)-coated CPG beads.

To 1 g Titanium(IV)-coated CPG beads, 100 mg of crude Glucose isomerase solution (20 mg/ml) in 0.05M phosphate buffer, pH 7 was added. 0.05M phosphate buffer, pH 7.Vacuum was applied for 15 minutes using a desiccator and water pump at room temperature Then, the reaction was allowed to continue for 4 hours in a refregerator, followed by filtration and the filtrate was kept for protein and activity measurements. Then the enzyme-bound beads were washed successively with d.d.H_2O, 1.0M NaCl , d.d.H_2O, and 0.05M phosphate buffer, pH 7. Finally, the immobilized enzyme preparation was kept in 0.05M phosphate buffer, pH 7.or 0.04M HEPES buffer, pH 7.7 in the refrigerator.

B. Coupling glucose isomerase to the carbonyl derivative of CPG beads

Glucose isomerase was covalently bound to the active carbonyl derivatives (silanized or aminated CPG beads activated with glutaraldhyde) according to the method of Weetall (1976).

C. Coupling glucose isomerase to the alkylamine derivative of CPG beads via carbodiimide.

Silanized derivatives of Titaniun(IV)-coated CPG beads were bound to glucose isomerase by simultaneous addition of EDC and the enzyme to the alkyleamine derivative of the coated CPG beads as follows:

To 2.5 ml of 4.6 mg/ml Glucose isomerase solution in 0.02M buffer of various pHs (acetate buffer, pH 5.0, phosphate buffer, pH 6.0 and pH 7.0), different amount of EDC (2.4, 4.8, and 9.6

mg which corresponds to concentrations of 5, 10, and 20 mM) were added. Immediately, 0.1 g silanized beads (547Å pore size) were added to the enzyme-EDC mixture.and vacuum was applied for 15 minutes to degas the system. The reaction was allowed to continue for 15 more minutes at room temperature. Then, the reactants were transferred to a refrigerator for 4 hours. At the end of refrigeration, the enzyme-bound beads were filtered, washed successively with d.d.H_2O, 1.0M NaCl, d.d.H_2O, and finally with 0.04M HEPES buffer, pH 7.7 in which the immobilized preparation was kept for later use.

Determination of moisture content:

Water content of the immobilized glucose isomerase preparations was determined by drying in vacuum oven (25 inch Hg.) at 90^{o}C for 5 hours.

Enzyme kinetics:

Km and Vm of free and bound glucose isomerase were determined. The Km of the forward reaction of the pure Glucose isomerase was reported (Bogulawski, 1983) to be 110 mM. Therefore, the enzymatic activity was determined under the assay conditions except that the substrate (glucose) concentration was varied over a range of 50 to 400 mM. The velocity was expressed in terms of glucose isomerase units. One unit of glucose isomerase activity is the amount of the enzyme which produces 1 µmol of fructose/min at 60^{o}C. Specific activity is calculated as units per milligram of protein.The appearent Km and Vm were computed from Lineweaver-Burk's plots using a computer program developed at the Biochemistery Department, Michigan Strate University.

Statistical analysis:

The means, standard error of means (SEM), regression analysis, analysis of variance (simple one way, simple two ways, and split plot repeated measure), and comparison between means of treatments that showed significant effect were carried out according to Gill (1981) in order to determine whether the obtained treatment differences were due to the treatment effect or to the experimental errors within treatments.

Diffusional limitation effects:

The flow rate dependence of diffusional limitation (external diffusion resistance) and particle size dependence of diffusional limitaion (internal diffusion resistance) were studied.

A. Flow rate dependence of diffusional limitation

Effect of the flow rate on the immobilized glucose isomerase activity was studied in a continuous isomerization process. When the enzyme deactivation rate was at the minimum level during the operational stability test, the flow rate of the substrate was changed and the concentration of the product at each flow rate was determined.

B. particle size dependence of diffusional limitaion

Effect of particle size on the immobilized glucose isomerase activity was studied in a batch process. The enzyme activity of the immobilized enzyme preparations before and after crushing, in the presence of 0.04M HEPES buffer, pH7.7 was determined as mentioned earlier in the enzyme assay section.

RESULTS AND DISCUSSION

I. Optimum conditions for the activity of soluble (unbound) glucose isomerase :

A. Optimum activity pH

The optimum pH for the activity of crude soluble glucose isomerase (D-glucose ketol-isomerase EC 5.3.1.5) from *Flavobacterium arborescens* was tested. The activity of the enzyme was measured as mentioned before in the enzyme assay section of materials and methods except that the pH was varied from 6.0 to 9.0.

As shown in Figure 3, after correction was made for high-pH non-enzymatic isomerization, the maximal enzymatic activity was found to be in the pH range of 7.5-8.5. However, the enzymatic activity decreased rather rapidly on either side of the optimum pH range. These results agreed with the results reported for most of glucose isomerases (GI's) which exhibited a pH optimum above 7.0 with a broad peak (Takasaki et al., 1969, Strandberg and Smiley, 1971, and Scallet et al., 1974). More specifically, Suekane et al.(1978) studied the physico-chemical and enzymatic properties of purified GI's from *Streptomyces olivochromogenes* and *Bacillus stearothermophilus* They found that the optimum pH was 8.0 to 10.0 for the Streptomyces enzyme and 7.5 to 8.0 for the Bacillus enzyme. Also, Callens et al.(1986) reported that the GI activity of *Streptomyces violaceoruber* was optimal between 7.5 and 9.5.

The effect of pH on enzyme activity is a complex phenomenon, involving compositional and structural effects. It is an acid-base behavior and inactivation or denaturation at extreme pHs. It is worth mentioning that Danno (1971) found that the rate of activation of the isomerization reaction by metal ion (Co^{2+} or Mg^{2+}) was pH dependent.

B.Optimum activity temperature

The enzymatic activity at the optimum pH was measured at various temperatures between 50°C and 90°C. As shown in Figure 4, enzymatic activity increased with temperature with maximal value at 70°C. The activity decreased as temperature increased above 70°C with a sharp decline at 90°C. This was due to the overall effect of two processes: 1) the usual increase in reaction rate with temperature, which dominated at temperatures below 70°C, and 2) the rate of thermal denaturation of enzyme molecules, which dominated at temperatures above 70°C (optimum activity temperature). It is important to state that 60°C was used for the enzyme assay because the enzyme was more stable at 60°C than at 70°C as will be later demonstrated in the thermal stabiliy section.

C. Metal ion requirements

1. Effect of magnesium ion concentration:

The effect of magnesium ion concentration on the activity of crude soluble GI was studied. The enzymatic activity was assayed as mentioned in the enzyme assay section, exept that Mg^{2+} concentration was varied from 0 to 200 mM

As shown in Figure 5, enzyme activity increased with increasing Mg^{2+} concentration. The optimal activity concentration was at 100 mM. These results agreed with the results reported by Boguslawski (1983) for the same, but pure enzyme. The activation effect of Mg^{2+} addition has been shown for most of known GI's (Antrim et al., 1979).

2.Effect of cobalt ion concentration:

The effect of cobalt ion concentration from 0 to 20 mM on the activity of GI was studied. as shown in Figure 6, the addition of Co^{2+} increased the enzymatic activity with optimal value at 2 mM. This result was higher than those (0.1 mM) reported earlier by Boguslawski (1983) for the pure enzyme. This may be because some of the added Co^{2+} reacted with the impurities associated with the crude enzyme and became unavailable.

Figure 7 showed that when both Mg^{2+} and Co^{2+} were absent from the reaction mixture, no enzymatic activity was detected. However, the enzyme expressed its full activity when both metals were present. This agreed with the findings of Boguslawski (1983). Looking at the activity expressed when only one metal ion was added, it was found that 62% of the activity was expressed when Mg^{2+} only was added at the optimal concentration vs. 40% when Co^{2+} only was added at the optimal concentration. These resuls varied from the results of Boguslawski (1983) who reported that the activity of pure GI was 23.8% when Co^{2+} only was added (0.1 mM) vs. 21.2% when Mg^{2+} only was added (100 mM). Kasumi et al. (1982) studied the role of magnesium and cobalt in the isomerization reaction of GI from *Streptomyces griseofuscus* S-41 and concluded that the affinity of the substrate for enzyme was enhanced by combining a metal with the enzyme and similarly, the affinity of the metal for enzyme was enhanced by combining a substrate with the enzyme. The authors found that magnesium was superior to cobalt as an activator, but was inferior as a protector.

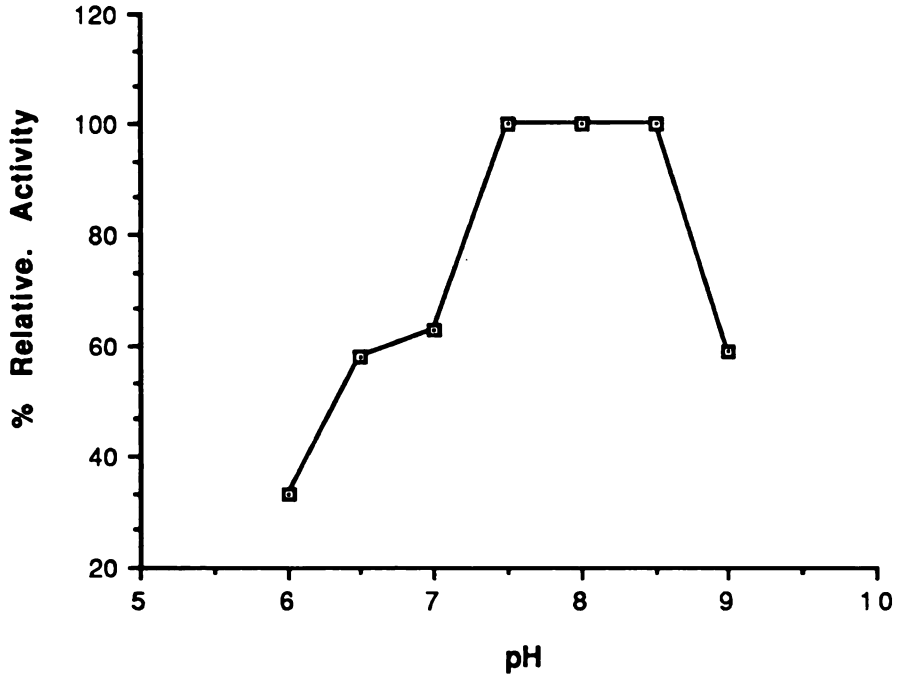


Figure 3. The effect of pH on the activity of soluble glucose isomerase under the enzyme assay conditions (0.1 M glucose solution containing 0.1 M Mg^{2+} and 2 mM Co^{2+} at 60°C for 30 min.).

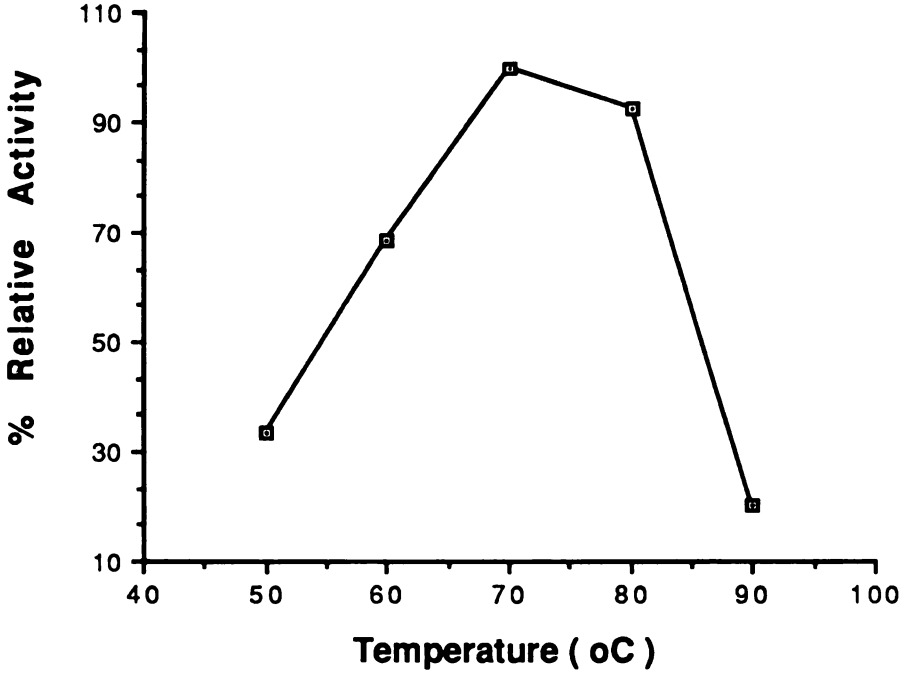


Figure 4. Effect of temperature on the activity of soluble (unbound) glucose isomerase under the enzyme assay conditions (0.04M HEPES buffer, pH 7.7 containing 0.1M glucose, 0.1M Mg^{2+}, and 2mM Co^{2+}) for 30 min.

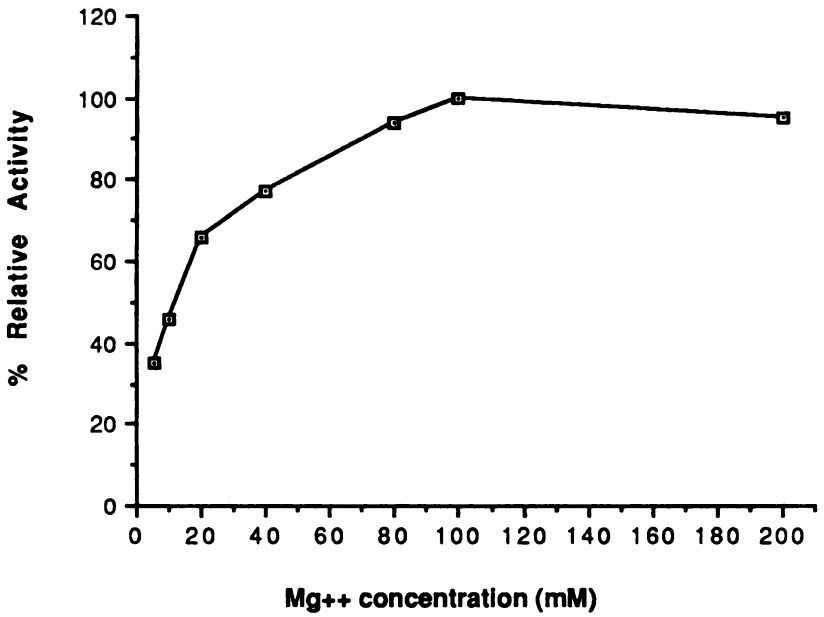


Figure 5. The effect of magnesium ion concentration on the activity of soluble glucose isomerase under the enzyme assay conditions (0.1M glucose solution in 0.04M HEPES buffer, pH 7.7 containing 2 mM Co^{2+} at 60°C for 30 min.).

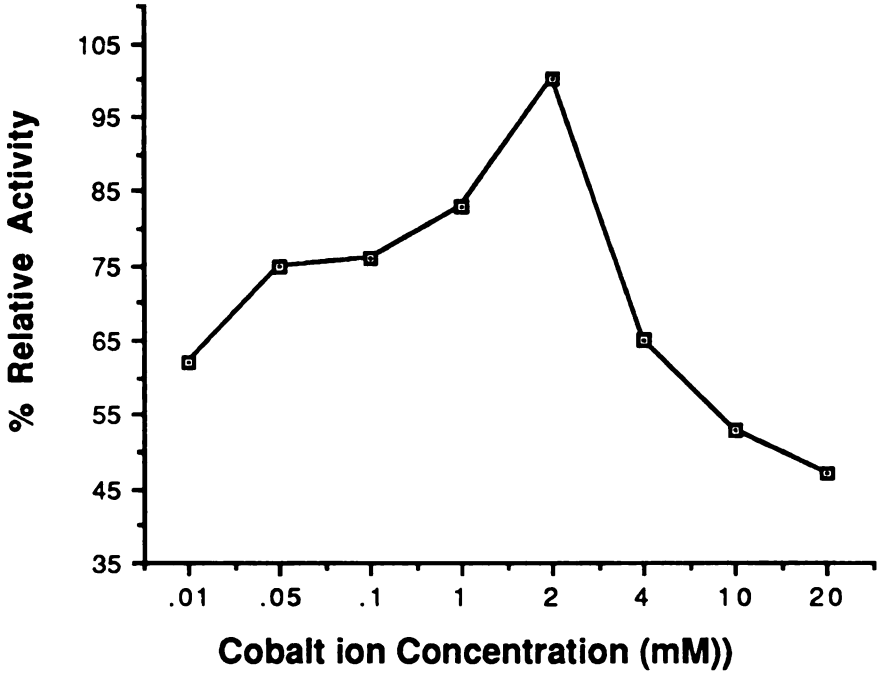


Figure 6. Effect of cobalt ion concentration on the activity of soluble glucose isomerase under the assay conditions (0.04M HEPES buffer, pH 7.7 containing 0.1M glucose and 0.1M Mg^{2+} at 60°C for 30 min.).

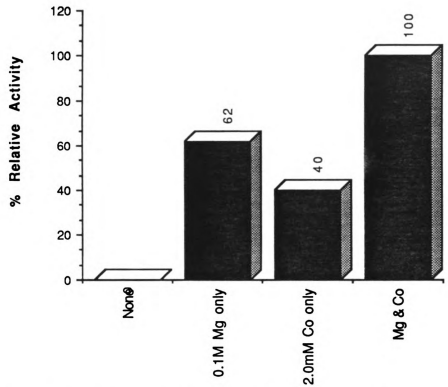


Figure 7. Effect of metal ions (none, Mg^{2+}only, Co^{2+}only, and Mg^{2+}&Co^{2+}) on the activity of glucose isomerase assayed using 0.1M glucose solution in 0.04M HEPES buffer, pH 7.7 at 60°C fo 30 min.

II. Soluble enzyme stability

A. pH stability of soluble glucose isomerase:

The enzyme was preincubated at room temperature (23^{o}C) for 30 minutes in various buffers covering a pH range from 3.5 to 11.5. 0.04M acetate, HEPES, and Glysine-NaOH buffers were used for the pH range of 3.5-5.5, 6.5-8.5, and 9.5-11.5, respectively.Then, the residual activity was assayed at pH 7.7 as mentioned in the enzyme assay section.

As shown in Figure 8, the enzyme was stable in a pH range of 5.5-11.5. The enzyme was able to retain 81.6% of its maximal activity at pH 4.5, but it became more labile at pH 3.5 at which the enzyme lost about 43% of its maximal activity. These results revealed that the enzyme under investigation showed a good pH stability at a wide pH range, especially at the alkaline side of the pH. These results were in consistent with many of the pH stability studies reported on GI's. For example, Streptomyces GI's were reported to be stable at a pH range of 4.5-11 0 (Takasaki et al., 1969), 5-12 (Suekane et al., 1978), 5-11 (Kasumi et al., 1981), or 5.7-12.0 (Callens et al., 1986). *Bacillus Stearothermophilus* GI was stable at a wider pH range (4-12) as reported by Suekane et al. (1978), while the Alkalophilic Bacillus No KX-6 GI was stable at narrower pH range (6-11) as reported by Kwon et al.(1987). The least pH stable GI was from *Lactobacillus xylosus* which was stable at a pH from 6.5 to 11.0 (Yamanaka and Takahara, 1977).

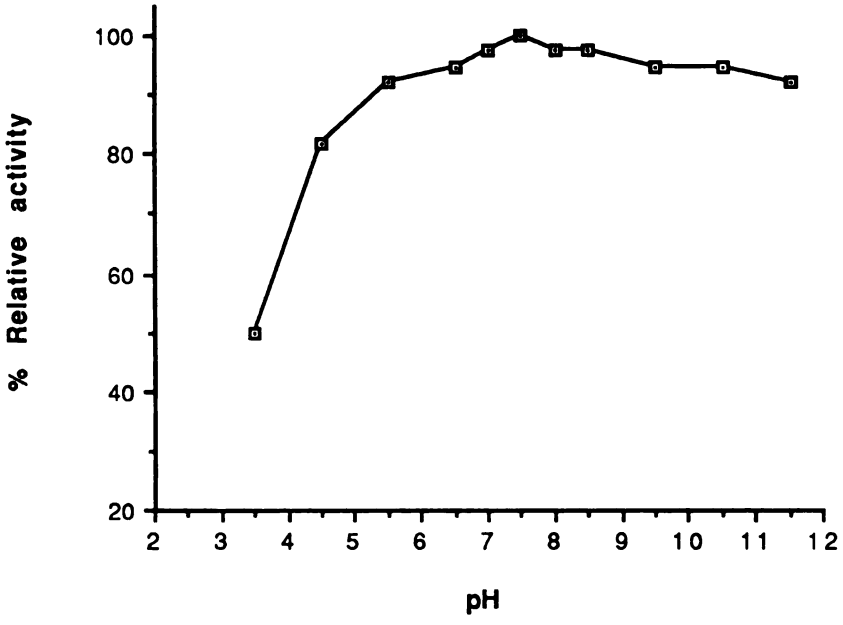


Figure 8. pH stability of soluble (unbound) glucose isomerase (0.04M acetate, HEPES, and Glysine-NaOH buffers were used for the pH range of 3.5-5.5, 6.5-8.5, and 9.5-11.5, respectively. Enzyme was incubated in the buffer for 30 min. at room temperature. Then, the residual activity was assayed at pH 7.7 using glucose solution containing 2 mM Co^{2+} and 0.1M Mg^{2+} at 60°C for 30 min.).

B. Thermal stability of soluble glucose isomerase:

The residual activity of soluble GI after incubating the enzyme (5 mg/ml) in d.d.H_2O or in 0.04M HEPES buffer, pH 7.7 in a thermostatic controlled water bath at temperatures from 55^oC to 90^oC for 30 and 60 minutes, was determined as indicated in the enzyme assay section.

As shown in Figure 9, The enzyme in either buffered or unbuffered solution was stable at 55 and 60^oC. The the enzyme gradually lost its activity at temperatures above 60^oC. Irrespective of the heating media or the time of exposure, the enzyme retained ~95% and ~84% of its activity at 65 and 70^oC, respectively. However, a rapid decrease in the enzymatic activity was noticed at temperatures of 75^oC or above. This trend was more noticable for the unbuffered enzymatic preparation which retained 70, 43, 23, and11% of its activity at 75, 80, 85, and 90^oC vs.80, 59, 42, and 18%, respectively for the buffered enzyme. The statistical analysis of the data in Figure (10) showed a significant effect ($p<0.001$) of the heat treatment (55-90^oC), the media (buffered vs. unbuffered) and the time (30 vs. 60 minutes). However the interaction of these effects was insignificant (Table 1 in Appendix A).

These results agreed with the results of Takasaki et al. (1969) for the *Streptomyces sp.* strain YT No.5 GI and the results of Yamanaka and Takahara (1977) for the *Lactbacillus xylosus* GI taking into consideration that the first authors preheated the enzyme for 10 minutes and the second authors included 1 mM Co^{2+} in the preheating buffer. This means that the investigated enzyme has more apparent thermostability than the aformentioned GI's. Also, the investigated enzyme in this regard is superior to GI from *Streptomyces olivochromogenes* which gradually lost its activity abve 55^oC even though it was preheated in the presence of 10 mM Mg^{2+} and 1 mM Co^{2+} (Suekane et al., 1978). However, the investigated enzyme was inferior to the GI from

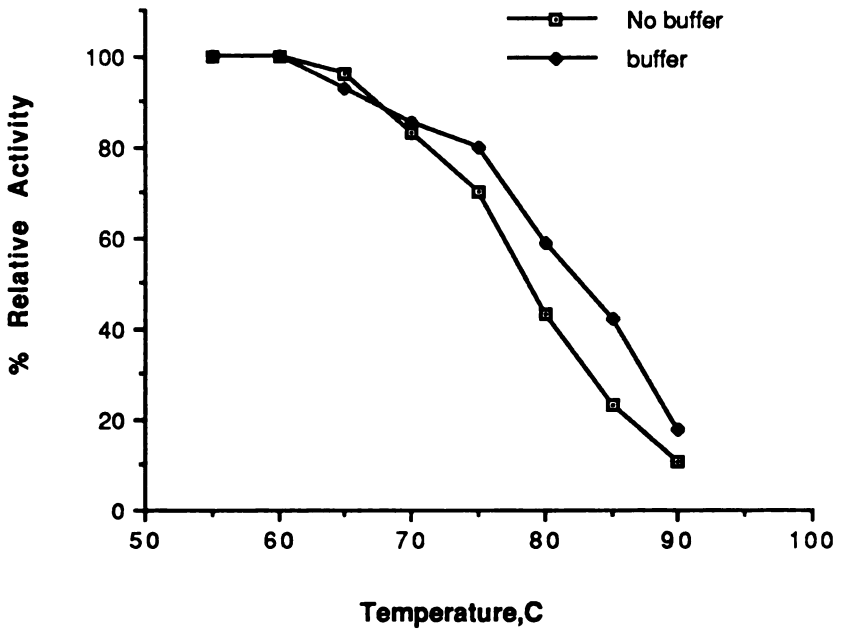


Figure 9. Thermal stability of soluble glucose isomerase in 0.04M HEPES buffer, pH 7.7, and in d.d.H_2O, no buffer (assayed using glucose solution in 0.04M HEPES buffer, pH 7.7 containing 2 mM Co^{2+} and 0.1M Mg^{2+} at 60°C for 30 min.).

