IMMOBILIZED ENZYMES:
STUDIES ON GLUCOAMYLASE (EC 3.2.1.3)

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CONTENT

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GENERAL INTRODUCTION

A marked revival of interest in enzymes as industrial catalysts has come about in recent years, mainly due to the rapid progress in techniques of enzyme immobilization.

Such immobilization is achieved either by restriction of enzymes within semipermeable membranes or by attachment to solid supports in a manner that the biological activity is retained. Enzymes are ideal catalysts on account of their high specificity and ability to bring about chemical conversions under mild pH conditions and at low temperatures. Immobilized enzymes in addition have the obvious advantage of repeated reuse. While the soluble enzyme can be used only in a single operation, being generally lost in processing, the immobilized catalyst is easily recovered. The economic benefits of reuse are particularly significant with intracellular enzymes whose isolation processes are generally tedious and expensive. Immobilization has also been reported in instances to stabilize and considerably extend enzyme half-life (Barker, 1978). Automated processing is possible in special reactors when immobilized enzymes are used. Product contamination by the added catalyst is also avoided and this has particular significance in the food and drug industries.

Amongst the earliest studies on the behaviour of immobilized enzyme systems are those of Nelson and Griffin (1916) on invertase adsorbed on charcoal and alumina. It was only after a lapse of some four decades that interest

in such immobilized systems was revived by the work of McLaren and Zittle (1953, 1954) who studied enzyme adsorption on inorganic carriers. Interest picked up further with the pioneering studies of Katchalski and his group, on the covalent attachment to polyaminoacid carriers (Silman and Katchalski, 1966). Extensive literature is now available on enzyme immobilization. Amongst the reviews and books the following may be mentioned: Chang (1977); Messing (1975); Mosbach (1976); Olson and Cooney (1974); Pye and Wingard (1974); Silman and Katchalski (1966); Stark (1971); Weetall (1975); Wingard (1972) and Zaborsky (1973) ~~(1973)~~, Since in the present study diverse immobilization procedures are compared and a new support material used, the standard methods of immobilization as well as carrier morphology are described briefly. The descriptions are only illustrative as a comprehensive review is outside the scope of this thesis.

I. METHODS OF IMMOBILIZATION

Methods of immobilization could be broadly grouped under the following five classes, dependent on the nature of attachment to supports or of restriction within semipermeable membranes:

1. Immobilization by Adsorption
2. Immobilization by covalent linkage to the matrix
3. Immobilization by intermolecular cross-linking
4. Gel entrapment of Enzymes
5. Encapsulation of Enzymes

I.1. Adsorption:

Classically adsorption of enzymes on solid supports was the earliest of the procedures used for enzyme immobilization. Amongst the early documented examples is the immobilization of invertase on activated charcoal by Nelson and Griffin (1916).

Adsorption on a wide variety of other surface-active solid supports such as titania (Messing, 1974), Bentonite (Ryu, et al., 1972) and hydrophobic materials (Hofstee, 1973) have also been reported extensively in the literature. The coating of otherwise inactive supports with a precipitate of surface-active substances like TiO_2 has also been achieved by addition of $TiCl_4$ to a suspension of the core material under hydrolysing conditions (Hasselberger et al., 1974). Ion exchange resins have also been used extensively. The earliest example of the industrial application of immobilized enzymes has been of such a system in which α-L-amino acid acylase was adsorbed on DEAE-Sephadex (Tosa et al., 1969b).

An advantage of this method is its general ease, simplicity and low cost as the support can generally be recycled with fresh charge of the enzyme. A major disadvantage, however, is the leakage of the activity, particularly under the influence of salts, high concentration of substrate, pH and ionic strength of the reaction medium. An example of substrate-induced desorption is of glucose-6-phosphate dehydrogenase (Goldman and Lenhoff, 1971). Desorption by a change in the

pH of the solution is reported for papain adsorbed on glass (Messing, 1970). Leucine aminopeptidase adsorbed on calcium phosphate gel leaks out with increase in ionic strength (Koelsch et al., 1970).