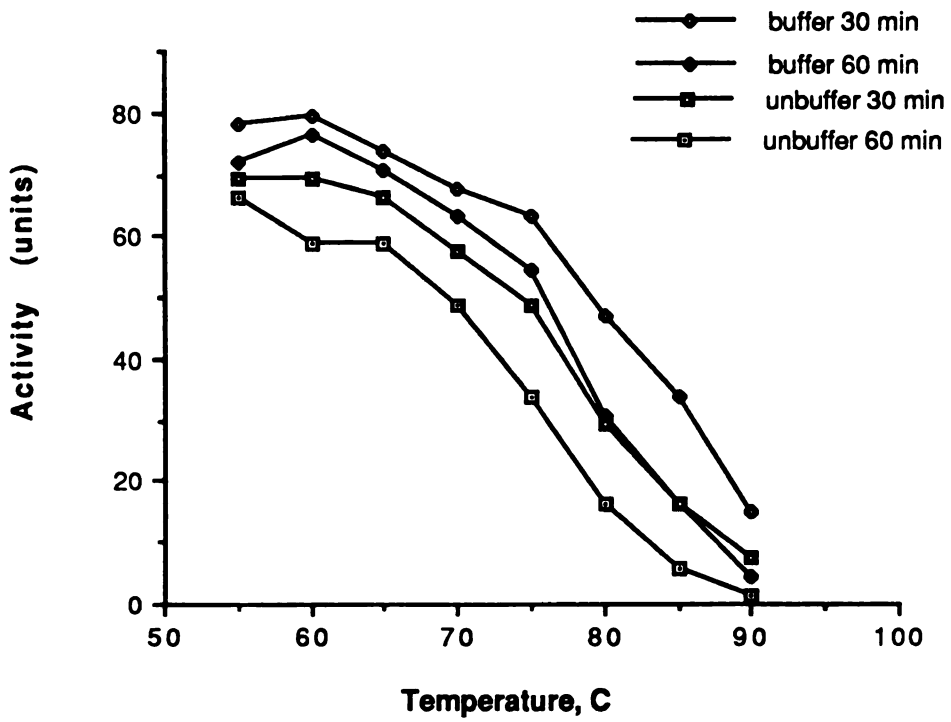


Figure 10. Thermal stability of soluble glucose isomerase in 0.04M HEPES buffer, pH 7.7 , and in d.d.H_2O (unbuffered) preincubated for 30 and 60 minutes at the corresponding temperatures. Then, the residual activity was assayed using glucose solution in 0.04M HEPES buffer, pH 7.7 containing 2 mM Co^{2+} and 0.1M Mg^{2+} at 60°C for 30 min.

Bacillus stearothermophilus which completely retained its activity up to 75^{o}C , when it was heated in the presence of cobalt and magnesium ions (Suekane et al., 1978). Again, the investigated enzyme was inferior to the GI from *Streptomyces griseofuscus* S-41 which lost no activity below 80^{o}C and retained 50% of its activity after it was preheated at 90^{o}C for 30 minutes (Kasumi et al., 1981).

C. Long-term stability (longevity) at 60^{o}C:

The long-term stability of the soluble enzyme was investigated by preincubating the enzyme (5 mg/ml) in 0.04M HEPES buffer, pH 7.7 or in the same buffer, but containing 20 mM Mg^{2+}, 2 mM Co^{2+}, or both 20 mM Mg^{2+} and 2 mM Co^{2+} at 60^{o}C and the residual activity was assayed at fixed intervals of time (0, 3, 6, 12, 24, ... up to 1032 hours).

As sown in Figure 11, when GI was incubated in 0.04M HEPES buffer, pH 7.7 without any metal addition, the enzyme rapidly lost about 75% of its original activity after 36 hours with a 12 hr half-life. The presence of 20 mM Mg^{2+} in the incubating buffer increased the stability of the enzyme ten times (the half-life became about 120 hrs). Not surprisingly, the addition of 2 mM $C0^{2+}$ greatly enhanced the thermostability of the enzyme with an 888 hr half-life. The addition of Mg^{2+} plus Co^{2+} did not change the stability of the enzyme compared to that exhibited when Co^{2+} only was added. Statistical analysis of the slope of the regression of the residual activity vs.time revealed that there was a significant difference ($p< 0.01$) between the slope of the regression of either the residual activity in buffer only or in buffer containing Mg^{2+} compared to those in buffer containing Co^{2+} or the combination of Co^{2+} and Mg^{2+} treatments (Table 2 and 3 in Appendix A). Generally, the results comply with the evidence presented by many researchers (Takasaki et al., 1969, Kasumi et al., 1982, and Callens et al., 1986) that Co^{2+} and to a lesser degree Mg^{2+} protect GI's against thermal denaturation.

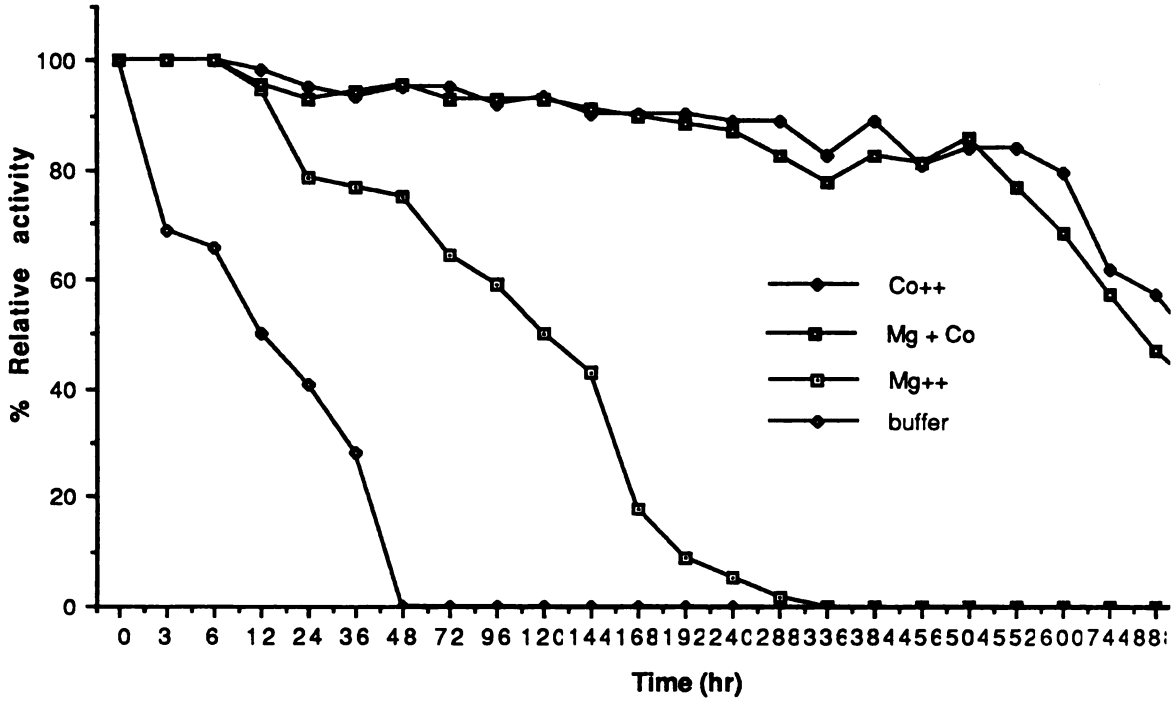


Figure 11. Thermal stability of soluble glucose isomerase in 0.04M HEPES buffer, pH 7.7 (B), B + 20 mM Mg^{2+}, B + 2mM Co^{2+}, and B + Co^{2+} + Mg^{2+} at various intervals of time.Then, the residual activity was assayed using glucose solution in 0.04M HEPES buffer, pH 7.7 containing 2 mM Co^{2+} and 0.1M Mg^{2+} at 60°C for 30 min.

When the longevity study was done by incubating the enzyme in 2.5 M glucose in 0.04M HEPES buffer, pH 7.7, there was no noticable improvement of the enzymatic stability (Figure 12).

D. The longevity of the investigated GI vs. a commercial GI preparation:

The longevity of the investigated GI was compared to that of a commercial GI preparation (Spezyme GI, M600) from *Streptomyces rubiginosus* , which was kindly donnated by Finnsugar Biochemicals, Inc., Illinois. Both of the enzymes were incubated under the same conditions (in 0.04M HEPES buffer, pH 7.7 at 60^{o}C).for up to 96hrs. As shown in Figure 13, the investigated enzyme was tremendously inferior to the commercial enzyme with regard to the long-term stability at 60^{o}C, which is the temperature used most by the HFCS industry. In another experiment similar to this one, but extending the time of incubation at 60^{o}C, the half-life of the commercial enzyme (without any metal addition) was 312 hrs (Figure 14)

All enzymes are proteins which have a very distinctive and precisely delinated structures. Activity is totally dependent on the presence of the appropriate structure at the "active site": i.e., the site where the substrate makes contact with the enzyme. Thus, structural integrity of at least one part of the enzyme alters the enzyme activity (Tombs, 1985). Structural alterations of enzyme molecules can occur from many causes, but heat denaturation is a very predominant cause. Extremes of pH and other parameters can also cause deactivation independently or by interacting with thermal denaturation (Lee et al., 1976). Tombs (1985) added that if the enzyme is one which is inherently unable to refold, there is little that can be done to preserve activity other than choosing conditions of maximum stability, or minimizing the factors which prevent refolding. Kasumi et al. (1982) reported that Co^{2+} tightly bound to GI molecules and had an essential role in holding the ordered conformation, especillay the quaternary structure of the enzyme, which resulted in a protective effect against thermal denaturation.

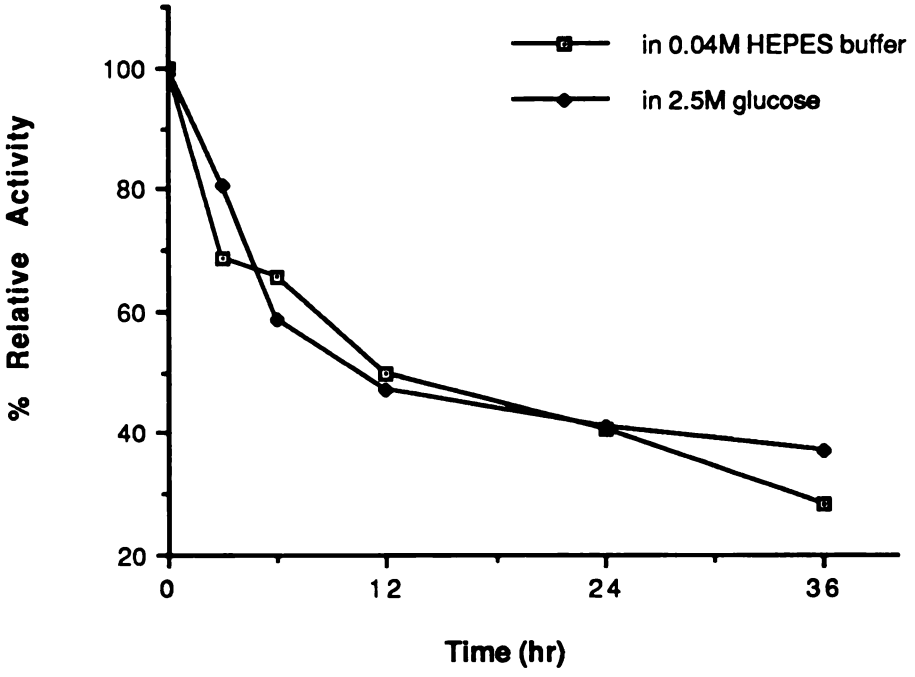


Figure 12. Long-term stability of the soluble glucose isomerase at 60°C when incubated in 0.04M HEPES buffer, pH 7.7 vs.in the same buffer, but containing 2.5M glucose.

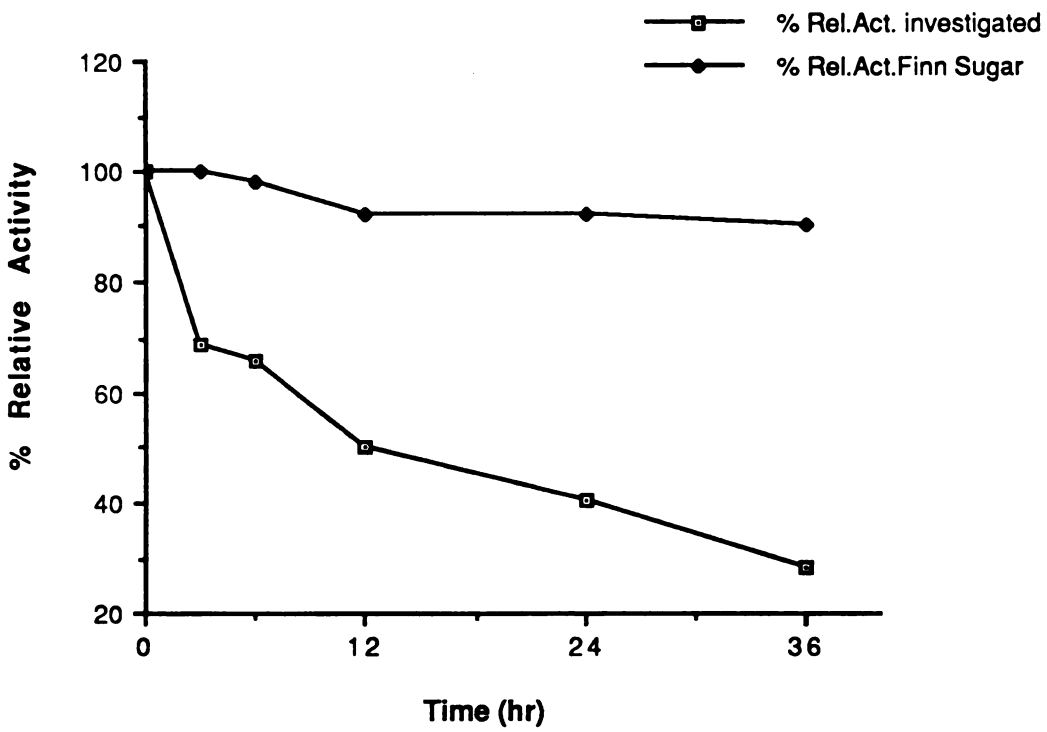


Figure 13. Long-term stability of the enzyme under investigation vs. Finnsugar enzyme at 60°C in 0.04M HEPES buffer, pH 7.7.

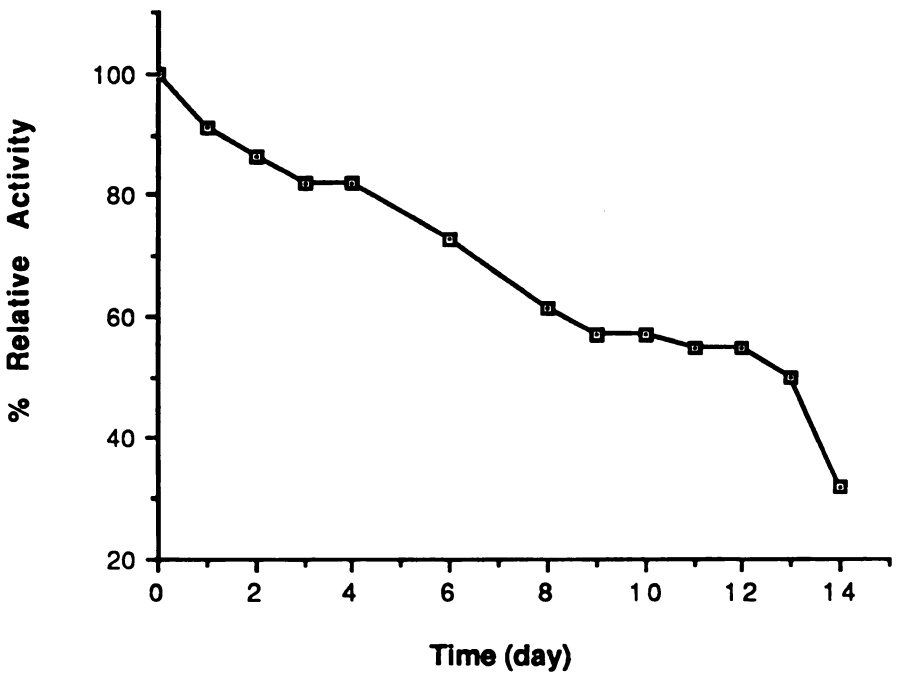


Figure 14. Long-term stability of Finnsugar GI in 0.04M HEPES buffer, pH 7.7 at 60°C.

E. Storage stability of soluble glucose isomerase:

The residual activity of soluble glucose isomerase (5 mg/ml) in 0.04M HEPES buffer, pH 7.7 containing 0.06% sodium azide at the refrigerated temperature (4-6^{o}C) was measured.

As shown in Table 4, the crude enzyme gradually lost its activity with time. The enzyme lost 13% of its original activity after 3 weeks and about 26% was lost after 7 weeks of storage. At the end of the storage period, the enzyme retained 65.2% of its original activity. However, the enzyme in lyophilized powder form (as supplied by Miles Laboratories, Inc.) hardly lost any activity over 2 years when kept at -20^{o}C (data is not shown). Boguslawski (1983) reported that the purified enzyme is exceptionally stable (less than 10% loss of activity after 1 year at 4^{o}C).

Proteolysis is a potential candidate for the cause of enzyme activity loss, during storage of the soluble enzyme, caused by the proteolytic enzymes which either was originally left in the crude enzyme or from a microbial contamination. Tombs (1985) in his enzyme stability review stated that proteases are certainly involved, and there is a rough correlation between half-life and susceptibility to proteolysis. He added that all evidence suggests that enzymes, before they can be degraded by intracellular proteases, must be in an unstructured (unfolded) form. In principle, such forms are always present in equilibrium with the folded form.

Table 4. Storage stability of soluble Glucose isomerase.in 0.04M HEPES buffer, pH 7.7 containing 0.06% sodium azide at refrigerated temperature (4-6^{o}C)

Time (week)	% Relative activity
0	100.0
1	93.5
2	89.1
3	87.0
4	78.3
5	73.9
7	73.9
9	65.2

Data standard deviation = ±3.

III. Enzyme Kinetics of soluble enzyme

Km and Vm

The apparent Michaelis-Menten constant "Km" (substrate concentration at half maximal velocity), and the apparent maximum velocity "Vm" of glucose as substrate were determined at $60^{o}C$ in the presence of 100 mM Mg^{2+} and 2 mM Co^{2+}.

From Lineweaver-Burk's plot (Figure 15), the apparent Km of the crude soluble glucose isomerase for glucose was 115 mM and the apparent Vm was 168 units. One unit equal to 1 micro mole fructose formed per minute under the assay conditions.

The obtained results for the Km was very close to the results (110 mM) reported by Boguslawski (1983) for the purified enzyme.

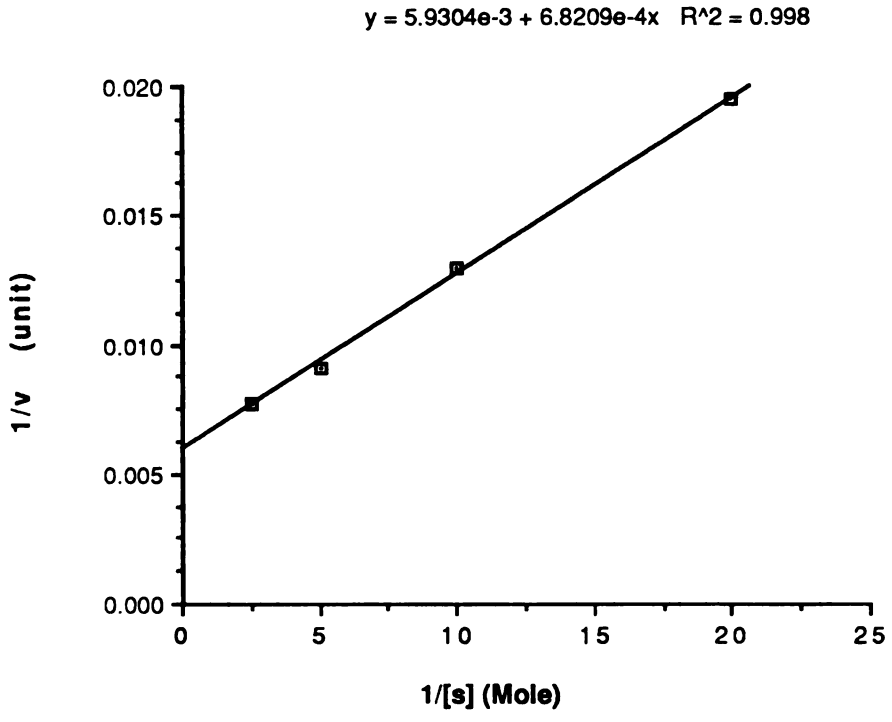


Figure 15. Lineweaver-Burk plot of soluble glucose isomerase (assay conditions: pH 7.7, 0.04M HEPES buffer containing 2 mM Co^{2+} and 0.1M Mg^{2+} at 60°C for 30 min.).

IV. Enzyme immobilization

Upon immobilization enzymes can change their thermal characteristics. These changes generally improve the thermal stability of the enzyme (Weetall, 1985). Initial activity and half life are the key to determining system performance and cost (Pitcher, 1979). The highest value of the reported densities of immobilized isomerase activity was for covalent immobilization of partially purified enzyme on coated porous glass (Havewala and Pitcher, 1974). Another study of using semipurified GI merits definite attention since purification of GI is very labor-consuming and expensive (Ananichev et al., 1984). Hamilton et al. (1974) reported that a coated porous glass is much more resistant to dissolution than uncoated glass. Moreover, the choice of an inorganic system as a support material was based upon several of the following perceived advantages cited by Weetall (1985): 1) inorganic materials can be prepared in most pore diameters (30-2000Å) and particle sizes (400 US mesh size to marble size). This allows one to optimize a process for diffusion limitation, pressure drop and other similar parameters. 2) inorganic materials are impervious to microbial attack by enzymes produced by contaminating bacterial systems, compared with some of the more common carriers such as celluloses, polydextrans and polyamines. 3) inorganic materials do not change morphology under different solvent and pH conditions, as do many organic polymers. 4) inorganic materials can be doped with activator ions which can then be eliminated from the feed. These advantages increase the ease and speed scale-up and decrease many operation problems. Based on the above reports a crude glucose isomerase was covalently bound to coated CPG beads as outlined in Figure 16.

Crude GI was immobilized on Titanium(IV)-coated, derivatized, and activated CPG beads. Two derivatization methods (amination and silanization) and two activation methods (using either glutaraldehyde or EDC) were tested. In addition, the enzyme was directly immobilized to the Titanium(IV)-coated CPG beads without further derivatization or activation steps.

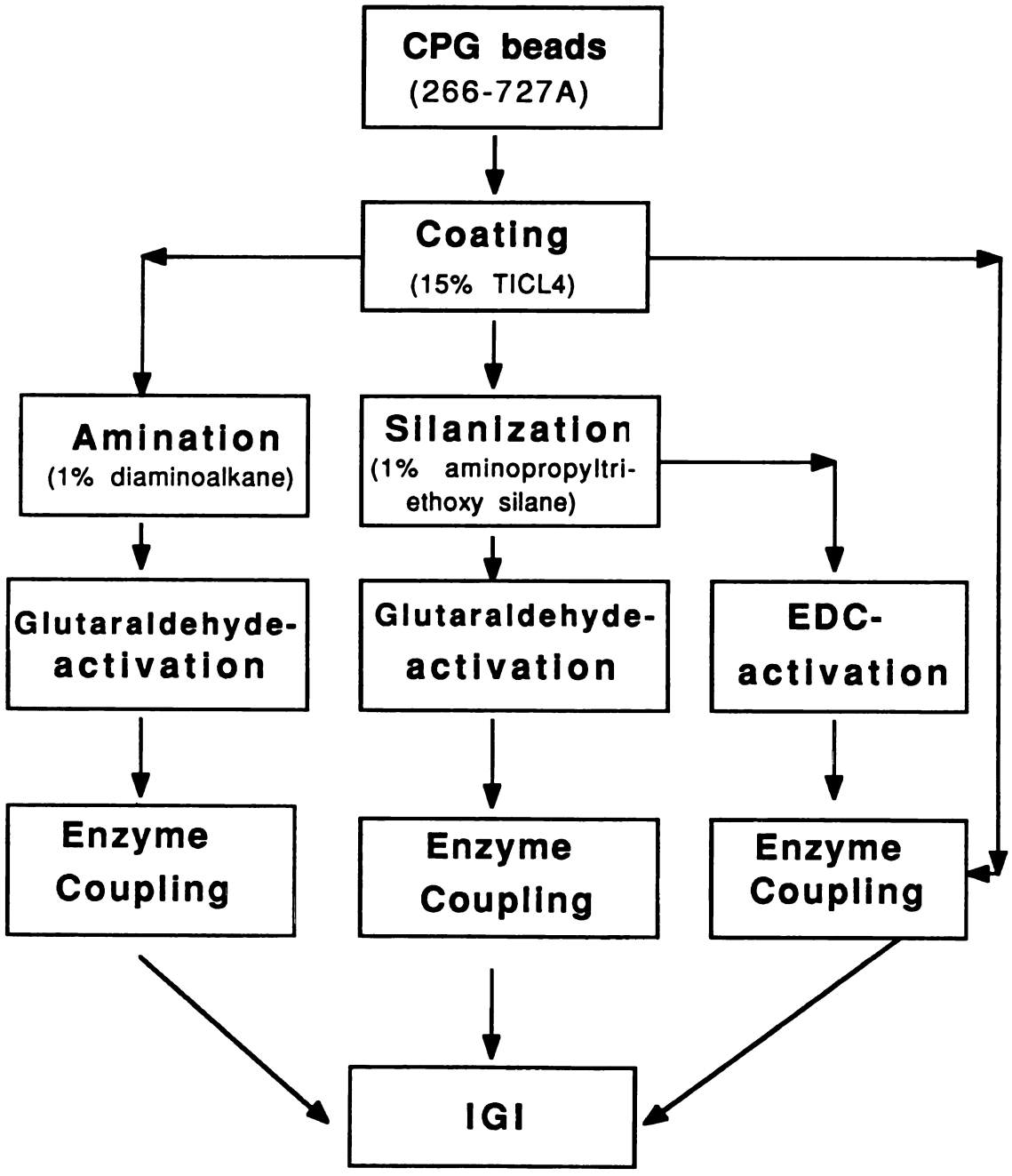


Figure 16. Outline of enzyme immobilization.

The enzyme was covalently bound to the activated CPG beads either through its residual epsilon amino groups to the glutaraldehyde-activated beads forming imine bonds (Schiff's base) or through its residual carboxyl groups to the EDC-activated beads forming amide bonds. In the direct immobilization technique, the enzyme is more likely to be adsorbed onto the surface of the beads or form a metal-chelate complex with the titanium coating.

A. Chemistry of immobilization:

The chemistry of GI immobilization is illustrated in Figures17, 18 and 19.

1. Coating of CPG beads:

As shown in Figure 17, CPG beads were coated with a layer of Titanium(VI) hydroxide or Titanium(IV) oxide/titanium(IV) chloride according to the degree of dryness. Residual chloride ions were removed when in contact with aqueous solutions. This paricular reaction was previously reported (Emery et al.,1972, Kennedy, 1979, and Cabral et al., 1982).

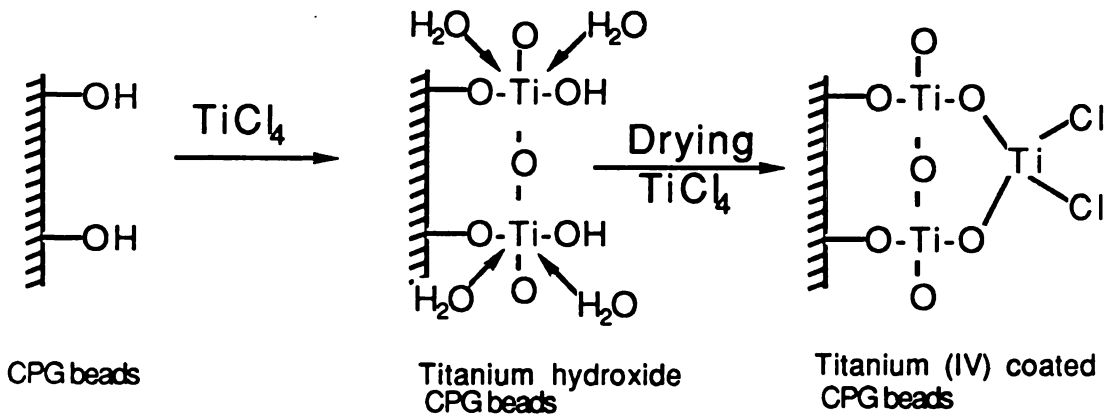


Figure 17. Titanium(IV)-coating of CPG beads

2. Amination of titanium(IV)-coated CPG beads

As shown in Figure 18, the titanium(IV)-coated CPG beads having residual chloride ions were then reacted with diamines (in chloroform) of various chain length (1,3 diaminopropane, 1,4 diaminobutane, 1,6 diaminohexane, 1,8 diaminooctane, and 1,10 diaminodecane) forming an aminoalkyl derivative. The absence of water was essential for the above reaction to proceed (Cabral et al., 1981).

3. Silanization of titanium(IV)-coated CPG beads

The coated beads were reacted with gamma aminopropyltriethoxy silane in acetone to form an amino derivatve (Fig.19). In addition, the silane may react with the titanium through the other chloride ion or the hydroxyl group according to the degree of dryness. The silane may also polymerize over the support surface (Weetall, 1985 and Lambrecht et al., 1987)

4. Activation of the derivatized CPG beads and enzyme coupling to the active beads

The alkylamine derivative, aminated or silanized beads were reacted with the common bifunctional reagent glutaraldehyde with the formation of a Schiff's base resulting in a derivative with an active aldehyde gruop Figure18 and 19.

a) Enzyme coupling to the glutaraldhyde-activated beads

The glutaraldehyde-activated beads were covalently bound to the enzyme. The residual amino groups of the enzyme being reacted with the aldhyde of the activated beads through a Schiff's base formation. A tan color formaion was an indication of the Schiff's base formation during the activation and the enzyme coupling steps Figure18 and 19.

b) Enzyme coupling using EDC

EDC was used for the simultaneous activation and enzyme coupling. The EDC was added to the enzyme solution so that the carboxyl groups of the enzyme were activated. Immediately, the silanized titanium-coated CPG beads were added. The amino groups of the silanized beads reacted with the residual activated carboxyl groups(from the aspartyl and glutamyl enzyme residues) to form an amide bond Figure 20.

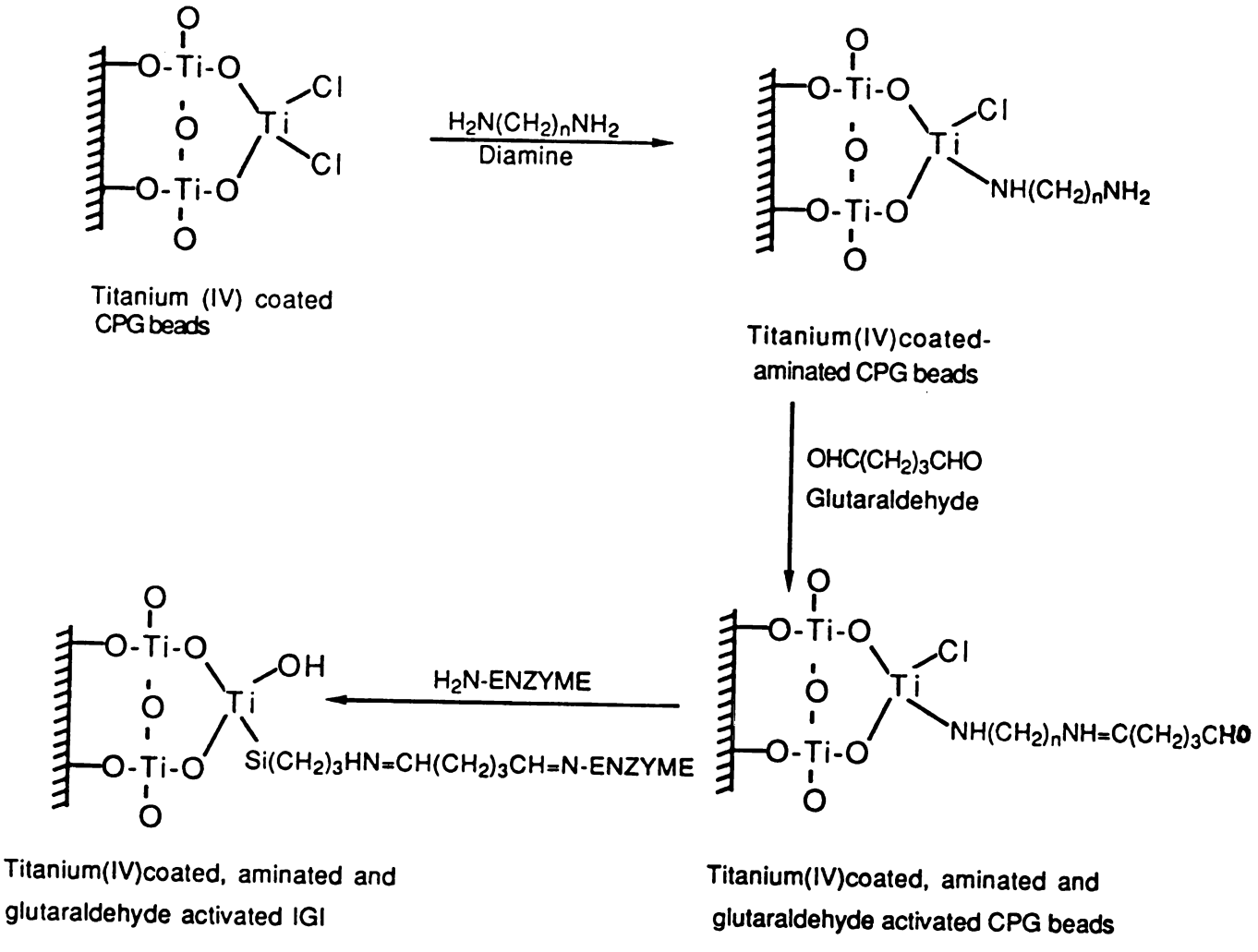


Figure 18. Amination, glutaraldhyde-activation, and enzyme coupling of Titanium(IV)-coated CPG beads.

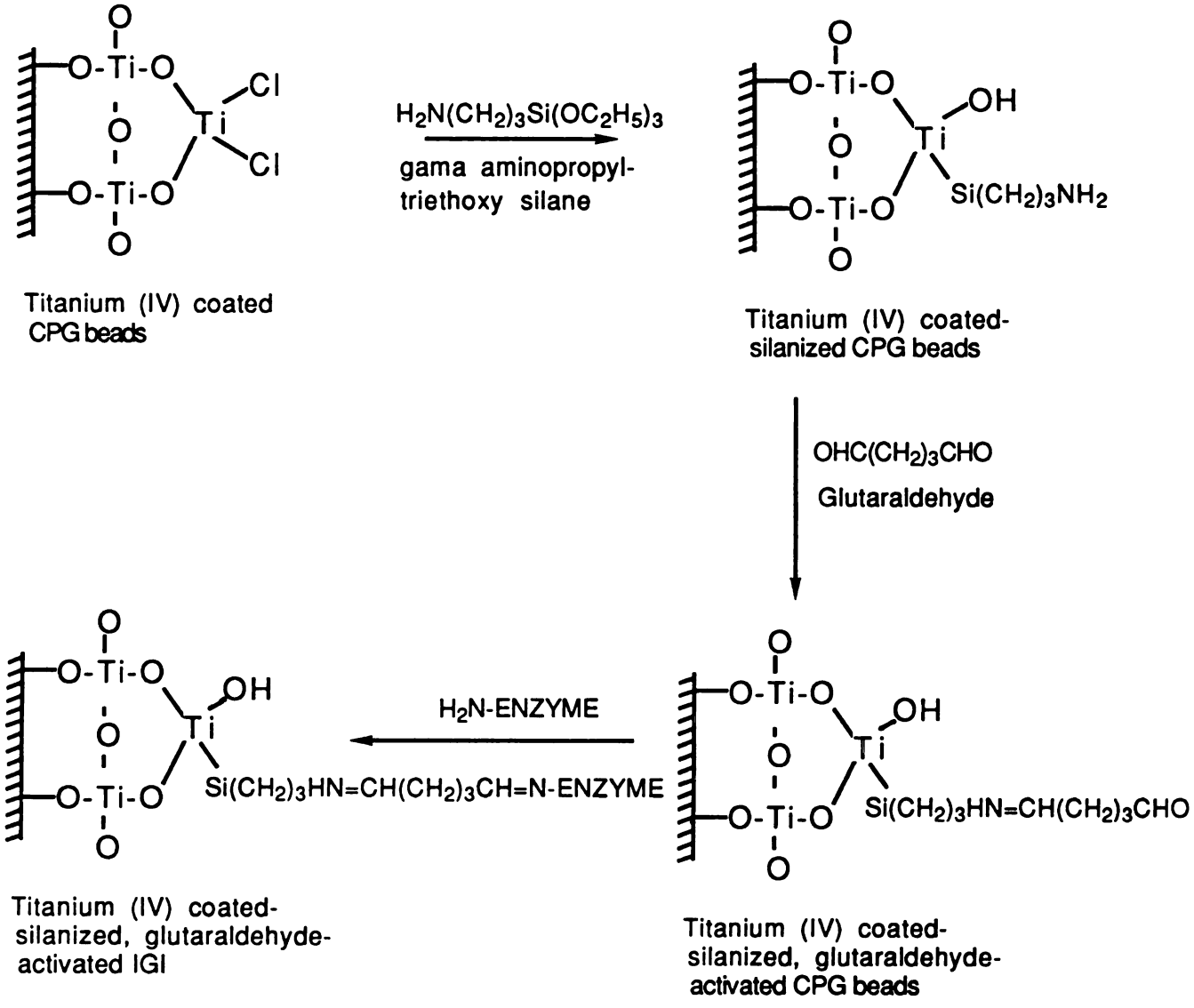


Figure 19. Silanization, glutaraldehyde-activation, and enzyme coupling to Titanium(IV)-coated CPG beads.

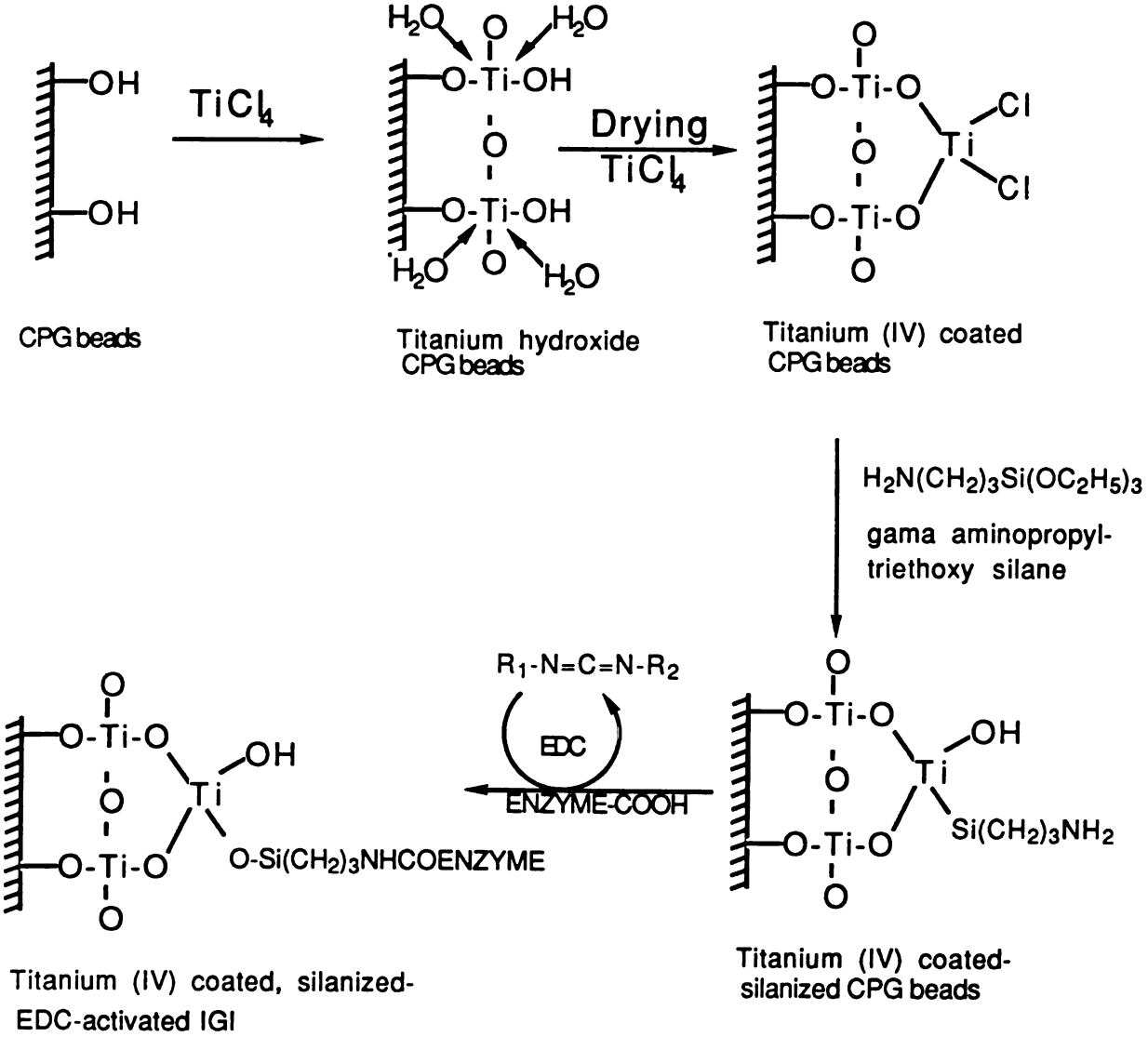


Figure 20. Titanium(IV)-coating, silanization, and enzyme coupling of CPG beads in the presence of EDC as an activator.

B. Optimization of glucose isomerase immobilization on Titanium(IV)-coated CPG beads:

The effect of pore size (diameter Å) and spacer length (number of carbon atoms) between GI and the Titanium(IV)-coated CPG beads were throughly investigated. Also, the effect of pH and EDC concentration on enzyme immobilization using EDC was studied to determine the optimum conditions for the covalent binding of the enzyme.

Since a crude enzyme was used in the immobilization, it is important to remember that extraneous protein could bind to the beads. The protein content of all immobilized GI preparations was determined directly by the Micro Kjeldahl method and was expressed as mg protein per g dry IGI preparation.

1. Effect of pore size (diameter) on the activity of bound glucose isomerase

Pore size is an important factor in choosing the proper carrier for the enzyme to be immobilized (Weetall, 1976). The author added that there will always be an optimal pore diameter for an enzyme. Therefore, Titanium(IV)-coated silanized glutaraldehyde-activated CPG beads of pore diameter from 266 Å to 727 Å were used to determine the optimum pore size for GI immobilization.

As shown in Figure 21, the highest activity of bound GI was obtained with beads of 350 Å pore diameter. The activity gradually decreased as the pore size increased. This trend coinsides with the activity of the unbound enzyme remaining in the filterate (unbound enzyme), i.e. there was an inverse relationship between the activity of the bound enzyme and the activity remaining in

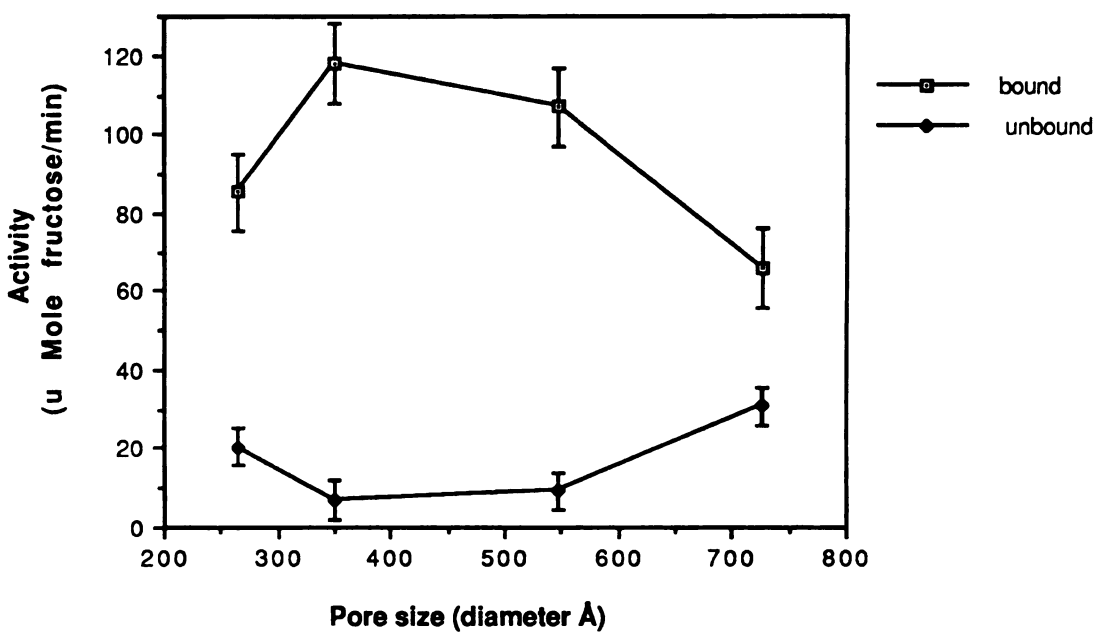


Figure 21. Effect of pore size on the activity of bound and unbound (left in filterate during immobilization) glucose isomerase.

the filterate, suggesting that the increase of the bound enzyme activity that was found at the lower side of the pore diameter range used was due to the increase in the amount of enzyme bound to the beads. Therefore, the effect of pore size on the amount of bound protein was investigated along with the activity of the corresponding IGI preparation As shown in Figure 22. The results confirmed that bound enzyme activity paralleled the amount of protein bound to the beads.The highest protein load was 44.6 mg/g beads for the 350 Å beads which gave the highest enzyme activity, as indicated earlier. The lowest protein load (26 mg/g beads) was seen with the the beads having the highest pore diameter (727 Å) which gave the lowest activity. The activity of GI bound to 266 Å beads was relatively low when it was compared to the amount of protein that it bound. This may be due to diffusional limitations which arose at that small pore size. The statistical analysis (one factor ANOVA) of the bound enzyme activity indicated a significant effect ($p<0.09$) of the pore size. However, when the means of bound enzyme activity were compared, a significant difference was found only for 350 Å vs. 727 Å and 547 Å vs.727 Å comparisons (Table 5 and 6 in Appendix A).

Since there is an inverse relationship between the pore diameter and the surface area, activity per unit surface area and activity per unit protein weight were examined. As shown in Figure 23, the specific activity (either per unit surface area, 1 m^2, or per unit protein weight, mg protein) remained relatively constant for the pore size from 350 Å to 727 Å. The smallest pore size (266 Å) was an exception to the above findings due to its extermely high surface area (94 m^2/g beads) and the low activity expression discussed earlier. From the above results, the optimum pore size for glucose isomerase immobilization was in the range of 350-547 Å under the assay conditions used in this study. The 350 Å CPG beads were selected for further enzyme immobilization due to its superiority over 547 Å CPG beads with regard to activity and amount of bound protein. These results were in agreement with the conclusions of Swaisgood (1985) that as the pore size increases one would expect less enzyme to bind per unit weight and the amount of immobilized enzyme per unit surface area to remain relatively constant. Moreover, CPG beads

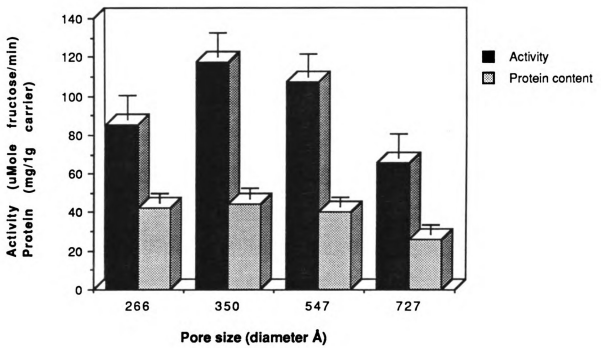


Figure 22. Effect of pore size on the activity and protein content of bound glucose isomerase.

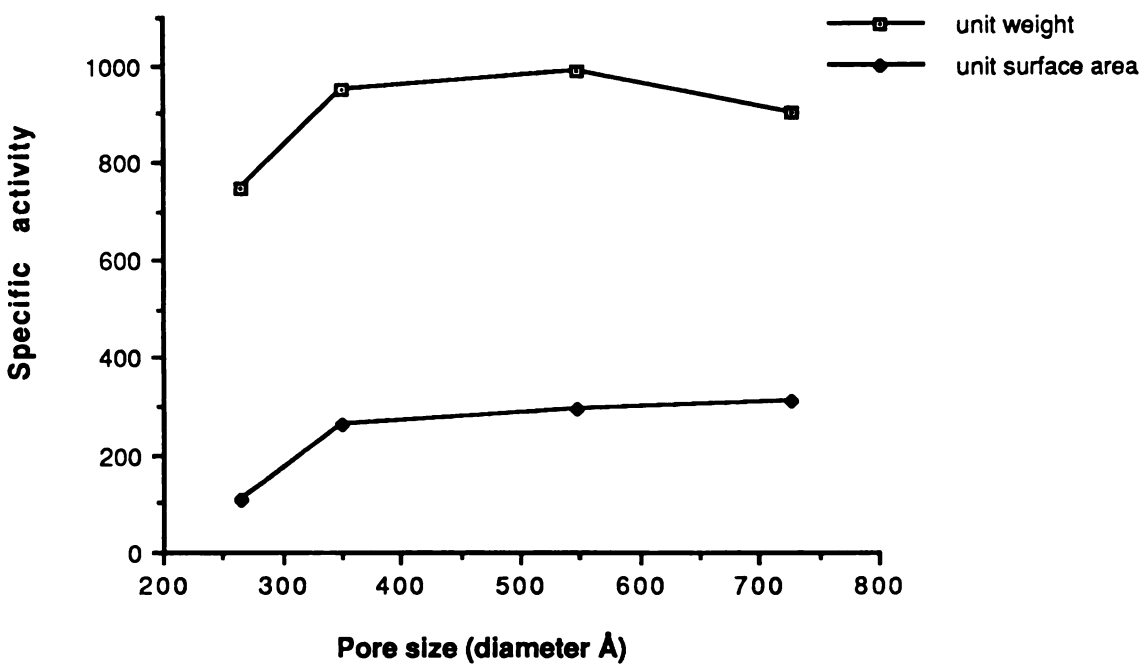


Figure 23. Effect of pore size on the specific activity of bound GI calculated per unit weight and unit surface area of immobilized glucose isomerase.

with pore diameter of 350 Å and 550 Å were used for GI immobilization by Havewala and Pitcher (1974) and Lee et al.(1976), respectively.