I.2. Covalent attachment:

A more permanent retention of enzymic activity on the matrix than that resulting through adsorption is generally obtained by chemically coupling an activated matrix to the enzyme through an amino acid residue (or residues) not involved at the active site. Various reagents and reactions have been used for the purpose dependent both on the chemical nature of the support and the type of the enzyme involved.

Usually the groups involved in this type of bonding are:

1. α- and ϵ-amino groups
2. α, β- and γ-carboxyl groups
3. sulfhydryl groups of cysteine and hydroxyl groups of serine
4. imidazole group of histidine
5. phenol ring of Tyrosine,

If required, the active site of the enzyme can be protected during the immobilization procedure by doing the reaction in the presence of a competitive inhibitor, or the substrate, or by chemically modifying the enzyme.

A few examples of the chemical reactions used for enzyme immobilization are described here.

(a) CNBr for activation of polysaccharide supports:

One of the most widely used reagents for activation

of polysaccharides like cellulose, agarose and dextrans is the CNBr procedure, first described by Porath and his group (Porath et al., 1967). The CNBr-activated polysaccharide is then coupled to the enzyme molecule through free amino groups.

(b) Aminoalkyl derivative of glass and ceramics:

Silane coupling agents, such as γ-aminopropyl-triethoxysilane, have been used for activating glass and ceramic surface (Weetall, 1975). Protein is coupled to the alkylamine-support via glutaraldehyde, thiophosgene or triazine or carbodiimide coupling (Weetall, 1976). The alkylamine-derivative can also be converted to arylamine by treatment with p-nitrobenzoyl chloride followed by reduction of the nitro group (Weetall and Filbert, 1976), and coupling to enzyme through the diazonium salt.

(c) Carboxymethyl-cellulose azide:

Mitz and Summaria (1961) first used this method for the immobilization of α-chymotrypsin and trypsin. Carboxymethyl cellulose can be treated with hydrazide and nitrous acid to yield the azide. The azide formed can be linked to the ϵ-aminogroup of lysine residues in the enzyme molecule.

(d) Cyanuric chloride (Trichloro-s-triazine):

Polysaccharides can be activated by reaction with cyanuric chloride or its dichloro derivatives (Kay and Crook, 1967).

(e) Carbodiimide coupling:

Carboxyl carriers can be activated by water soluble carbodiimides and then covalently linked to enzyme via α- or ϵ-aminogroups of enzymes (Weliky and Weetall, 1965).

(f) Ethylene maleic anhydride copolymer:

Immobilization can also be achieved with the use of reactive synthetic polymers like ethylene maleic anhydride (EMA) copolymer (Levin et al., 1964).

I.3. Intermolecular Cross-linking:

Intermolecular cross-linking is often achieved with relative ease through the use of multifunctional reagents to give enzyme conjugates with a three dimensional cross-linked network. Examples of the commonly used bi or multifunctional reagents are: Glutaraldehyde (Quiocho et al., 1964), Cyanuric chloride (trichloro-s-triazine) (Surinov and Monoilov, 1966), 1,5-difluoro 2,4-dinitro-benzene (Marfey and King, 1965).

The basic principle of the method is coupling of the protein molecules to each other, although intra-molecular linkages are unavoidable side reactions of the immobilization reactions. To minimise the intramolecular linkages, the concentration of enzyme as well as that of multifunctional reagent and pH of the reaction mixture and ionic strength could be controlled.

I.4. Gel Entrapment of Enzymes:

Physical entrapment of an enzyme within a gel lattice restricts the macromolecules to the aqueous phase within

the gel. While small molecular weight substrates and products can move through with relative ease, the enzyme is restricted within the gel. In practice the monomer, a cross-linking agent and the enzyme to be immobilized are taken in buffered solution and polymerization initiated with addition of a catalyst system. Gel entrapment in polyacrylamide has been extensively reported on in the literature (Koch-Schmidt, 1977). In this the physical properties such as porosity can be tailored by varying monomer concentrations, small pore gels being obtained at high monomer concentrations and large pore ones at relatively lower monomer concentrations. The polymer with entrapped enzyme can be reduced to convenient particle size for actual use in reactors. A medium in which a preformed polymer is used for gel entrapment is the natural polymer collagen which occurs as a structural protein. In this case, membranes of collagen are readily obtained by adding enzyme to collagen suspension and casting as a membrane and then drying to facilitate macromolecular complexation between enzyme and collagen (Wang and Vieth, 1973). Collagen enzyme preparation have also been obtained by electrocodeposition (Constantinides *et al*., 1973; Suzuki *et al*., 1974).