2. Effect of spacer length on the activity of bound glucose isomerase

The effect of spacer length , in terms of number of carbon atoms between the enzyme and the carrier (Titanium(IV)-coated CPG beads of 350 Å pore diameter) on the activity of bound glucose isomerase was studied. The carrier was derivatized with diamines of various chain length (3-10 carbon atom long) and all derivatives were coupled to the enzyme using glutaraldehyde as explained earlier in the chemistry of immobilization section.

As shown in Figure 24, the activity of bound GI was greatly affected by the spacer length between the carrier and the enzyme. The highest activity (94.2 units) was obtained when diaminohexane (C_6) was used as a spacer. Diaminobutane (C_4) and diaminooctane (C_8) were next to diaminohexane giving relativity the same activity (89.5 and 84.3 units for C_4 and C_8, respectively). However, diaminodecane gave only (50.4 units) about half of the activity that was obtained with the C_6 spacer. Interestingly, diaminopropane (C_3), which had the highest NH_2 group concentration (100 uMole) among the diamines tested, gave no activity when used as a spacer. In addition, the activity of the unbound enzyme left in the filterate after immobilization, using the aforementioned spacers, increased as the activity of bound enzyme decreased, suggesting that the variation in the activity of bound enzyme preparations of different spacer length may be attributed to the differences in the amount of bound protein (enzyme).

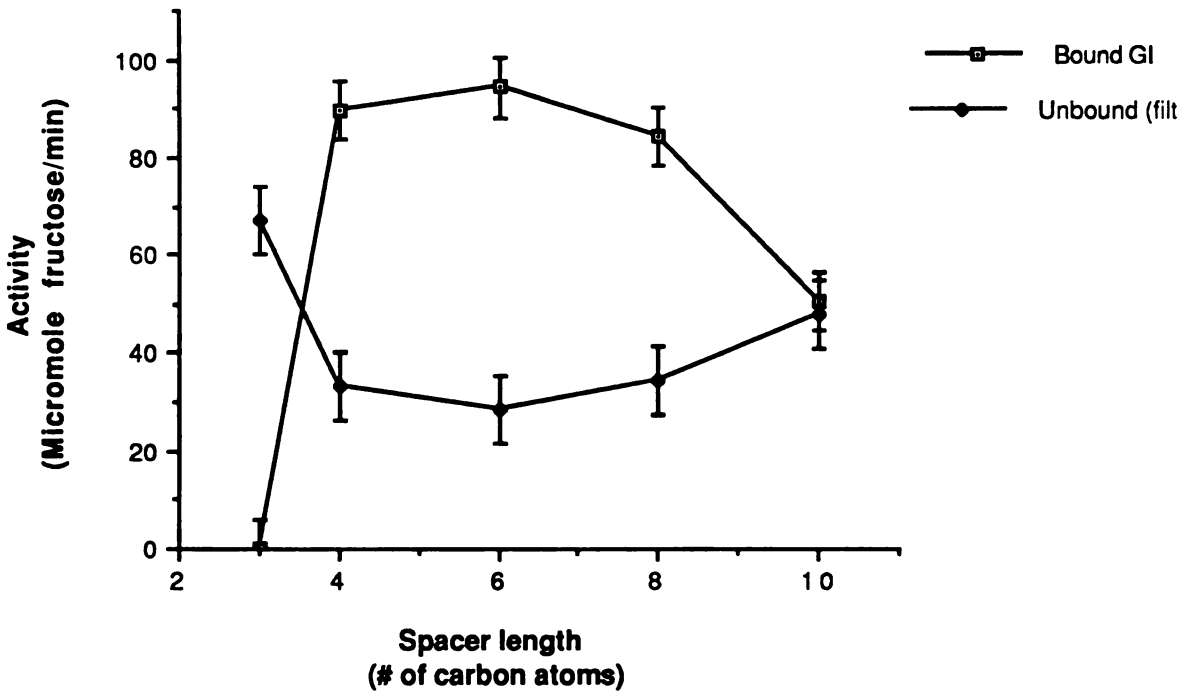


Figure 24. The effect of spacer length (number of carbon atoms) on the activity of bound and unbound glucose isomerase.

The statistical analysis (one factor ANOVA) of the activity of bound enzyme indicated a significant ($p<0.001$) effect of the spacer length. Therefore, the activity means of the bound enzyme preparations were compared. The comparisons showed that there was no significant difference for C_6 vs. C_8, C_6 vs. C_4, and C_8 vs. C_4 comparisons. However, a significant difference did exist for C_6 vs. C_{10} ($p<0.01$), C_4 vs C_{10} ($p<0.02$), and C_8 vs. C_{10} ($p<0.05$) comparisons (Table 7 and 8 in Appendix A)

The amount of bound protein decreased as the spacer length increased from C_4 to C_{10} (Figure 25). However, no protein was found when C_3 was used as a spacer.explaining the absence of activity that was noticed for that spacer. This is thought to be due to the high possibility of 6 member ring formation (Carey and Sundberg, 1984) during the amination reaction of the Titanium(IV)-coated CPG beads with the diamino propane Figure 26. The cyclic derivative formation would prevent the formation of active carbonyl derivative, when reacted with glutaraldehyde, and consequently prevent the binding of the enzyme. In addition, the activity of the bound enzyme was correlated ($r=0.98$) to the amount of bound protein (Table 9 in Appendix A). Thus, the specific activity of the bound enzyme was relatively constant with regard to the spacer length in the range (C_4-C_{10}) studied. From the results discussed above (Figure 24, 25, and 27), the proper spacing for GI immobilization ranged from C_4 to C_8 with C_6 as the optimum one.

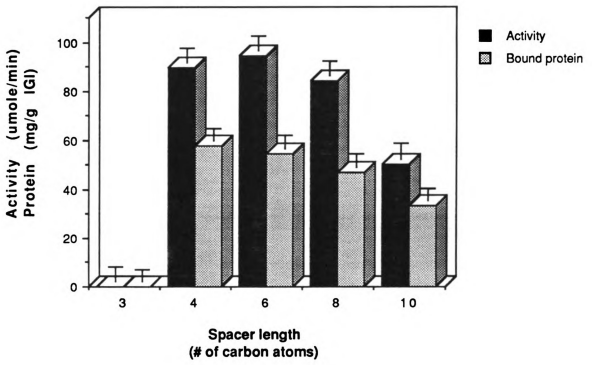
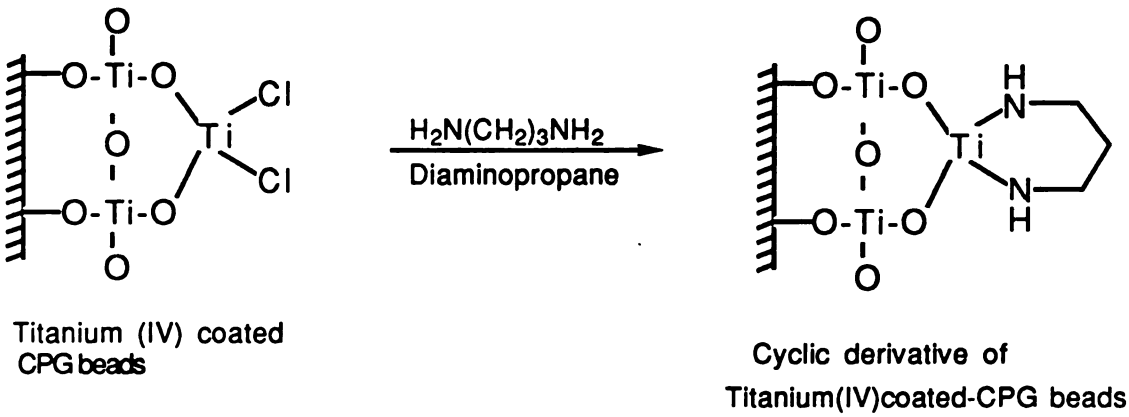


Figure 25. Effect of spacer length (number of carbon atoms) on the activity and the amount of bound glucose isomerase.



Titanium (IV) coated CPG beads

Cyclic derivative of Titanium(IV)coated-CPG beads

Figure 26. Projected ring formation upon the reaction of Titanium(IV)-coated CPG beads with diaminopropane.

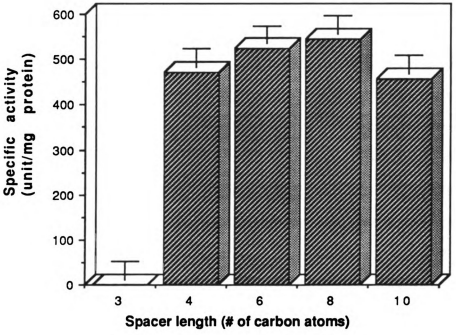


Figure 27. Effect of spacer length (number of carbon atoms) on the specific activity of bound glucose isomerase.

3. Effect of pH and EDC concentration on the activity and amount of protein bound to EDC-activated silanized Titanium(IV)-coated CPG beads

EDC can be used for enzyme immobilization in two main methods. In the first method (simultaneous method), the EDC is added to the enzyme and the mixture is immediately added or circulated to the amino-containing or the carboxyl-containing carriers. In the second method (sequential method), the carrier is first activated by adding or circulating the EDC solution to the carrier followed by washing the excess of EDC with a suitable buffer. Then, the enzyme solution is added or circulated to the activated carrier.The EDC concentration used with the simultaneous method is 1/10 to 1/100 of that commonly used with the sequential method. The reported results using the two methods varied according to the type of enzyme and the conditions of immobilization used. While equivalent activity were obtained with either of the methods for sulfhydryl oxidase immobilized on succinamidopropyl glass beads (Swaisgood and Horton, 1976 and Janolino and Swaisgood, 1982), only the sequential technique was successful in preserving the activity of fibrinolysin on polyacrylamide gel, while a complete enzyme inactivation occured when the simultaneous method was used.(Papisov et al., 1985). These authors concluded that the only reliable optimization technique is to plan the conditions for every case.

In order to establish the optimum condition for glucose isomerase immobilization using EDC as an activator, the amount of EDC and the pH were varied. As shown in Table 10, the amount of protein bound and the enzyme activity increased with the increasing of either pH (from 5 to 7) or EDC concentration (from 5 to 20 mM) except that at pH 5 and 20 mM EDC, the activity of the bound enzyme was minimal due to the combined effect of low pH and high EDC concentration. The highest activity (111.1 uMole fructose/minute) was obtained at pH 7 and 20

mM EDC followed by an activity of 106.5 at the same pH, but with 10 mM EDC. However, the specific activity of the bound enzyme at 10 mM EDC was higher (513 units/mg protein) than that obtained (496 units/mg protein) at 20 mM EDC at the same pH (7) as shown in Figure.28. Statistical analysis (2 factor ANOVA, Table 11 in Appendix A) of the effect of pH and EDC concentration on the activity of the bound enzyme indicated a significant effect of the pH ($p < 0.01$) and the EDC concentration ($p < 0.01$). The analysis also indicated a significant interaction between the two effects ($p < 0.01$). Comparisons of the means of the bound enzyme activity between the pH levels used indicated that at 5 mM EDC there was no significant difference for pH 5 vs. pH 6 comparison. However, at 10 and 20 mM EDC, significant variations were obtained for pH 5 vs pH 6 and pH 5 vs. pH 7, but no significant difference was found for the comparisons of pH 6 vs. pH 7 at the two EDC levels (10 and 20 mM) as shown in Table 12 in Appendix A. Moreover, it is more economical to use the lower concentration of EDC and the neutral pH which is more gentle on the enzyme than the acidic pH's. Therefore, pH 7 and 10 mM EDC were chosen as the optimum condition for subsequent immobilization using EDC.

Table 10. Effect of pH and EDC cocentration on the activity and amount of protein bound to EDC-activated silanized Titanium(IV)-coated CPG beads

EDC (mM)		pH 5	pH 6	pH 7
5	activity[1]	80.3	76.4	92.6
	Protein[2]	46.3	48.9	56.6
10	activity	81.0	101.8	106.5
	protein	51.1	56.5	62.9
20	activity	69.4	99.5	111.1
	protein	53.0	60.7	67.9

1 micromole fructose/minute

2 mg protein/g IGI preparation, dry weight basis.

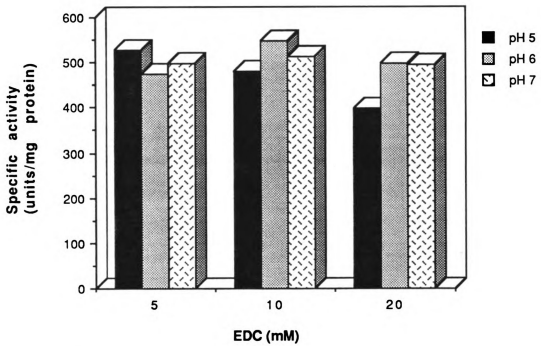


Figure 28. Effect of pH & EDC concentration on the specific activity of bound GI.

C. Effect of immobilization techniques on the amount and percentage of bound glucose isomerase

1. Effect of type of alkylamine (aminated vs.silanized) on the amount and percentage of bound enzyme

As shown in Table 13, Titanium(IV)-coated aminated and Titanium(IV)-coated Silanized CPG beads which were activated by the same method (using glutaraldehyde) bound the same amount (about 49 mg/1g IGI, dry weight bases) of protein. The percentage of binding reached about 57% of the offered crude soluble enzyme. This may be because the amount of functional amino groups on both aminated and silanized beads were not greatly varied (84 vs. 95 uM/g carrier for aminated and silanized beads, respectively). These results were in accordance with the results of Lee et al.(1976), who reported GI loading of 48 mg/1g zirconium-coated porous glass beads. However, the results were higher than those reported by Strandberg and Smiley (1971), who reported that 40% of the cell-free enzyme added was entrapped in polyacrylamide gel, and Goldberg et al.(1979), who reported 50% binding of GI covalently bound to PVC-silica support devolped at Amerace Corporation. Higher enzyme load (60 mg/1g carrier) was reported by Voroshilova et al.(1984), for GI bound to macroporous silica carriers having pore size of 500-800Å.

Table 13. Effect of type of alkylamine (aminated vs. silanized CPG beads) on the amount and percentage of binding of glucose isomerase

Alkylamine-CPG bead derivative	uMole NH_2 groups	Bound protein mg/1 g IGI means ± Std[1]	% of binding mean ± Std.
Aminated	84	48.7 ± 3.18	56.7 ± 3.72
Silanized	95	49.1 ± 4.29	57.0 ± 5.17

1 Standard deviation of data

2. Effect of method of activation on the amount and percentage of bound GI bound to Titanium(IV)-coated silanized CPG beads.

As far as the type of activation methods used, the EDC-activated beads significantly ($p<0.01$), as shown in Table 14 in Appendix A, bound more protein (63.2±3.1 mg/1g IGI) than the glutaraldehyde-activated beads (49.1±4.3 mg/1g IGI). Consequently, the percentage of bound protein varied for the two activation methods with 57.0±5.2% for the glutaraldehyde activator and 73.5±3.6% for the EDC activator (Table 15). Glutaraldehyde is a common bifunctional reagent that has been used for the binding of many ligands (having any group that can react with the aldehyde group in the glutaraldehyde reagent) in the area of affinity chromatography, immunoassay, and enzyme immobilization (Robenson, 1971, Mosbach, 1976, and Weetall, 1976). On the other hand, EDC which catalyzes the amide bond formation between a ligand having carboxyl group and another reactant having an amino group. This catalyst was relatively not as common as glutaraldehyde especially in the area of enzyme immobilization. Recently, EDC become a good choice for enzyme immobilization over a wide range of pH (Swaisgood et al., 1976, Kjellen and Neujahr, 1979, Janolino and Swaisgood, 1982, and Papisov et al., 1985).

D. Effect of immobilization techniques on the percentage of activity retained and specific activity of bound glucose isomerase.

As shown in Table 16, the percentage of activity retained upon immobilization on Titanium(IV)-coated aminated (A) or Titanium(IV)-coated silanized beads (S), activated by glutaraldehyde, was from 73.6 to 76.8. A higher percentage of activity retained (about 90%) was obtained when the Titatanium(IV)-coated silanized CPG beads were activated by EDC (E) The increase in % activity retained noticed for E preparation was due to the high protein load of EDC-activated beads as discussed earlier. The specific activity of the bound enzyme was 586, 600, and

545 units/mg protein for A, S, and E preparations, respectively. The specific activity of the free enzyme was lower (441units/mg protein) than that of the bound enzyme preparations. The above results were obtained when both free and bound GI were assayed at the same conditions (optimum conditions for the free GI). This increase of specific activity seems to be not only due to the selective immobilization of enzymatic protein over the extraneous protein in the crude enzyme extract added to the beads, but also could be due to the activation effect of immobilizing the enzyme on a very high surface area carrier (53.45 m^2/g carrier) which prevented a protein-protein interaction that might occur in the free enzyme. Moreover, the activation increase could be because of the structural effect that the enzyme might experience upon immobilization. The results obtained were in agreement with the results of Goldberg et al. (1979), who reported a 90-100% activity yield of GI bound to macroporous PVC-silica support and Chen et al. (1981) who reported a 70% activity retained for GI adsorbed on DEAE-cellulose. However, the results obtained were higher than many other reported results. For example, 32% activity retained for chitosan-bound GI (Kasumi et al., 1977), 41-47% for diazotized CPG beads bound GI (Stanely and Smiley, 1972), and 56% for zirconium-coated CPG beads bound GI (Lee et al., 1976).

When the enzyme was directly immobilized on Titanium(IV)-coated CPG beads, no activity was detected. This inactivation may be due to the spacing effect (i.e. there was no spacer arm between the titanium layer and the enzyme) or due to traces of $TiCl_4$ left on the beads which was not completely washed from the beads. In addition, this method bound only 10% of the added enzyme. In this case, the enzyme was more likely to be chemically adsorbed rather than covalently bound to the beads as with the rest of the immobilization techniqes used. The adsorbed enzyme is, of course, more readily washed away from the beads under the conditions applied (1 M NaCl). Titanium(IV)-coated bound GI.

Table 15. Effect of method of activation on the amount and percentage of bound GI to Titanium(IV)-coated silanized CPG beads

Activator	Bound protein	
	mg/g IGI ± Std[1].	% of Binding ± Std.
Glutaraldehyde	49.1 ± 3.2	57.0 ± 5.2
EDC	63.2 ± 3.1	73.5 ± 3.6

1 Standard deviation of the data.

Table 16. Effect of immobilization techniques on the percentage of activity retained and specific activity of bound glucose isomerase

Enzyme preparation	Activity (units[5]) added	bound	%Activity retained	Specific activity[6]
A[1]	37965	27930	73.6 ±8.5	586 ± 27
S[2]	37965	29176	76.8 ± 9.7	600 ± 40
E[3]	37965	34016	89.6 ± 6.5	545 ± 30
T[4]	37965	0	0.0	0

1 Titanium(IV)-coated aminated glutaraldehyde-activated CPG bound GI

2 Titanium(IV)-coated silanized glutaraldhyede-activated CPG bound GI

3.Titanium(IV)-coated silanized EDC activated-CPG bound GI

4 Titanium(IV)-coated CPG beads bound

5 Micromole fructose/minute

6 Units/mg protein, specific activity of added enzyme was 441 umits/mg protein.

E. Optimum activity conditions for bound glucose isomerase

1.Optimum activity pH

As shown in Figure 29, the activity of all IGI preparations (A, S, and E) reached its maximum at pH 8. Not surprisingly, the activity of the IGI preparations was stable in the alkaline pH (up to 11.5). However, below pH 8 the activity gradually decreased as the pH decreased (H^+concentration increased). Therefore, pH 8 was considered the optimum pH for subsequent IGI assay.

Comparing the pH activity profile of the bound GI to that of the soluble enzyme, Figure 30 showed that the bound enzyme had a wider pH activity range. This may be due to the increase of structural rigidity of the bound enzyme molecules upon immobilization,.for example.the unfolding and other pH dependent structural changes of the enzyme molecules became more restricted for IGI than that for the soluble enzyme. This explanation is supported by the evidence found during the pH stability study, which will be discussed later. A wider pH activity for the immobilized enzymes than the free enzymes was reported by Weetall (1985).

2. Optimum activity temperature

The activity of IGI preparations (A, S, and E) at the optimum pH, was studied at various temperatures (50-90^{o}C). As seen in Figure 31, the activity of the IGI preparations increased as the temperature increased reaching the heighest activity at 70^{o}C. About 95% relative activity was obtained at 80^{o}C, then the activity markedly decreased above 80^{o}C.

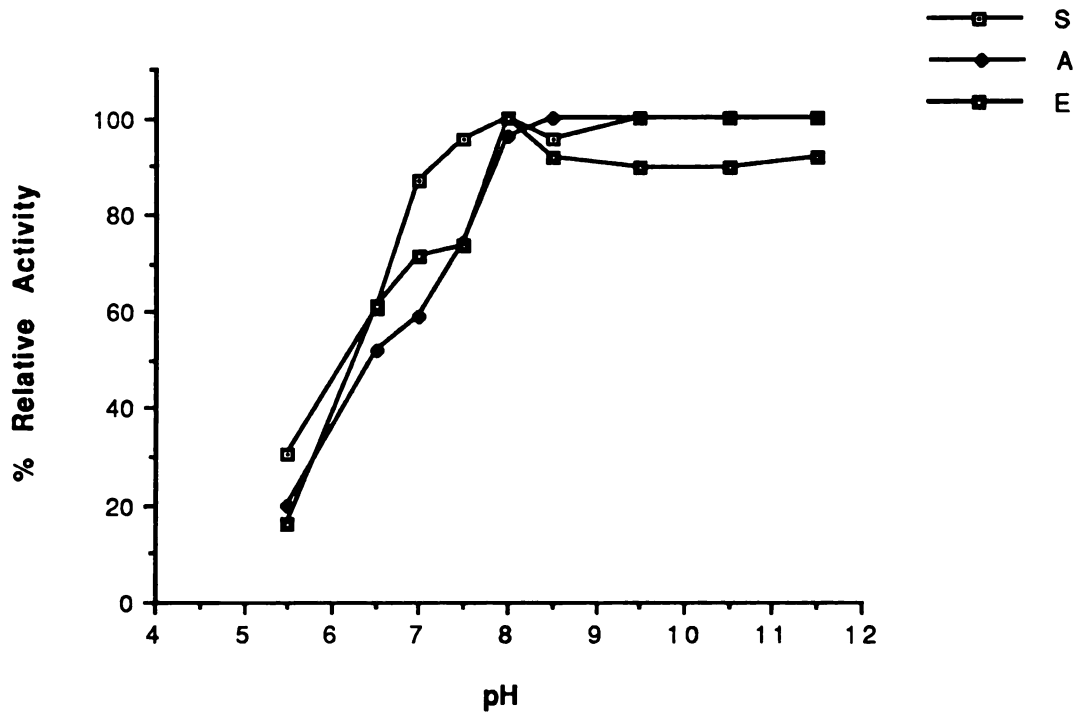


Figure 29. Optimum activity pH of bound glucose isomerase preparations A, S, and E which correspond to enzyme bound to aminated glutaraldehyde-activated, silanized glutaraldehyde-activated, and silanized EDC-activated Titanium(IV)-coated CPG beads, respectively.

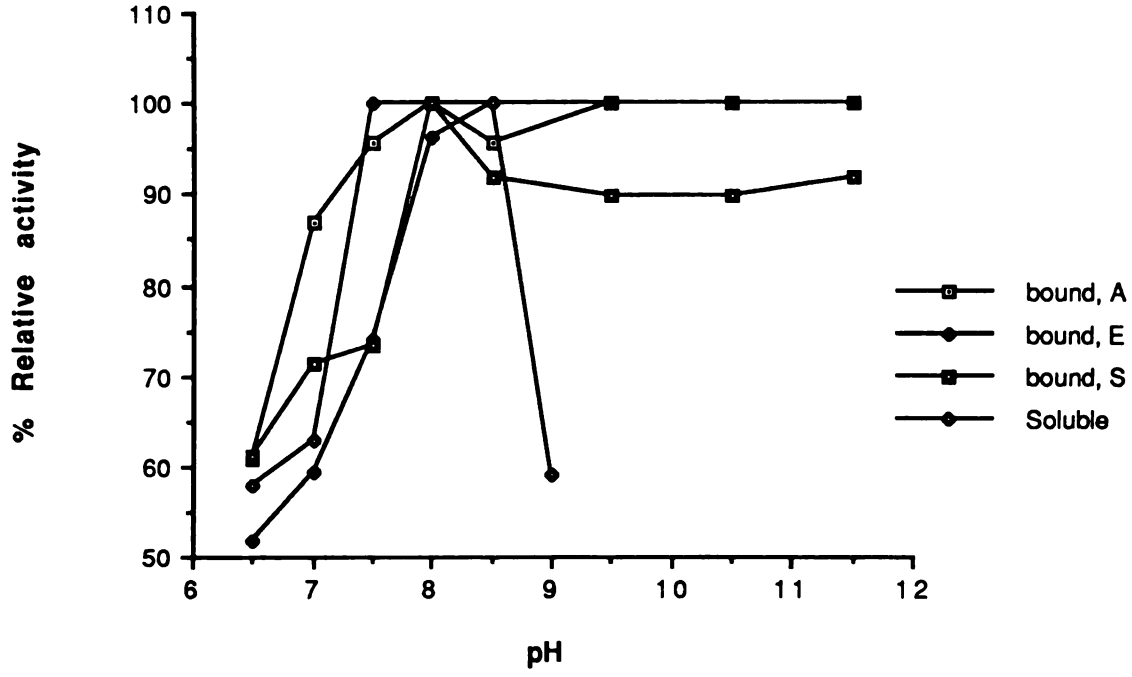


Figure 30. Effect of pH on the activity of bound (A, S, and E which correspond to enzyme bound to aminated glutaraldehyde-activated, silanized glutaraldehyde-activated, and silanized EDC-activated Titaniuum(IV)-coated CPG beads, respectively) and soluble glucose isomerase.

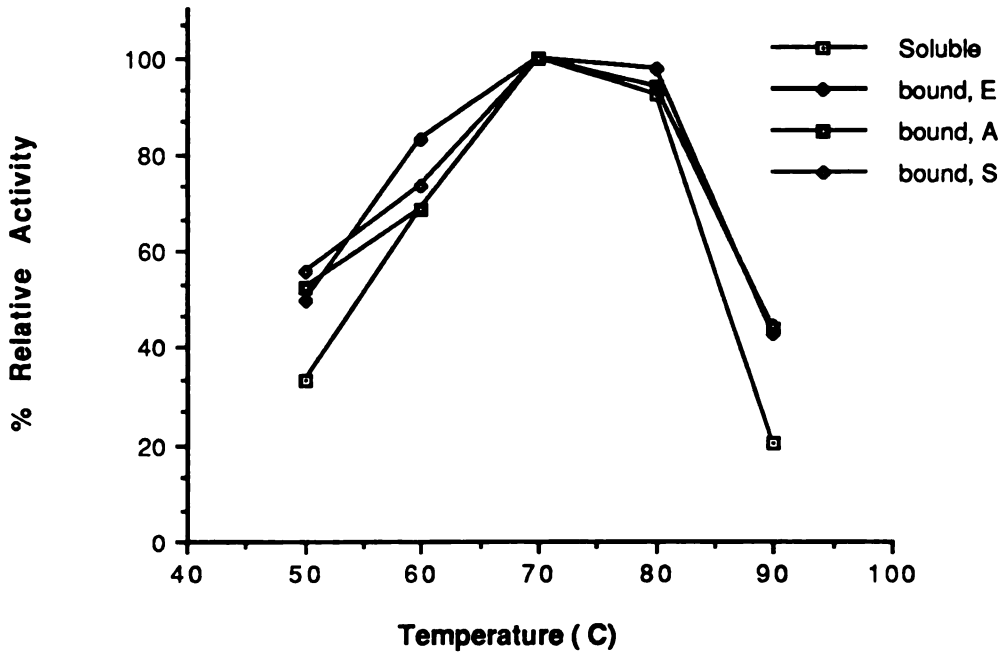


Figure 31. Effect of temperature on the activity of bound (A, S, and E which correspond to enzyme bound to aminated glutaraldehyde-activated, silanized glutaraldehyde-activated, and silanized EDC-activated Titaniuum(IV)-coated CPG beads, respectively) and soluble glucose isomerase

Comparing the above results for IGI with the results of free GI, once again, as seen with the pH results, a wider activity temperature profile was obtained for the bound enzyme than that for the soluble one (Figure 31). This effect, which was noticed for most of the industrial enzymes was explained by Weetall (1985). The author stated that the increase in the thermotolerance was due to the increase in molecular rigidity, thus preventing changes in the molecule's tertiary structure upon heating.

3. Metal ion requirements of bound glucose isomerase

a) Effect of magnesium ion concentration:

As for soluble GI, magnesium ion was required for activation of the bound enzyme. As shown in Figure 32, the activity of bound GI increased with the increasing of Mg^{2+} with maximal observed activity at 80 mM. Interestingly, the bound enzyme preparations (A, S, and E) expressed about 75-80% of their maximum activity when just 10 mM Mg^{2+} was used and about 85-90% relative activity at 20 mM. However, at the same Mg^{2+} concentration (20 mM) the soluble enzyme gave about 65% relative activity, as discussed earlier. Thus, the bound enzyme required less Mg^{2+}concentration for full activity than that needed by the unbound enzyme. This is a very important finding since huge amounts of substrate solution are handled in the manufacture of HFCS using IGI, and therefore large savings could be achieved when low Mg^{2+}-requiring enzyme are used.

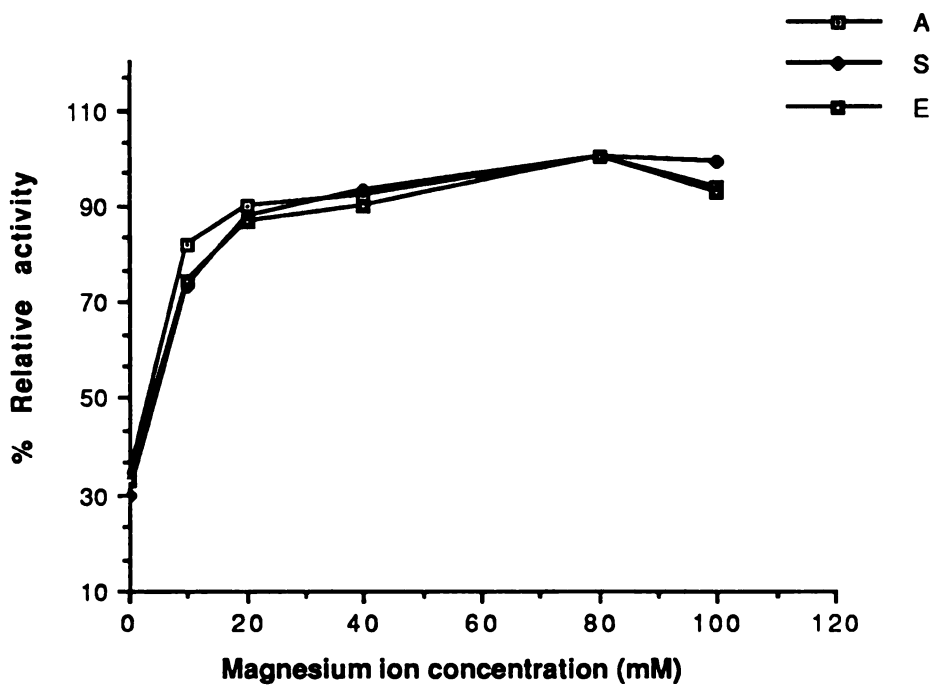


Figure 32. Effect of Mg^{2+} concentration on the activity of immobilized glucose isomerase preparations (A, S, and E which correspond to enzyme bound to aminated glutaraldehyde-activated, silanized glutaraldehyde-activated, and silanized EDC-activated Titanium(IV)-coated CPG beads, respectively).

b) Effect of cobalt ion concentration:

As shown in figure 33, the activity of bound GI preparations (A, S, and E) increased with increasing Co^{2+} concentration with maximal value at 0.2 mM. Then, the activity gradually decreased with the increasing of Co^{2+}. The optimum activity concentration of Co^{2+} for the bound enzyme was close to that (0.1 mM) of the pure enzyme as mentioned by Bogulawski (1983),but it was 10 times less than that (2 mM) found for the crude soluble enzyme as discussed earlier.

Full activty of the bound GI was obtained when both Mg^{2+} and Co^{2+} were present at their optimal concentration. However, and contrary to that observed for the soluble enzyme, about 20-35% relative activity was obtained for the bound enzyme when both metal ions were absent (Figure 34). This may be due to structural changes upon immobilization, which resulted in activation of the bound enzyme. When only Co^{2+} was added (0.2 mM), about 30-35% relative activity was obtained. This activity percentage was close to that (40%) obtained with the soluble enzyme as discussed earlier. However, when only Mg^{2+} was added (0.08 M), about 93-98% of the full activity was obtained. Thus, eliminating the need for Co^{2+} addition as an activator. This is a very important since cobalt is no longer accepted, as an activator, by the fructose-containing syrup manufacturers and is considered as a hazardous material with regard to food or food ingredients.

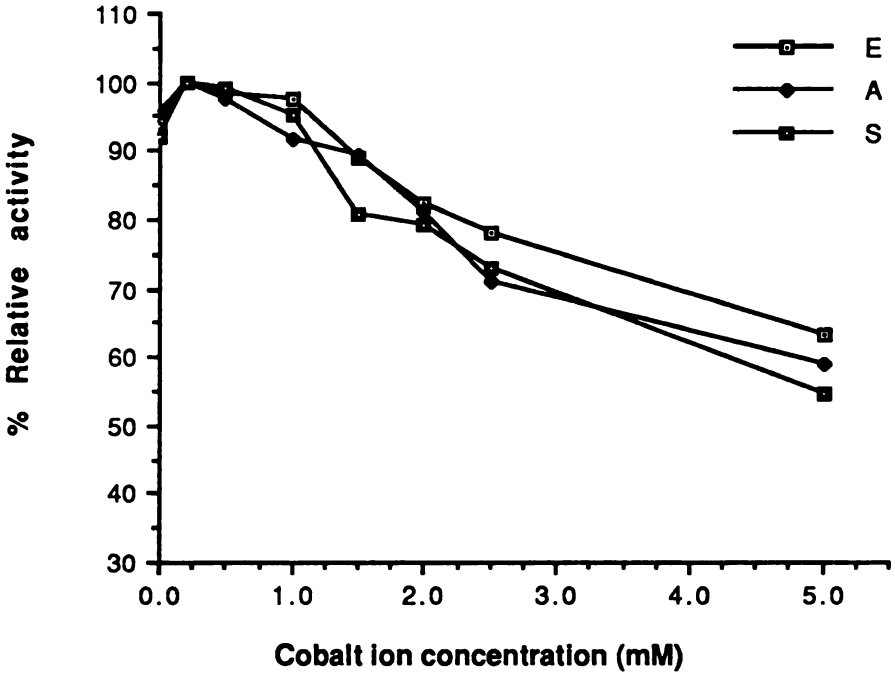


Figure 33. Effect of cobalt ion concentration on the activity of bound enzyme preparations (A, S, and E which correspond to enzyme bound to aminated glutaraldehyde-activated, silanized glutaraldehyde-activated, and silanized EDC-activated Titanium(IV)-coated CPG beads, respectively).

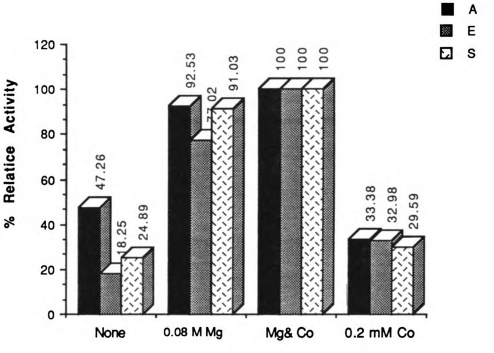


Figure 34. Effect of activator(s) addition (only 0.08M Mg^{2+}, only 0.2 mM Co^{2+}, or both 0.8 M Mg^{2+} and 0.2 mM Co^{2+}) on the activity of bound enzyme preparations A, S, and E which correspond to enzyme bound to aminated glutaraldehyde-activated, silanized glutaraldehyde-activated, and silanized EDC-activated Titaniuum(IV)-coated CPG beads, respectively.

F. Kinetics of bound glucose isomerase

The effect of substrate (glucose) concentration on the activity of bound enzyme preparations (A, S, and E) was studied. Glucose concentration varied from 0.05 to 0.4 M and the activity of the bound enzyme preparations was assayed at the optimum conditions, except that 60^{o}C was used instead of 70^{o}C.

K_m and V_m :

From Lineweaver-Burk plots, the apparent Michaelis-Menten constant (K_m) and the apparent maximal velocity (V_m) were obtained for each IGI preparation. The double reciprocal plot (Lineweaver-Burk plot) yields a straight line with slope K_m/V_m and ordinate $1/V_m$.

From Lineweaver-Burk plots, Figures 35, 36 and 37, the apparent K_m was 0.120, 0.145, and 0.135 M for A, S, and E bound GI preparations, respectively. These results were higher than that (0.115 M) obtained for the soluble enzyme. This may be due to some degree of diffusional limitations from pore blockage and/or a high protein load. These results were in agreement with the conclusion of Weetall (1985) that , generally, the apparent K_m for an immobilized enzyme is greater than that of soluble form. The author added that this increase is usually related to various parameters including change of the substrate and carrier diffusion effects, and even mode of operation.

The apparent V_m was 1665, 1768, and 2214 units for A, S, and E bound GI preparations, respectively as shown in Figures 37, 38 and 39.
these results were about 10 times higher than that (169 units) for the soluble enzyme. This increase in the bound enzyme activity may be due to the selective binding of the enzymatic over extraneous protein of the crude soluble GI used in the immobilization process. Structural changes

upon immobilization could be another possible cause of bound enzyme activation. Kostner and Kreen (1974) reported that in some instances, the enzyme is activated during the process of attachment.

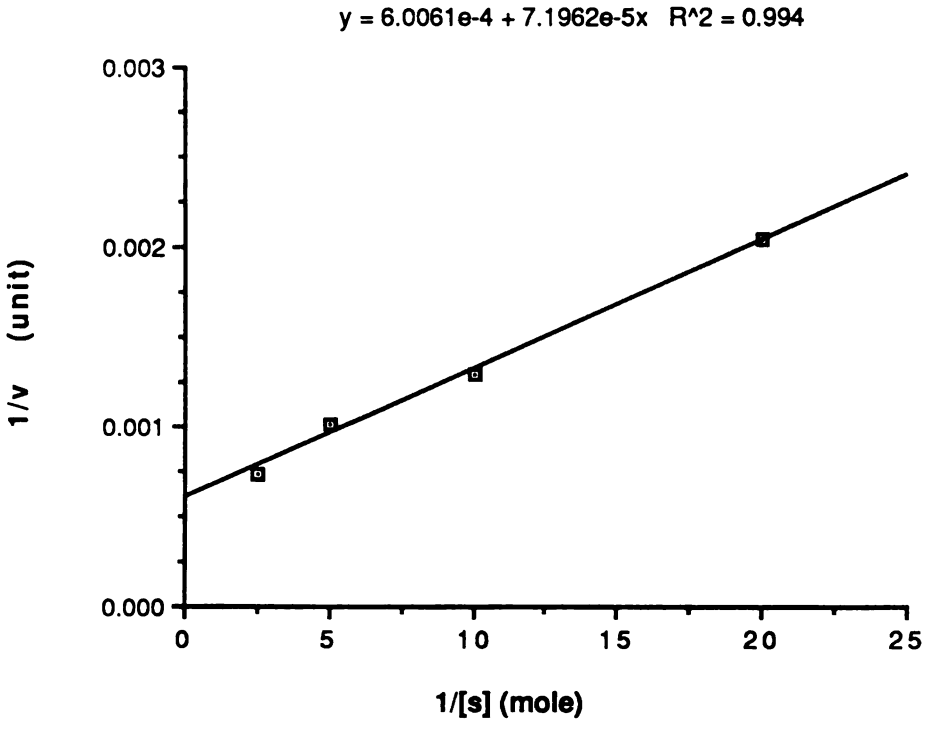


Figure 35. Lineweaver-Burk plot of bound GI (A), glucose isomerase covalently bound to Titanium(IV)-coated aminated glutaraldehyde-activated CPG beads.

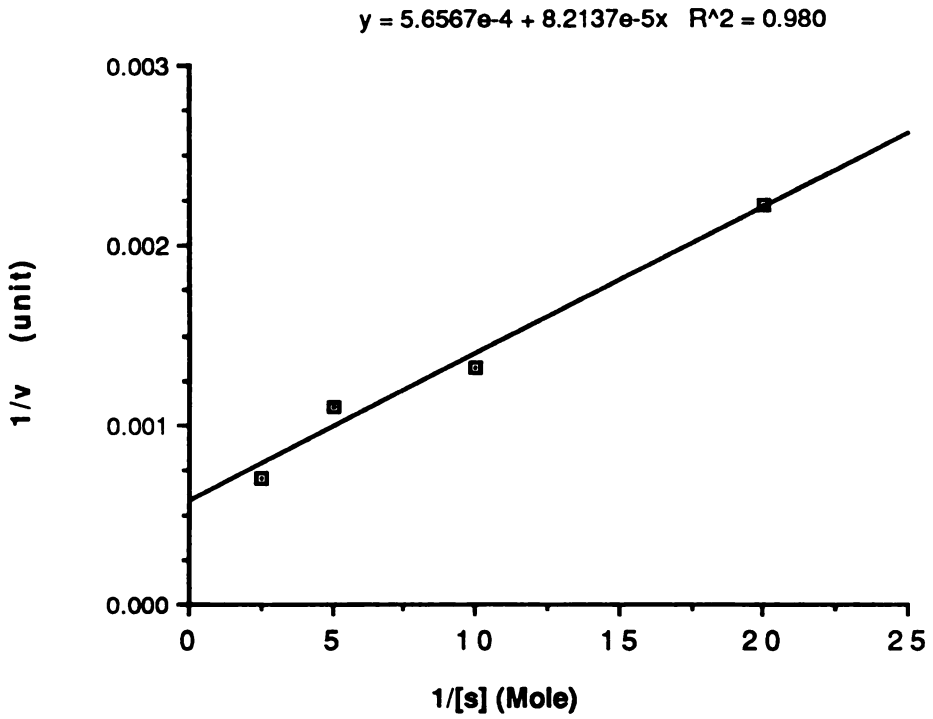


Figure 36. Lineweaver-Burk plot of bound GI (S), glucose isomerase covalently bound to Titanium(IV)-coated silanized glutaraldehyde-activated CPG beads.

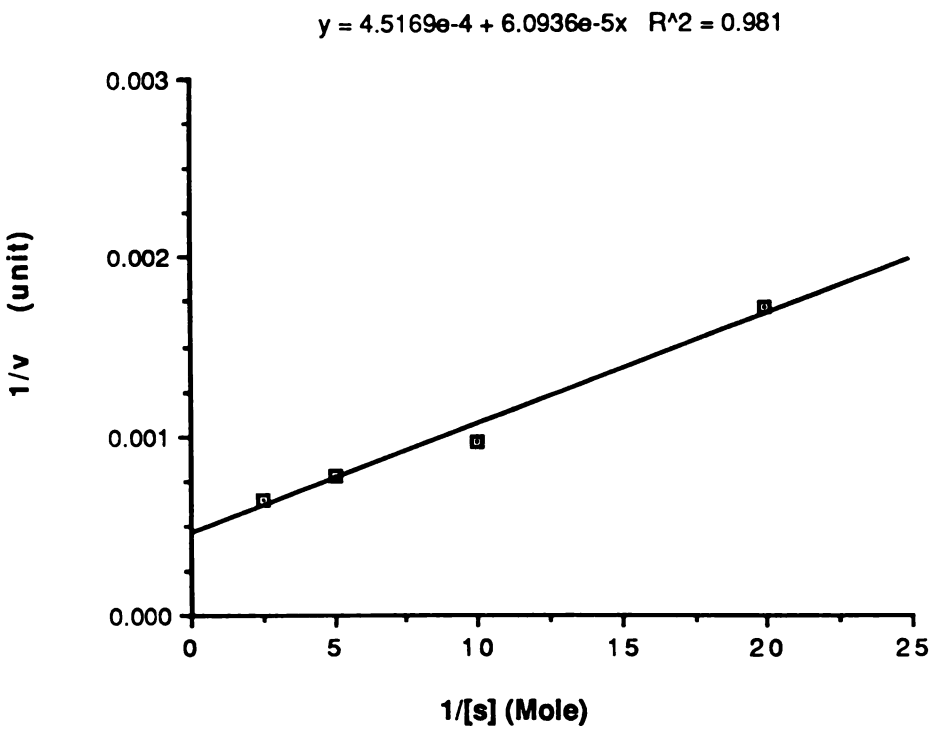


Figure 37. Lineweaver-Burk plot of bound GI (E), glucose isomerase covalently bound to Titanium(IV)-coated silanized EDC-activated CPG beads.

G. Stability of bound glucose isomerase

pH, storage, thermal and operational stabilities of the IGI preparations (A, S, and E) were studied.

1. pH stability of bound glucose isomerase:

Exactly as for the soluble GI, the bound enzyme preparations were preincubated at room temperature (23^{o}C) for 30 minutes in 0.04 M buffer solutions covering a pH range from 3.5 to 11.5 (acetate, HEPES, and glysine-NaOH buffers were used for the pH ranges of 3.5-5.5, 6.5-8.5, and 9.5-11.5, respectively). Then, the residual activity was assayed, at the optimum activity pH (8.0) of the bound enzyme, as mentioned in the enzyme assay section.

As shown in Figure 38, the bound enzyme preparations A and S were relatively stable over the entire pH range (3.5-11.5). However, preparation E was stable at a narrower pH range (3.5-9.0), i.e.it was unstable at pH's above 9.0. Interestingly, the soluble enzyme showed instability in the opposite direction (below pH 5.5) to that of preparation E. The stability of bound GI(preparation A and S) in the acidic side of the pH may be attributed to the increase of molecular rigidity of the bound enzyme molecules upon immobilization so that changes in hydrogen ion concentration did not give rise to substantial changes from the proper conformational structure required for enzymatic activity. The activity decline of bound GI (perparation E) after preincubation in buffers having pH's above 9.0, is thought to be due to the fact that this preparation was covalently bound through the enzyme carboxyl groups. Thus, at least part of these groups was used up during immobilization. This may result in changing the overall charges on the bound enzyme molecules. Such a change would be critical for the ordered molecular structure and consequently for the enzymatic activity at pH's above 9.0.

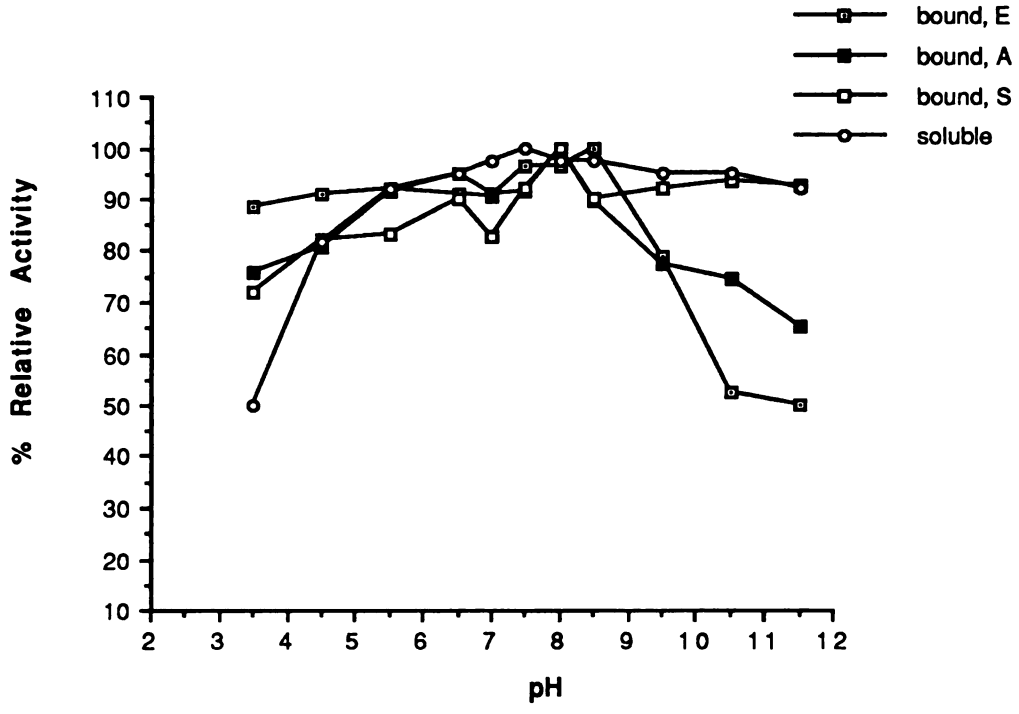


Figure 38. pH stability of soluble and bound glucose isomerase preparations A, S, and E which correspond to enzyme bound to aminated glutaraldehyde-activated, silanized glutaraldehyde-activated, and silanized EDC-activated Titanium(IV)-coated CPG beads, respectively.

The obtained pH stability results were in agreement with those reported by Ananichev et al.(1980), who immobilized GI on various carriers (cellulose, CM-cellulose, DEAE-cellulose, spheron, silochrome, porous glass, and nylon) and found that for all preparations, except that immobilized on spheron, a rather extended zone of pH values with preservation of maximum activity (pH from 6.0 to 8.5) was observed. They added that the highest stability of glucose isomerase on change of pH value from 5 to 9 was observed on its immobilization on aminated porous glass and silochrome, as well as nylon activated with $TiCl_4$, which was stable at a very low pH. Also, Voroshilova et al.(1984) concluded that the immobilized GI (on macroporous silica carriers) was more stable than soluble enzyme in a wider pH range. Moreover, Kasumi et al.(1979) reported that the stability of GI was expanded by immobilization (*Streptomyces* cells containing GI treated with chitosan, entrapped in gelatin, and cross-linked with glutaraldehyde). However, a narrow pH stability range (6-8) was reported by Ananichev et al. (1986) for gelatin entrapped *Lactobacillus brevis* 74 cells containing GI which was cross-linked with glutaraldehyde and stabilized with tannin.

2. Storage stability of bound glucose isomerase:

As shown in Figure 39, the immobilized enzyme preparations (A, S, and E) retained 76-79% of their original activity over 9 weeks storage at the refrigerated temperature (4-6^{o}C) without any addition of stabilizers which would extend the storage stability. The bound GI stabilty was better than that observed for the soluble enzyme which retained 65% of its original activity over the same period of time. These results were better than the results of Kent and Emery (1974) who reported a retention of 53% of the initial specific activity of GI bound to microcrystalline cellulose after 8 weeks of storage at 4^{o}C in 0.02 M Tris buffer, pH 7.

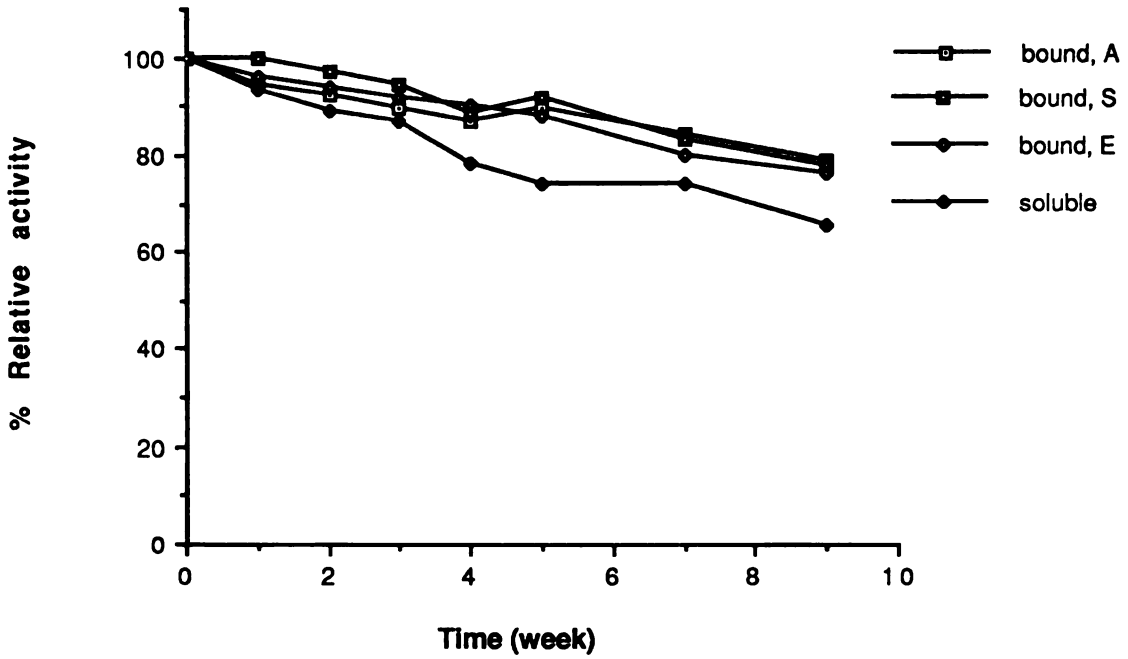


Figure 39. Storage stability of bound (A, S, and E preparations which correspond to enzyme bound to aminated glutaraldehyde-activated, silanized glutaraldehyde-activated, and silanized EDC-activated Titanium(IV)-coated CPG beads, respectively) and soluble glucose isomerase at refrigerated temperature.

3. Thermal stability of bound glucose isomerase:

The residual activity of bound glucose isomerase (preparation, S), after preincubation in 0.04M HEPES buffer, pH 7.7 in a thermostatic controlled water bath at various temperatures (50-90^{o}C) for 30 minutes, was determined as mentioned in the enzyme assay section.

As shown in Figure 40, the bound enzyme exhibited an excellent stability at temperatures from 50 to 80^{o}C. However, a rapid decrease of the enzyme activity was obtained at 90^{o}C. As far as the comparison between the soluble and bound enzyme is concerned, both enzymes were stable at temperatures of 50 and 60^{o}C. Also, a rapid inactivation was noticed at 90^{o}C for enzymes. However, the bound enzyme was more thermostable than the soluble one at temperatures of 70 and 80^{o}C.

4. Long-term stability (longevity) of bound glucose isomerase:

The residual activity of bound glucose isomerase (preparation, S), after preincubation in 0.04M HEPES buffer, pH 7.7 at 60^{o}C for various periods of time (up to 48 hrs), was determined as mentioned in the enzyme assay section. As shown in Figure 41, the activity of the bound GI was relatively stable at the first 12 hours, then it gradually decreased with time. About 50% of the original activity was retained after 48 hrs. However, the soluble enzyme activity decreased rather rapidly. About half of the original activity was lost after 12 hrs of incubation.

When the bound enzyme was cross-linked with glutaraldehyde, its longevity profile did not show substantial change from that without cross-linking (Figure 42). This may be because the enzyme was already "fixed" by the covalent immobilization. This molecular "fixation", which did not permit much conformational changes of the enzyme molecules upon heating, is thought to be the reason behind the enhanced thermal stability of bound GI over the soluble enzyme. Protein-protein interaction and aggregation, which are likely to

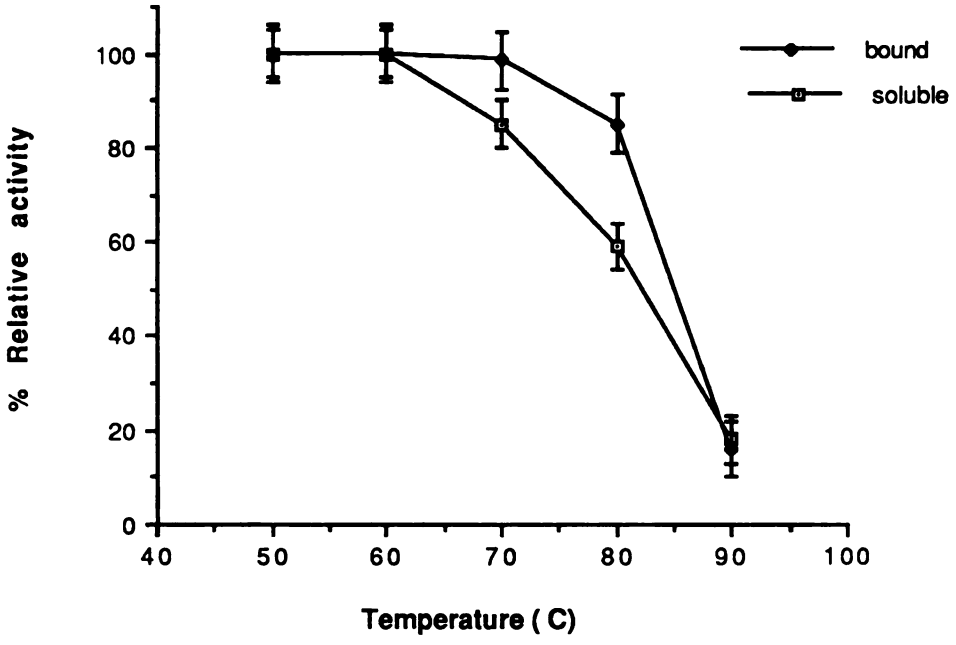


Figure 40. Thermal stability of soluble and bound (covalently bound to silanized glutaraldehyde-activated Titanium(IV)-coated CPG beads) glucose isomerase.

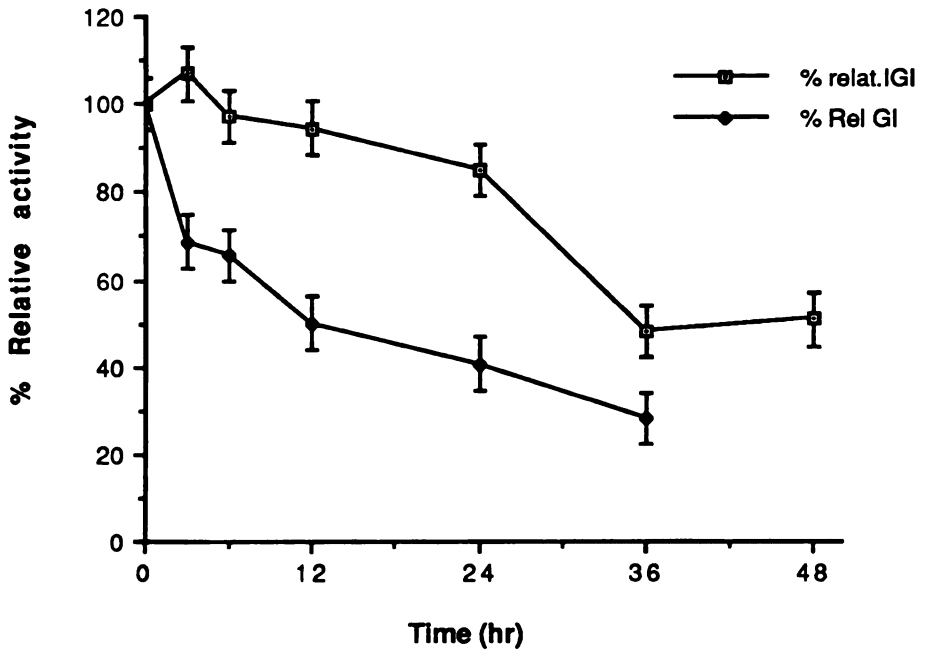


Figure 41. Long-term stability (at 60°C) of soluble and bound (covalently bound to silanized glutaraldehyde-activated Titanium(IV)-coated CPG beads) glucose isomerase.

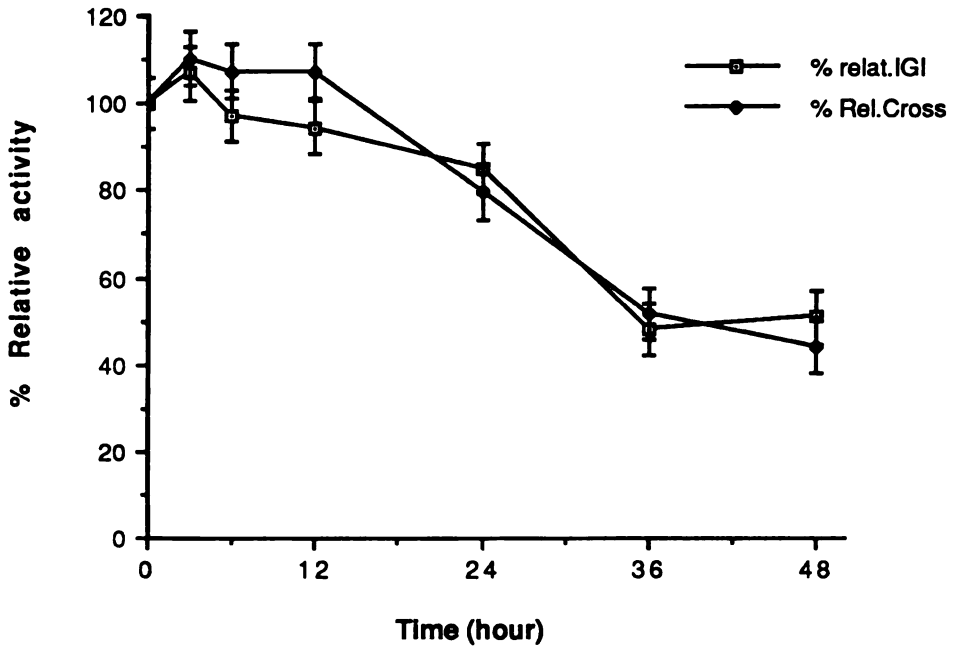


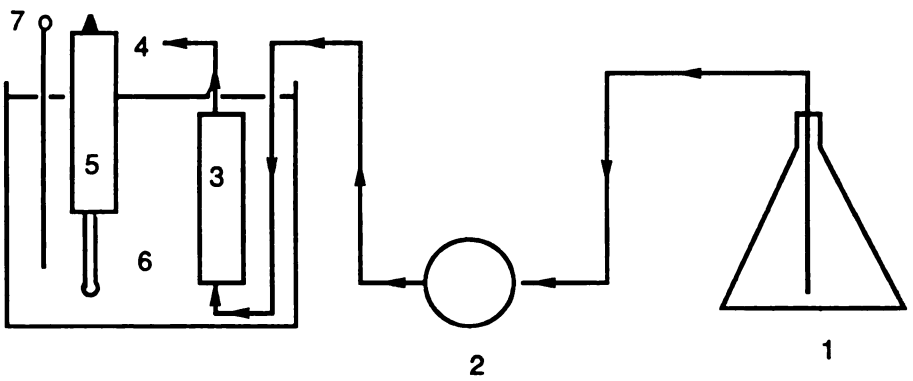
Figure 42. Effect of cross-linking on the long-term stability, at 60°C, of bound (covalently bound to silanized glutaraldehyde-activated Titanium(IV)-coated CPG beads) and soluble glucose isomerase.

occur on a faster rate for soluble than bound enzymes (especially with heating), may be considered in this regard. The above results were in agreement with those of Robinson et al.(1971), who reported that glass -chymotrypsin derivative was more heat stable than free enzyme, and Havewala and Pitcher (1974), who found increased stability for GI (from *Streptomyces* organism) immobilized on zirconium-coated CPG beads as compared with the soluble enzyme (one day half life).

5. Operational stability of bound glucose isomerase

The stability of bound glucose isomerase preparations (A, S, and E) in a continuous isomerization process was studied. As shown in Figure 43, an immobilized enzyme preparation (~1.2 g) was packed in 9 x 50 mm (inner diameter x length) glass column and the substrate solution was passed upward at about 20 ml/hour into the column, which was immersed in a thermostatic controlled water bath at 60^{o}C. The substrate consisted of 45% glucose solution in 0.04M HEPES buffer, pH 7.7 containing 20 mM Mg^{2+} and 10 mM Na_2SO_3.

Cooney (1983) reported that, in a continuous process, the relation between product concentration, p, and operating conditions is $p = S_a X V/F$, where S_a is the specific activity of the catalyst (enzyme), X is the catalyst concentration, and F is the substrate flow rate. Thus the product concentration is inversly related to substrate flow rate, but proportional to the enzyme activity (S_a x $X_)$. Since the operational stability of the bound enzyme preparations was done at a constant flow rate, the time required for the percent relative conversion (conversion/maximum conversion x 100) to reach half of its initial value was considered as the apparent half life.



1 substrate reservoir
2 variable speed polystaltic pump
3 column reactor
4 Effluent
5 heat regulator
6 water bath
7 thermometer.

Figure 43. Flow diagram of reactor system used in the operational stability of bound glucose isomerase.

Regarding the operational stability of bound enzyme preparation A (glucose isomerase was covalently bound to Titanium(IV)-coated aminated glutaraldehyde-activated CPG beads), Figure 44 showed that the apparent half life was only 103 hours (about 4 1/2 days). However when the column effluent was checked for protein discharge from the column (Figure 45), it indicated that indeed there was a protein discharge from the enzyme bed. This discharge was relativly high (580μg/ml) at the first 10 minutes of operation, then sharply decreased to 50 μg/ml after 3 hrs. After that, the protein leaching gradually decreased until it reached zero μg/ml after 56 hours of continuous isomerization. It was found that the enzyme bed weight at the end of the isomerization process was lower than the initial enzyme bed weight. In addition no fines were detected in the column effluent. Thus, the rapid loss of enzyme activity and the protein discharge from the column may be due to the dissolution of the exposed surface of the porous glass where, presumably, the enzyme is covalently bonded. The alkaline pH (7.7) , high temperature, and large surface area of the glass would tend to cause relatively rapid dissolution. A Similar conclusion was reported by Strandberg and Smiley (1972) who studied the operational stability of covalently bound glucose isomerase, from *Streptomyces phaeochromogenes* NRRL B-3559, on porous glass beads under similar conditions (pH 8, 60^{o}C) except that lower glucose concentration (0.5 M) and lower flow rate (5 ml/hr) were used. In addition, the authors stated that the actual pH of the isomerization process was about 7 due to the decrease of pH noticed when the substrate was left for 2 days at room temperature before use (NH_3-NH_4Cl buffer was used). The lower pH and lower flow rate would tend to reduce the rate and, therefore, the amount of enzyme discharged from the column as compared to those found in this study. Consequently the half life was lower than that(12-14 days) of Strandberge and smiley (1972). When GI was covalently bound to zirconium-coated CPG beads (Lee et al., 1976), 16 day half-life was obtained at 60^{o}C. However, the substrate pH was 7 and Co^{2+} (0.1 mM) was added. Results obtained by Havewala and Pitcher (1974) at similar conditions (60^{o}C &1 mM Co^{2+}) of the previous study gave 16-20 day half-life, but the pH was a little lower (6.8). The titanium coating was not as good as expected in preventing carrier dissolution. In this study evidence was presented (and will be more convincing) that carrier

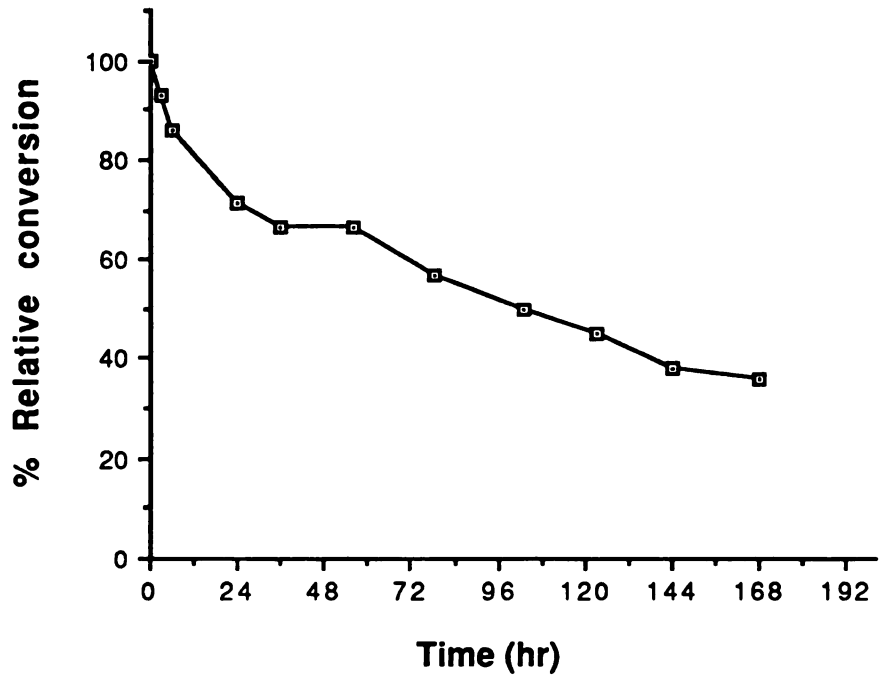


Figure 44. Operational stability of bound glucose isomerase preparation A (enzyme bound to aminated glutaraldehyde-activated Titanium(IV)-coated CPG beads).

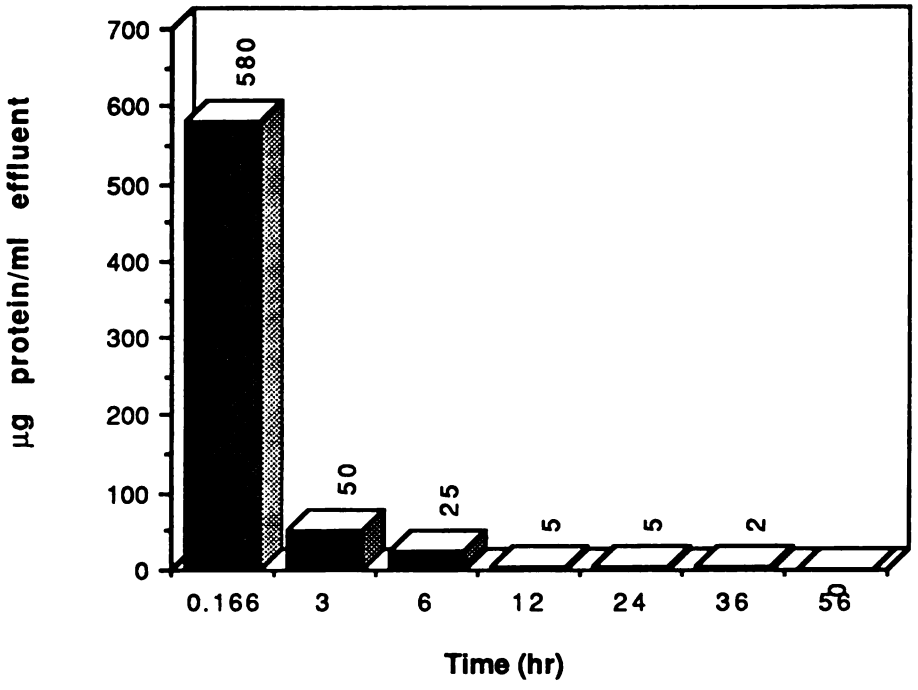


Figure 45. Protein discharge from enzyme bed during the operational stability of bound glucose isomerase preparation A (enzyme bound to aminated glutaraldehyde-activated Titanium(IV)-coated CPG beads).

dissolution is a major cause of activity loss of covalently bound GI on CPG beads. Also, titanium coating did not prevent such loss.

Regarding the operational stability of bound enzyme preparation S (glucose isomerase was covalently bound to Titanium(IV)-coated silanized glutaraldehyde-activated CPG beads), Figure 46 shows that the apparent half life was only 100 hours (about 4 days). However when the column effluent was checked for protein discharge from the column (Figure 47), it again indicated that indeed there was a protein discharge from the enzyme bed. This discharge was relativly high (180μg/ml) at the first 30 minutes of operation, then sharply decreased to 20 μg/ml after 3 hrs. After that, the protein leaching gradually decreased until it reached zero μg/ml after 96 hours of continuous isomerization. As indicated earlier for bound enzyme preparation A, an enzyme bed weight loss was occured. Therefore, the rapid loss of enzyme activity, especially at the first 24 hrs of operation was due to the dissolution of the exposed surface of the porous glass where the enzyme is attached.

From the above results, the type of carrier derivative (aminated vs. silanized) did not affect the operational stability of covalently bound glucose isomerase on Titanium(IV)-coated CPG beads. Also, the dissolution of the exposed surface of the porous glass under the isomerization conditions was a major factor, along with the thermal inactivation effect, contributing to the low half life obtained.

The effect of Co^{2+} addition on the operational stability of bound glucose isomerase was examined. Two mM Co^{2+} was added to the substrate (45% glucose in 0.04M HEPES buffer, pH 7.7 containing 20 mM Mg^{2+} and 10 mM Na_2SO_3) and the change of percent relative conversion with time was followed. As shown in Figure 48, the addition of Co^{2+} increased the half life of the bound enzyme from about 4 days (without Co^{2+} addition) to about 10 days. Interestingly, the percent relative conversion gradually inraesed during the first 34 hrs of operation, then started to

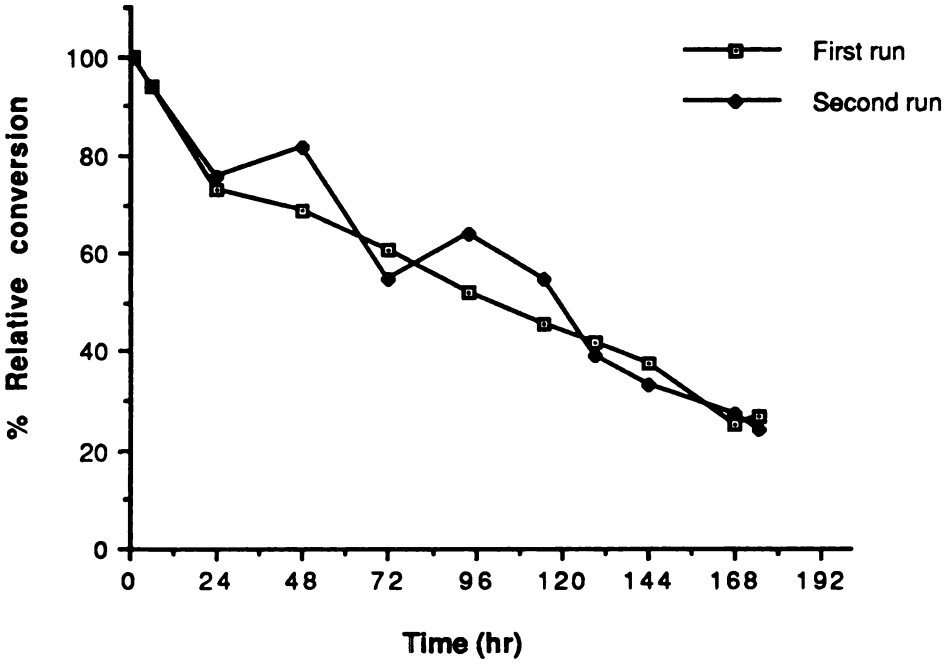


Figure 46. Operational stability of bound glucose isomerase preparation S (enzyme bound to silanized glutaraldehyde-activated Titanium(IV)-coated CPG beads).

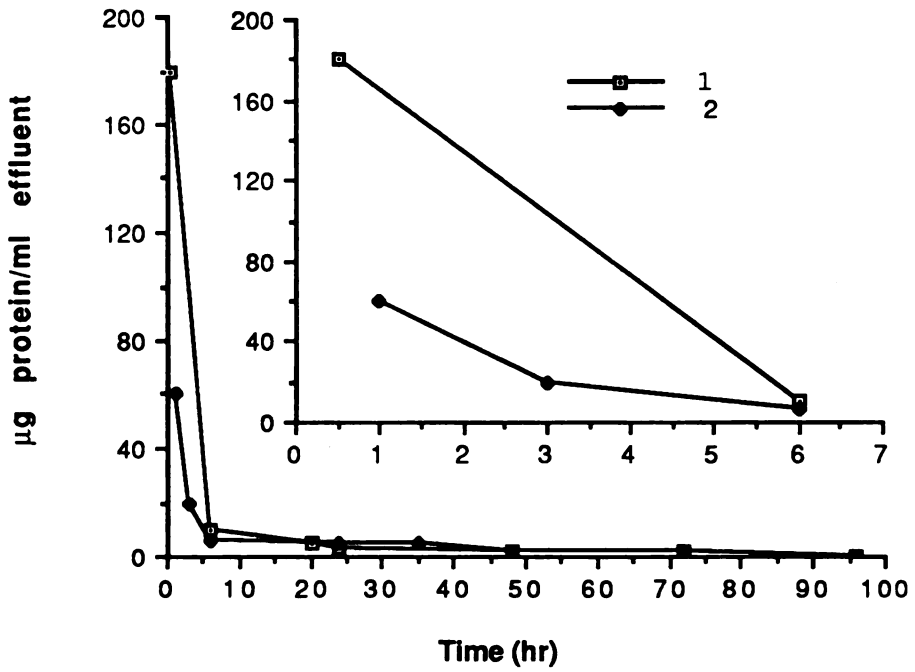


Figure 47. Protein discharge from enzyme bed during the operational stability of bound glucose isomerase preparation S (enzyme bound to silanized glutaraldehyde-activated Titanium(IV)-coated CPG beads), two runs.

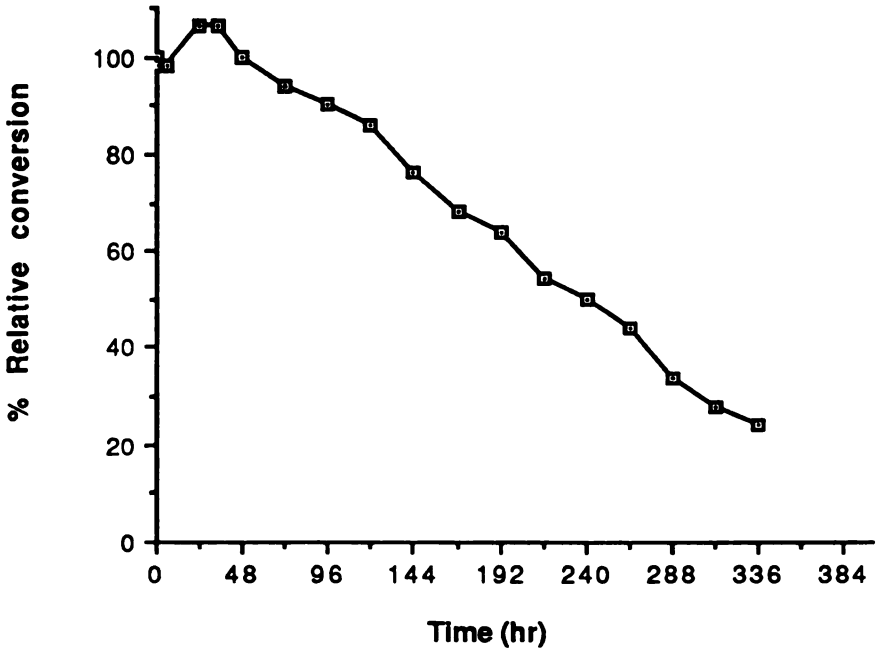


Figure 48. Operational stability of bound glucose isomerase preparation S (enzyme bound to silanized glutaraldehyde-activated Titanium(IV)-coated CPG beads) when cobalt ions (2 mM) were added to the substrate.

decrease after 48 hrs. Also the color of the enzyme bed changed from sand-like color to dark purple as the isomerization progressed. At the same time, the column effluent was colorless differing from the light pink color of the substrate (due to the presence of $CoCl_2$). This indicated that the cobalt ions were retained by the enzyme bed. The increase in the relative conversion during the first 34 hrs was mainly because the activation rate of the bound enzyme, by Co^{2+} (as discussed earlier in the optimum activity conditions of bound enzyme), was higher than the activity loss rate that might have occured due to carrier dissolution, thermal inactivation (denaturation), and poisoning of the enzyme by inhibitors. Protein discharge (caused by carrier dissolution) from the enzyme bed was far less than when Co^{2+} was omitted as seen in Figure 49. Therefore, the increase in the bound enzyme stability, which has been reported (Lantero, 1977; Rosevear et al., 1977; Kasumi et al., 1982; and Callens et al., 1986)), was due to at least two reasons: 1) the direct protective effect of Co^{2+} against heat denaturation 2) the role of Co^{2+} in holding the ordered conformation, especially the quaternary structure of the enzyme i.e. holding the enzyme subunits together which in turn reduced the rate of enzyme discharge during the continuos isomerization process.

When the bound glucose isomerase preparation S was pretreated with Co^{2+} (about 1.5 g of bound enzyme in 0.04M HEPES buffer, pH 7.7 were incubated at $60^{o}C$ for 1 hour, then filtered, and washed with d.d.H_2O and 0.04M HEPES buffer, pH 7.7), its operational stability substantially increased. The half life was 22 days. However, the repeated run gave 19 day half-life. In both runs, the percent relative conversion increased in the first 4 days of operation, then gradually decreased for the rest of the run(Figure 50). As with cobalt ions added to the substrate, the protein discharge from the enzyme bed (Figure 51 a & 51b)was not as high as was found without cobalt treatment (Figure 49). Thus, the Co^{2+}-pretreated bound glucose isomerase, without cobalt addition to the substrate, was more stable than that with Co^{2+} added to the substrate. This was because in the latter case, the accumulation of Co^{2+} on the enzyme-bound CPG beads would tend to block the carrier pores preventing or at least decreasing the diffusion

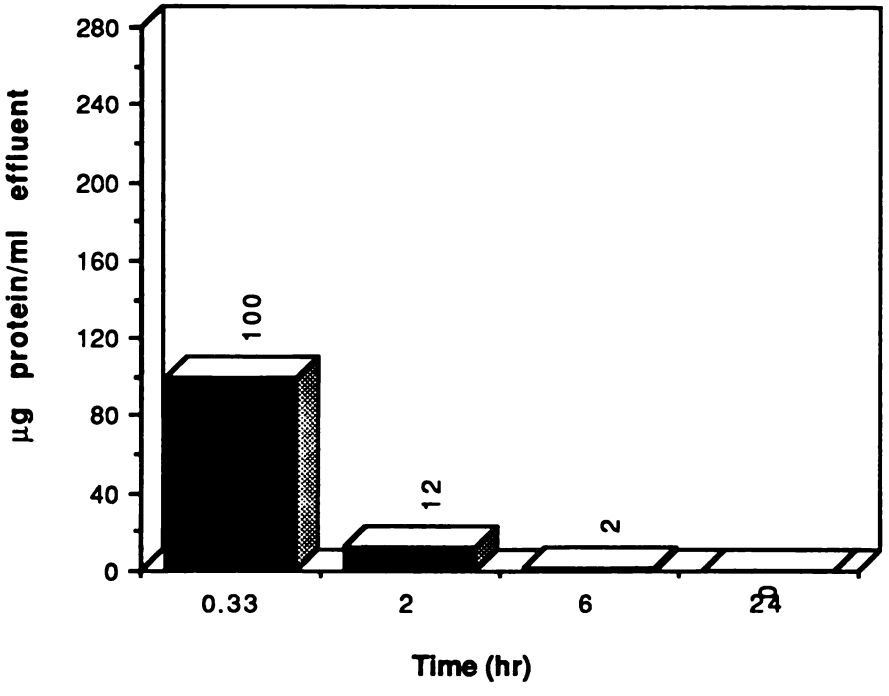


Figure 49. Protein discharge from enzyme bed during the operational stability of bound glucose isomerase preparation S (enzyme bound to silanized glutaraldehyde-activated Titanium(IV)-coated CPG beads), when cobalt ions (2 mM) were added to the substrate.

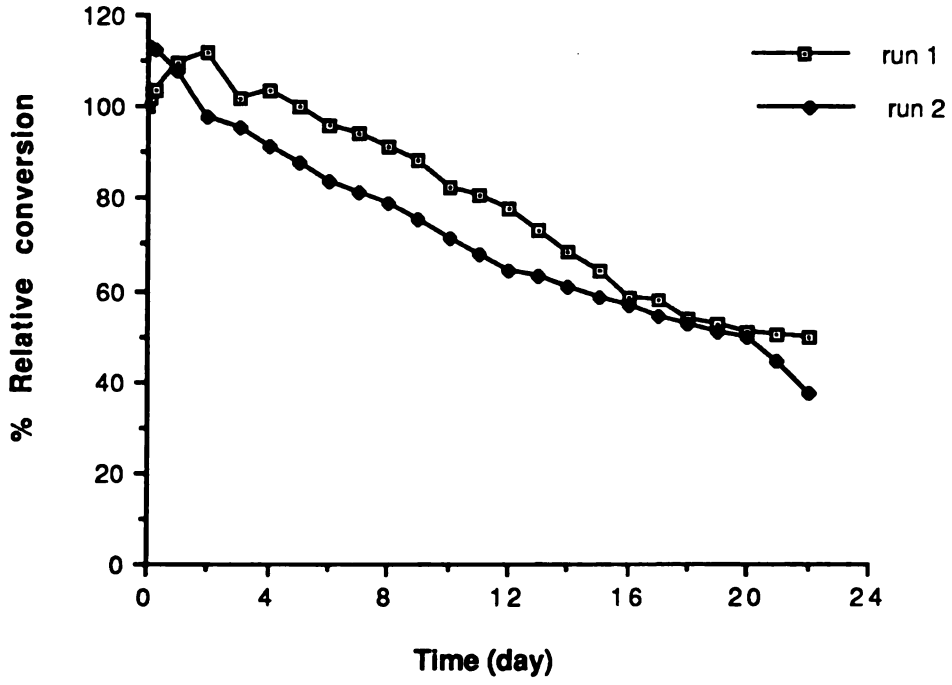


Figure 50. Operational stability of bound glucose isomerase preparation S (enzyme bound to silanized glutaraldehyde-activated Titanium(IV)-coated CPG beads) when the enzyme was pre-treated with cobalt.

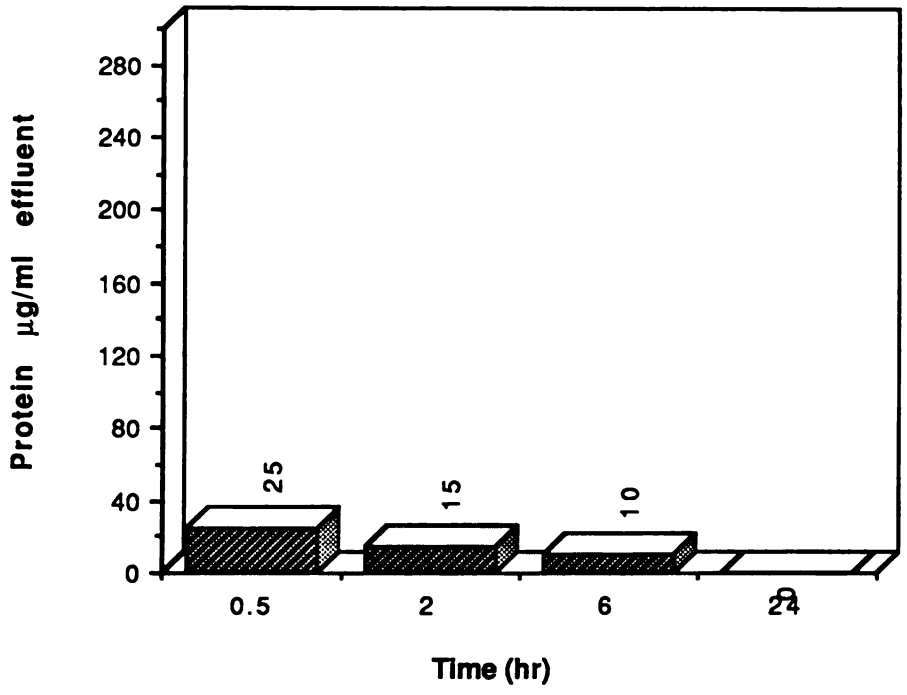


Figure 51a. Protein discharge from enzyme bed during the operational stability of bound glucose isomerase preparation S (enzyme bound to silanized glutaraldehyde-activated Titanium(IV)-coated CPG beads) when the enzyme was pre-treated with cobalt, first run.

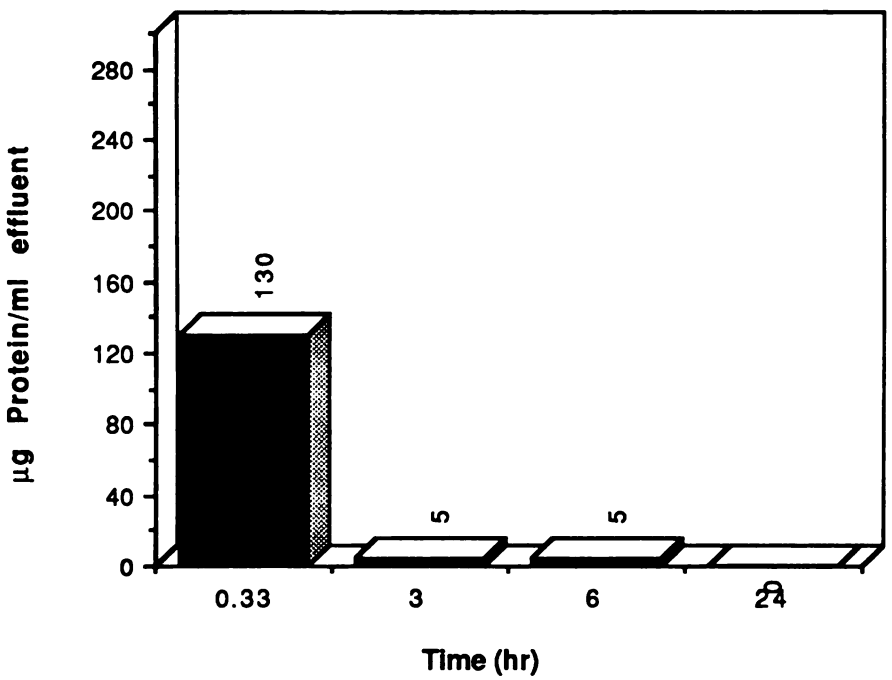


Figure 51b. Protein discharge from enzyme bed during the operational stability of bound glucose isomerase preparation S (enzyme bound to silanized glutaraldehyde-activated Titanium(IV)-coated CPG beads) when the enzyme was pre-treated with cobalt, second run.

rate of glucose and fructose to and from the enzyme particles. The enzyme bed loss was more noticable (decreased from 1.2 g at the begining to 0.55 g at the end of the operational stability experiment) than that found when the bound enzyme preparation was not pre-treated with cobalt.since the operational stability using Co-treated enzyme lasted longer (23 days) than that without cobalt pre-treatment (7 days). The increase in enzyme stability with Co^{2+}-pretreatment is an important finding since cobalt was eliminated from the substrate and it was still possibe to get a relatively stable bound enzyme preparation under operational conditions. It is worth mentioning that the Co^{2+}-pretreatment increased the bound enzyme activity (31 $\pm$ 8%) over the untreated enzyme. Such an increase in the stability and activity was not surprising since the stability of the whole-cells bound enzyme from *Flavobacterium arborescens* (same source as the investigated cell-free enzyme) commercially prepared at Miles Laboratories, Inc. known as Takasweet FM was reported (Lantero, 1982) to have a high stability. Boguslawski (1983) who purified the investigated enzyme reported that even though Co^{2+} was required by the solubilized enzyme, the activity in the whole cells remained almost unchanged when cobalt was omitted.
The author added this suggested that the cells were capable of scavenging Co^{2+} from the medium.

Regarding the operational stability of glucose isomerase covalently bound to Titanium(IV)-coated silanized CPG beads using EDC as an activator, Figure 52 shows that the stability of Co^{2+}-treated enzyme (2 runs) was higher (> 96 hr half life) than that (48 hr half life) of the untreated enzyme. The protein discharge from the enzyme bed during the continuous isomerization process for the untreated enzyme was pretty high (400 µg/ml effluent) for the first 2 hours of operation, then decreased gradually until no protein was detected in the effluent at 72 hours of operation. However, the Co^{2+}-treated enzyme showed relatively lower protein leakage (300µg/ml effluent at the first 15 minutes of operation, then decreased to 250 µg/ml effluent after 2 hr and no protein was detected after 72 hours of operation). Therefore, pretreatment of the bound

enzyme with cobalt seemed to have a role in lowering the protein leakage (caused by carrier disolution) and consequently increasing the bound enzyme stability (Figure 53).

It should be noted that both Co^{2+}-treated and untreated preparations of bound enzyme E were inferior to Co^{2+}-treated and untreated preparations of bound enzyme S, respectively. This may be due to the difference in methods of binding. Enzyme preparation S was bound through the enzyme residual amino group(s), while enzyme preparation E was bound through the enzyme residual carboxyl group(s). The enzyme carboxyl groups seemed to have a role (with Co^{2+} treatment) in holding the enzyme subunits together and/or form an inter- or intramolecular coordination with the Co^{2+} which may have played a role in reducing the protein discharge from the column during continuous isomerization.

Generally, in all cases there was a protein leakage from the carrier. Although, such leakage has been reported (Strandberg and Smily, 1972, Lasch and Koelach, 1978, and Weetall, 1985) from inorganic and organic carriers, CPG beads which serve as a very good carrier with respect to mechanical stability; flexibility and availabilty in various sizes, pore sizes, and surface area; ability to be doped with various (almost any) reactive groups; and resistance to microbial attack, still suffer the drawback of being unstable in the alkaline pH, especially, at high temperature and under shear stresses. Though highly stable immobilized glucose isomerase preparations have been reported, in most cases the effect of diffusional limitations, which increase the apparent bound enzyme stability and decrease its efficiency(Giovenco et al., 1973), was not made clear. Therefore, the effect of external and internal diffusional limitations on the bound glucose isomerase prepared in this study have been examined, as will be discussed in the next section.

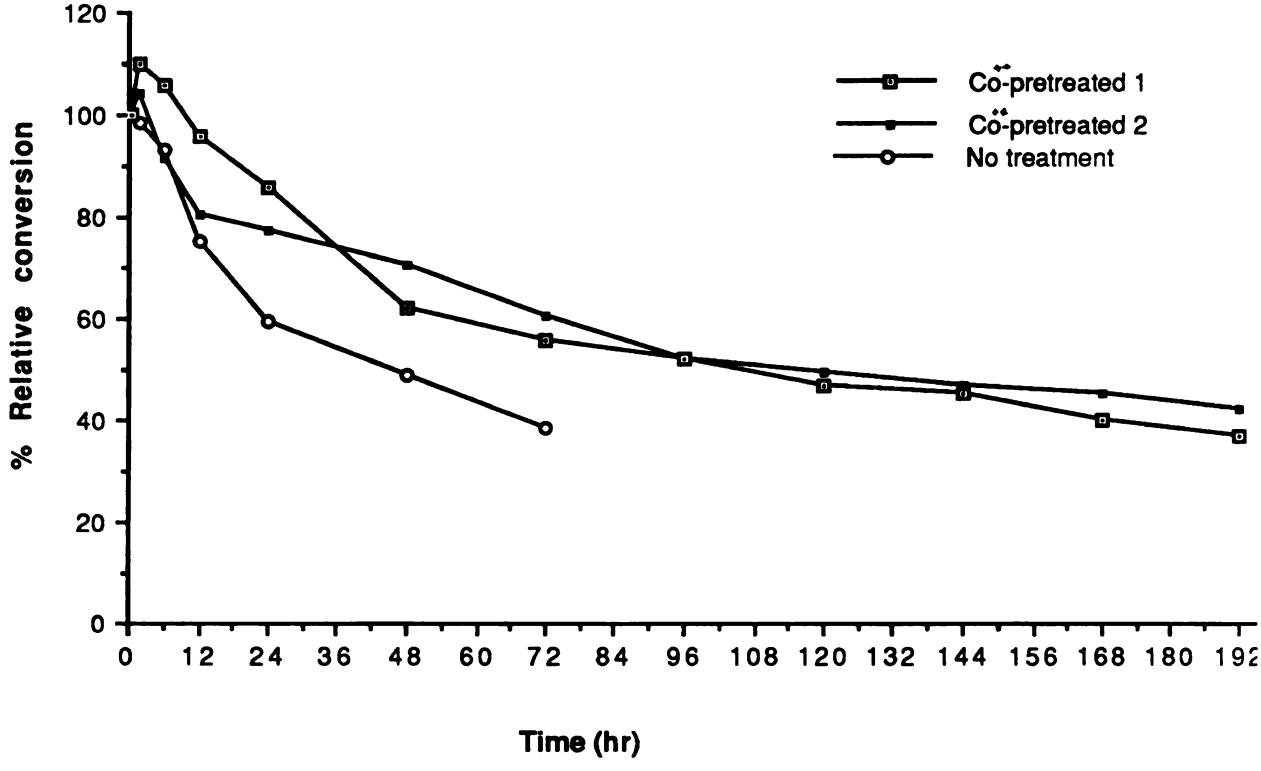


Figure 52. Operational stability of bound glucose isomerase preparation E (enzyme bound to silanized EDC-activated Titanium(IV)-coated CPG beads) when the enzyme was pre-treated with cobalt (2 runs) vs. without Co^{2+} treatment (1 run).

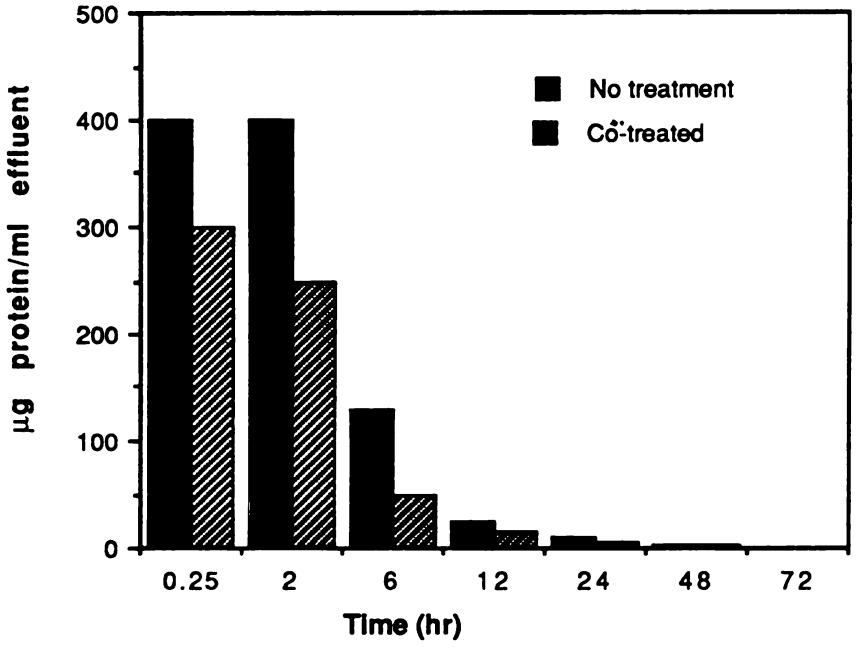


Figure 53. Protein discharge from enzyme bed during the operational stability of bound glucose isomerase preparation E (enzyme bound to silanized EDC-activated Titanium(IV)-coated CPG beads) when the enzyme was pre-treated with cobalt vs.untreated enzyme.

F. Effect of diffusional limitations

1. External diffusion limitation:

As shown in Figure 54, a straight line was obtained when the conversion (percent of initial) was plotted versus the reciprocal of flow rate indicating that there was no significant external diffusion resistance for the two runs. Had there been a significant external diffusion resistance (film diffusion), the percent conversion versus reciprocal flow rate plot should not have shown a straight line (Huitron and Limon-Lason (1978). The high substrate concentration (45% glucose) and the low range of flow rate (0.33-1.53 ml/minute) used made the above results more convincing. The absence of pore diffusion, as will be discussed in the next section, was another evidence of the validity of the above conclusion. These results are in agreement with the results reported by Havewala and Pitcher (1974) who found that the external diffusion resistance was not significant for glucose isomerase covalently bound on zirconium-coated CPG beads.

2. Internal diffusion limitation:

The internal diffusion limitation, which is also known as internal mass transfer resistance or particle size dependent diffusional limitation, was studied by grinding the bound enzyme particles (10 times smaller than the unground particles as determined microscopically). The specific activity of each enzyme preparation (A, S, and E), before and after grinding, was determined under the same conditions described in the enzyme assay section. As shown in Figure 55, the specific activity of the ground and unground enzyme preparations was relatively unchanged. Had there been a significant internal diffusion limitation, the specific activity of the bound enzyme would have greatly changed by grinding (changing the catalyst particle size) as reported by Reagan et al. (1974). The above results were obtained using 0.1 M substrate concentration. Conducting the

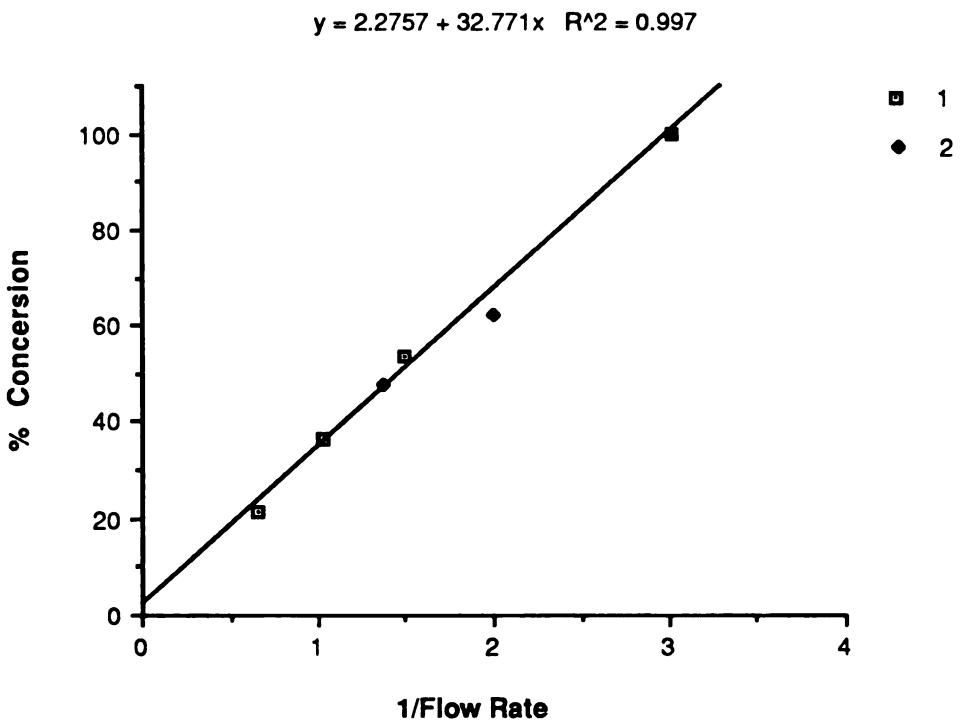


Figure 54. Dependence of conversion on reciprocal flow rate. Substrate solution passing through the column contained 45% glucose, 20 mM Mg^{2+}, and 10 mM Na_2SO_3 in 0.04 M HEPES buffer, pH 7.7 at 60°C.

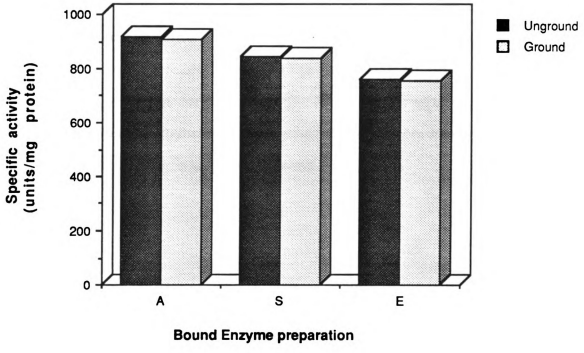


Figure 55. Effect of grinding of CPG beads-containing glucose isomerase on the specific activity of the bound enzyme preparations (A, S, and E which correspond to enzyme bound to aminated glutaraldehyde-activated, silanized glutaraldehyde-activated, and silanized EDC-activated Titaniuum(IV)-coated CPG beads, respectively).

above test at various substrate concentrations would give more insight about the internal diffusion limitations that might exist. However, the above results were obtained using low substrate concentration where the internal diffusional effects are most significant if any. Moreover, Reagan et al. (1974) stated that when diffusional effects are insignificant, a straight line is obtained with Lineweaver-Burk plots. This was the case, as discussed earlier in the bound enzyme kinetics section. These straight lines, which were obtained using a range of low substrate concentration (0.05-0.40 M), are another evidence of the absence of significant internal diffusional limitations of the prepared bound glucose isomerase preparations. The obtained results were in agreement with the results of Havewala and Pitcher (1974) who found no significant pore diffusion limitations for glucose isomerase bound to zirconia coated CPG beads.

SUMMARY AND CONCLUSIONS

Summary:

In this study, a crude glucose isomerase from *Flavobacterium arborescens* was covalently immobilized on Titanium(IV)-coated CPG beads using several different techniques. Two derivatization methods (amination vs. silanization) and two activation methods (glutaraldehyde vs. EDC) were used. In addition, the enzyme was directly immobilized to the titanium-coated CPG beads. The enzyme was bound either through its residual epsilon amino group(s) to the glutaraldehyde-activated beads forming Schiff's base or through its residual carboxyl group(s) to the EDC-activated beads forming amide bonds.

The optimum activity conditions (pH, temperature, and metal requirements), pH, thermal, operational, and storage stabilities of the soluble enzyme were studied. The enzyme had an optimum activity pH range of 7.5-8.5, optimum activity temperature of 70^{o}C and required 0.1 M Mg^{2+} and 2-mM Co^{2+} for full activity expression and the two cations acted synergistically. The enzyme was stable in a pH range of 5.5-11.5 and exhibeted a good thermal stability (stable at 50 and 60^{o}C). However, the long-term stability at 60^{o}C indicated that the enzyme had a very low half life (12 hours). The long-term stability substantially increased in the presence of Co^{2+} (888 hr half life) and to a lesser degree when Mg^{2+} was added (36 hours half life). Also, the enzyme was inferior to a commercial enzyme (Spezyme GI M600) with respect to the long-term stability (12 vs.312 hours half life) conducted under the same conditions. The soluble enzyme retained 74.0% and 65.2% of its original activity after 7 and 9 weeks of storage at 4-6^{o}C, respectively. The

K_m and V_m of the enzyme for glucose, as calculated from the Lineweaver-Burk plot, were 115 mM and 168 micromole fructose/minute, respectively

The immobilization conditions were optimized with regard to pore size, spacer length, EDC concentration, and pH. The optimum pore diameter of the coated CPG beads was found to be 350Å, when the beads were silanized and glutaraldehyde-activated. The optimum diamine (spacer & amination reagent) when followed by glutaraldehyde activation was found to be diaminohexan which has a 6 caron chain length. The optimum conditions for the enzyme immobilization using EDC were 10 mM EDC and pH 7. With regard to the percent of protein (enzyme) binding, about 57% was obtained regardless of the type of alkylamine used (aminated vs silanized beads). However, the percent of enzyme binding and activity retained significantly varied by the type of activation (glutaraldehyde vs.EDC). The percent of activity retained was about 74-90%. At the optimum Mg^{2+}concentration, > 90% of the full activity was obtained when Co^{2+} was omitted from the reaction mixture. No activity was detected when the enzyme was directly bound to Titanium(IV)-coated CPG beads under the applied conditions. The K_m and V_m of glucose for the bound enzyme preparations A, S, and E calculated from the Lineweaver-Burk plots were 0.120 and 1665; 0.145 M and 1768; and 0.135 and 2214 units, respectively.

As far as the stability of bound enzyme preparations, bound enzyme preparations A and S, which were bound through the enzyme residual amino group(s), were relatively stable at the entire pH range studied (3.5-11.5). However, bound enzyme preparation E, which was bound through the enzyme residual carboxyl group(s), was stable under a narrower pH range (3.5-9.0). Also, the bound enzyme preparations in 0.04M HEPES buffer, pH 7.7 containing 0.06% sodium azide retained 76-79% of their original activity over 9 weeks of storage at 4-6^{o}C. Moreover, the bound enzyme preparations exhibited an excellent thermal stability at temperatures from 50 to 80^{o}C. However, the long-term stability at 60^{o}C in 0.04M HEPES buffer indicated that the enzyme lost 50% of its original activity after 48 hours regardless of whether the enzyme was cross-linked

with glutaraldehyde or not. The half life of the bound enzyme preparations in a continuous isomerization process (45% glucose in 0.04M HEPES buffer, pH 7.7 containing 20 mM Mg^{2+} and 10 mM Na_2SO_3) was 4-5 days during which a protein (enzyme) discharge from the column was detected in the column effluent. However, the addition of 2 mM Co^{2+} to the substrate increased the half life to 10 days. Interestingly, the enzyme pre-treatment with cobalt before loading in the column increased the half life to 19-22 days without cobalt addition to the substrate for bound enzyme preparation S, but did not improve the operational stability of bound enzyme preparation E. The cobalt addition or cobalt pre-treartment reduced the protein (enzyme) dischage from the column during the continuous process. The carrier dissolution under the operational conditions (pH 7.7 and 60^{o}C) was a major factor in lowering the half life of the bound enzyme preparations and the Titanium(IV)-coating did not prevent this problem.

The soluble enzyme was stabilized upon immobilization as indicated by the following : 1) the bound enzyme had a wider pH activity range, pH stability range, activity temperature profile, and a better storage stability than the soluble enzyme 2) the bound enzyme was more thermostable than the soluble enzyme at temperatures of 70 and 80^{o}C. 3) the bound enzyme showed better (48 hour half life) long-term stability at 60^{o}C than the soluble enzyme (12 hour half life) 4) regardless of the lower thermal stability of the soluble enzyme compared to a commercial enzyme (Spezyme GI, M600) and the carrier dissolution releasing the enzyme from the column bed, a half life of up to 22 days was obtained in a continuous isomerization process.

Diffusional limitation study indicated no significant external or internal diffusion resistance as the percent conversion versus reciprocal flow rate gave a straight line and the specific activity of the bound enzyme preparations did not change with bead size change (ground vs. unground enzyme particles).

Conclusions:

The conclusions of this study can be drawn as follows:

1. The crude glucose isomerase was covalently bound to Titaniun(IV)-coated derivatized and activated CPG beads.

2. The conditions of glucose isomerase immobilization on the titanium coated CPG beads were optimized.

3. The percentage of protein (enzyme) binding was the same regardless of the type of the alkylamine used (aminated vs. silanized beads).

4. The percentage of protein binding and enzyme activity significantly varied by the activation methods used (glutaraldehyde vs. EDC).

5. No activity was detected when the enzyme was directly immobilized to the titanium coated CPG beads under applied conditions.

6. The bound enzyme required less magnesium and cobalt ions for full activity expression, and cobalt can be omitted with retention of >90% full activity.

7. K_m and V_m of glucose for the bound enzyme were higher than those obtained for the soluble enzyme.

8. The soluble enzyme was stabilized upon immobilization as shown by the following evidence:

   a) the bound enzyme had a wider pH activity range, pH stability range, activity temperature profile, and a better storage stability than the soluble enzyme

   b) the bound enzyme was more thermostable than the soluble enzyme at temperatures of 70 and 80^{o}C

   c) the bound enzyme showed better (48 hour half life) long-term stability at 60^{o}C than the soluble enzyme (12 hour half life)

   d) regardless of the lower thermal stability of the soluble enzyme compared to a commercial enzyme (Spezyme GI, M600) and the carrier dissolution releasing the enzyme from the column bed, a half life of up to 22 days was obtained in a continuous isomerization process.

9. The protein found in the column effluent confirmed an enzyme leakage due to carrier dissolution.

10. Cobalt pre-treatment reduced the amount of enzyme discharged from the column and therefore improved the enzyme operational stability.

11. Cobalt pre-treatment substantially increased the half life of bound enzyme preparation S,but not of preparation E. Thus,the kind of enzymatic residual group involved in the binding to the carrier may play a role in this respect.

12. Carrier dissolution was a major factor in lowering the half life of the bound enzyme preparations and titanium coating did not prevent such dissolution.

13. An excellent thermal stability does not always mean a good operational stability. At least the long-term stability should be conducted under the conditions that the bound enzyme might be used in order to get a better assessment of the enzyme stability.

14. No significant external or internal diffusional limitations were found for the bound enzyme preparations.

DIRECTIONS OF FUTURE RESEARCH:

1. More studies are needed to increase the chemical stability of CPG beads under the operational conditions most commonly used for the isomerization process ($60^{o}C$ and pH > 7.0).

2. Amino acid analysis of the free and bound enzyme would give more insight about how many points of attachment and which amino acids actually participated in the binding process so that these information may be related to the stability results and gave more understanding about the inactivation process.

3. Genetic engineering is a potential candidate to improve the stability and more importantly, at this time, to obtain glucose isomerases with optimum pH (in the acidic side) compatible with the other enzymes (alpha-amylase and glucoamylase) used in the production of fructose-containing syrups.

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Appendix A. Tables of statistical analysis

Table 1. Analysis of variance (split plot repeated measure) of soluble enzyme thermal stability data

Source	Degree of Freedom	Sum of Squares	Mean Square	F Value	Prob.
Temperature (A)	7	86485.52	12355.075	168.35**	<0.01
Medium (B)	1	5355.84	5355.84	72.98**	<0.01
Ineraction AB	7	550.17	78.596	1.07	0.404
$Error_1$ Units/AB	32	2348.50	73.391		
Time (C)	1	3125.54	3125.541	41.05**	<0.01
Interaction AC	7	530.63	75.804	1.00	
Interaction BC	1	4.83	4.829	0.06	
Interaction ABC	7	165.20	23.601	0.31	
$Error_2$ Units/C	32	2436.29	76.134		

** Significant @ p = 0.01 level

Table 2. Regression analysis (Beta coefficients) of long-term stability data (residual activity vs. time) of soluble enzyme

Treatment	Slope of Regression	Standard Error of Slope	t-Value	Prob.
Control (buffer[1])	0.961	0.2342	4.104	0.015
0.1 M Mg^{2+}	0.344	0.0264	13.044	0.001
2 mM Co^{2+}	0.050	0.0032	15.616	0.001
0.1M Mg^{2+}&2mM Co^{2+}	0.067	0.0038	17.310	0.001

1 0.04 M HEPES buffer, pH 7.7.

Table 3. Comparisons between regression slopes (Beta coefficients) of the long-term stability data of soluble enzyme

Comparison	Slope Difference	t-Value	Prob.
Control vs. Mg^{2+}	0.617	2.62	< 0.10
Control vs. Co^{2+}	0.911	3.89**	< 0.01
Control vs. Mg^{2+} & Co^{2+}	0.894	3.82**	< 0.01
Mg^{2+} vs. Co^{2+}	0.294	11.05**	< 0.01
Mg^{2+} vs. Mg^{2+} & Co^{2+}	0.277	10.37**	<0.01
Co^{2+} vs. Mg^{2+} & Co^{2+}	0.017	3.42*	< 0.02

* Significant @ p = 0.05 level
** Significant @ p = 0.01 level

Table 5. Analysis of variance (one Factor) of the effect of pore size (diameter Å) on the activity of bound enzyme

Source	Degree of Freedom	Sum of Squares	Mean Square	F Value	Prob.
Pore size[1]	3	4802.65	1600.88	3.14*	0.087
Error	8	4077.98	509.75		
Total	11	8880.63			

1 Pore size (diameter Å) were 266, 350, 547, and 727Å.
* Significant @ p = 0.09.

Table 6. Comparisons between means of the effect of pore size (266, 350, 547, and 727Å in diameter) on the activity of bound enzyme

Comparison[1]	Mean difference	Fisher PLSD[2]	t-Value
266Å vs. 350Å	32.4	34.28	1.75
266Å vs. 546Å	21.6	34.28	1.17
266Å vs. 727Å	19.5	34.28	1.06
350Å vs. 547Å	10.8	34.28	0.38
350Å vs. 727Å	51.93	34.28*	2.81*
547Å vs. 727Å	41.13	34.28*	2.23

* Significant @ p = 0.10
1 Standard error of means (SEM) = 13.035
2 PLSD = Protected least significant difference.

Table 7. Analysis of variance (one Factor) of the effect of spacer (carbon chain length) on the activity of bound enzyme

Source	Degree of Freedom	Sum of Squares	Mean Square	F Valu	Prob.
Spacer length[1]	4	18753.764	4688.44	48.113**	0.001
Error	10	974.460	97.45		
Total	14	19728.224			

1 spacers of 3, 4, 6, 8, and 10 carbon atom-long were used.
** Significant @ p = 0.01

Table 8. Comparisons between means of the effect of spacer length(3, 4, 6, 8, and 10 carbon atom-long) on the activity of bound enzyme

Comparison[1]	Mean Difference	Fisher PLSD[2]	t-Value
C_8 vs. C_{10}	33.90	17.96*	04.21*
C_8 vs. C_3	84.33	17.96**	10.46**
C_8 vs. C_4	05.16	17.96	00.64
C_8 vs. C_6	09.80	17.96	01.21
C_{10} vs. C_3	50.43	17.96**	06.25**
C_{10} vs. C_4	39.06	17.96**	04.85†
C_{10} vs. C_6	43.70	17.96**	05.42**
C_3 vs. C_4	89.50	17.96**	11.10**
C_3 vs. C_6	94.13	17.96**	11.68**
C_4 vs. C_6	04.63	17.96	00.57

1 Standard error of means (SEM) = 5.699
* Significant @ p=0.05
** Significant @ p=0.01
† Significant @ p=0.02

Table 9. Regression analysis of amount of bound protien (enzyme) vs. activity of the bound enzyme preparations using various spacers (diamines)

Source	Degree of Freedom	Sum of Squares	Mean Square	F Value	Prob.
Regression	1	2120.2	2120.2	69.98**	0.003
Residual	3	0090.9	0030.3		
Total	4	2211.1			

Correlation coefficient = 0.9792
** Significant @ p = 0.01

Table 11. Analysis of variance (two Factor) of the effect of pH level and EDC concentration on the activity of bound enzyme

Source	df.[1]	Sum of Squares	Mean Square	F Value	Prob.
pH (A)	2	1377.34	688.67	215.13**	0.0006
EDC (B)	2	0459.38	299.69	071.75**	0.0029
AB (Interaction)	4	0564.86	141.21	044.11**	0.0053
Error	3	0009.60	003.20		

1 Degree of Freedom
** Significant @ p = 0.01
Unbalanced analysis.

Table 12. Comparisons between the means of bound enzyme activity at various levels of pH and EDC concentration

Comparison		Mean Difference	t-Value
@ 5 mM EDC	pH5 vs. pH6	03.91	01.784
	pH5 vs. pH7	12.19	05.61**
	pH6 vs. pH7	16.21	06.40**
10 mM EDC	pH5 vs. pH6	24.31	11.09**
	pH5 vs. pH7	27.78	10.98**
	pH6 vs. pH7	03.47	01.58
20 mM EDC	pH5 vs. pH6	34.72	13.72**
	pH5 vs. pH7	41.20	18.80**
	pH6 vs. pH7	06.48	02.96
@ pH 5	5mM vs. 10mM	00.72	00.33
	5mM vs. 20mM	10.85	04.95**
	10mM vs. 20mM	11.57	04.57*
pH 6	5mM vs. 10mM	28.94	13.21**
	5mM vs. 20mM	27.78	10.98**
	10mM vs. 20mM	01.16	00.53
pH 7	5mM vs. 10mM	16.20	06.4**
	5mM vs. 20mM	18.05	08.24**
	10mM vs. 20mM	01.58	00.84

* Significant @ p = 0.05 level
** Significant @ p = 0.01 level
Data standard error of means (SEM) = 3.201, unbalanced analysis.

Table 14. Analysis of variance (one Factor) of the effect of activation methods (glutaraldehyde vs. EDC) on the amount of bound (protein) enzyme

Source	Degree of Freedom	Sum of Squares	Mean Square	F Value	Prob.
Activator	1	298.215	298.15	21.357**	0.0099
Error	4	055.853	013.963		
Total	5	354.068			

** Significant @ p = 0.01

Appendix B. Standard curves of fructose and protein analyses

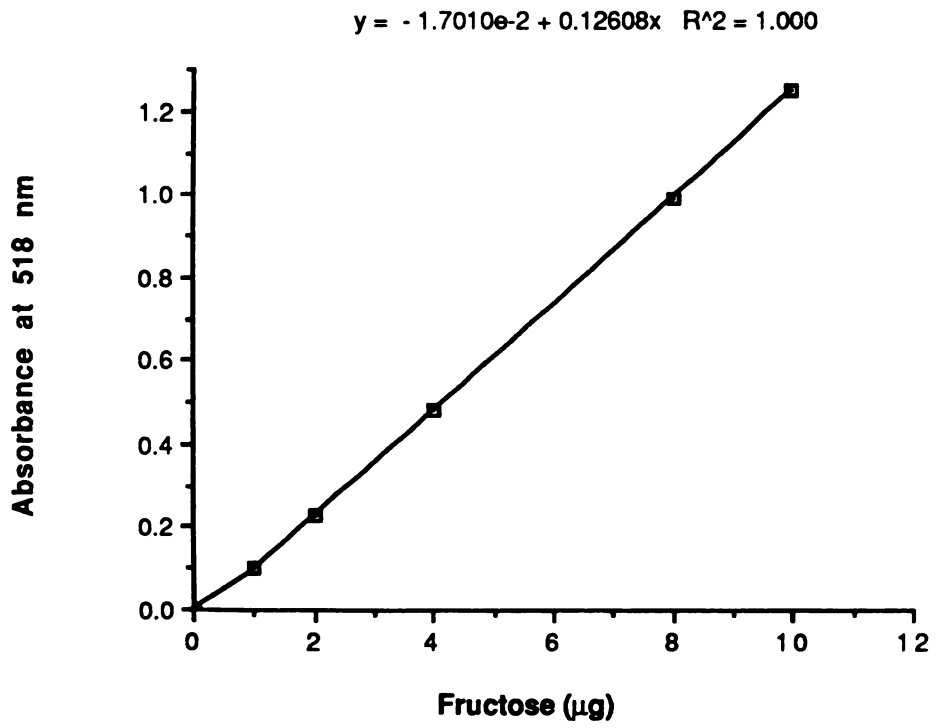

Figure 56. Standard curve of fructose determination.

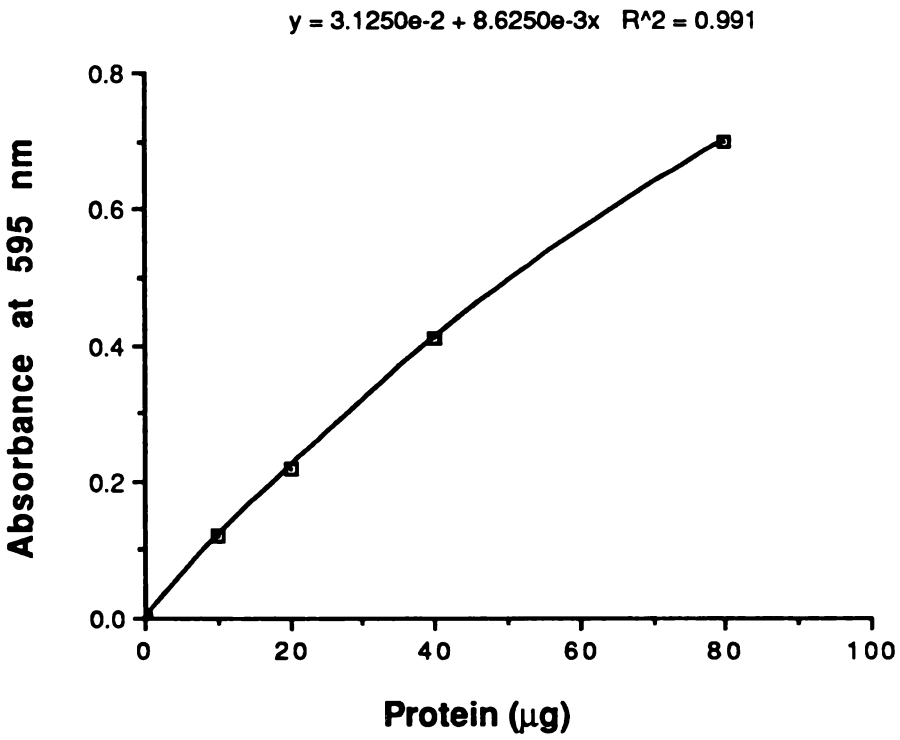

Figure 57. Standard curve of protein determination using Bio-Rad Method.

Appendix C. Table of Moisture content of bound enzyme

Table 17. Moisture content of immobilized glucose isomerase on Titanium(IV)-coated CPG beads

Carrier	Activator	% H2O	
		Wet Dasis	Dry Basis
Aminated beads[1]	Glutaraldhyde	66.8	202.5
Silanized beads[1]	Glutaraldhyde	65.8	202.0
Silanized beads[1]	EDC	66.3	202.2

1 Titanium(IV)-coated controlled pore glass (CPG) beads.

Appendix D. Physical characteristics of CPG beads used for the immobilization of glucose isomerase

Table 18. Physical characteristics of CPG beads used for the immobilization of glucose isomerase

Mesh size	Mean pore Diameter (Å)	Pore Distribution (+ %)	Pore Volume (cc/g)	Surface Area (m^2/g)
20-80	266	6.2	0.85	94.00
20-80	350	6.5	0.91	53.45
20-80	547	6.3	1.06	44.50
20-80	727	6.4	0.77	24.90