The procedure of gel entrapment is also an extensively used procedure for entrapment of whole microbial cells (Chibata *et al*., 1974). Commercial use of such entrapped whole cells for the production of L-malic acid from

fumaric acid with entrapped Brevibacterium ammoniagenes and of L-aspartic acid from fumaric acid with entrapped aspartase-containing Escherichia coli has been exploited for the first time by Tanabe Seiyaku Company of Japan (Chibata, 1974).

I.5. Encapsulation of Enzymes:

This method of immobilization is distinguished from the others in that the enzyme solution is packaged within a membrane envelope system in a manner that the enzyme is enclosed within the intramembrane environment and prevented from leaking into the external environment by the semipermeable membrane layer. Substrates and products, provided they are of low enough molecular weight, can diffuse rapidly across the membrane. Unlike gel entrapment the enzyme is in its native environment and not entrapped in polymer lattices. The earliest examples of encapsulation have been in form of artificial cells. Such artificial microcapsules of cellular dimensions have been obtained by containment with spherical ultrathin semipermeable membranes enveloping a solution of enzymes (Chang, 1957; 1964; 1972).

The preparation of microcapsules have been carried out by two distinct procedures based either on physical coacervation or on chemical interfacial polymerization. In the former case a preformed polymer such as collodion (cellulose nitrate) is made to separate at the interface of microdroplet between an organic phase such as ether

and an aqueous phase. Such separation of polymer to yield a membrane envelope is due to its lower solubility at the microdroplet interface. All enzymes tested with this procedure have been found to retain their activity. In the alternate procedure of interfacial polymerization, water-soluble diamines such as 1,6-diaminohexane, an organic soluble diacylhalide such as 1,10-decanoyl chloride (sebacoyl chloride) are made to react in presence of enzyme molecules to give microcapsules of nylon (-6,10) (Chang, 1964). The application of interfacial polymerization procedure is restricted to only a few enzymes such as urease and asparginase, which are stable in the high pH conditions of the polymerization reactions.

Wet spun fibres of cellulose triacetate have been used for entrapping several enzyme solutions. This procedure first described by Dinelli (1972) has potential for extensive industrial application. Although termed as fibre "entrapment", in this procedure the enzyme solution is enclosed within the fibre.

An encapsulation procedure which permits direct therapeutic administration of enzymes is through use of Liposome systems consisting of microscopic bilayer lipid spheres within which the aqueous enzyme solution is encapsulated (Gregoriadis et al., 1971).

In several procedures of immobilization, a combination of the methods described above could play a part in

immobilizing the enzyme. Thus CNBr-activated polysaccharides used for covalent attachment also adsorb proteins through charge effects as well as through hydrogen bonding and hydrophobic interactions, particularly where spacer arms separate protein from the carrier.

II. CARRIERS AND SUPPORTS

A broad spectrum of supports ranging from inorganic materials including metals like steel, etc. to synthetic and natural polymers have been used for immobilization of enzymes. A review of support materials is outside the scope of this thesis and only support morphology is briefly described here.

It is possible to use the same carrier in diverse forms e.g. particulate, fibre, membrane, etc. The brief review that follows is only to describe the nature and advantages of some of the carriers used in the present study, such as controlled-pore ceramic and magnetic γFe_2O_3. This review is based on articles and recent publications by Messing (1974; 1975).

The morphological characteristics of carriers has been classed in the following manner:

1. Non porous carriers
2. Porous carriers:
   (a) Controlled pore
   (b) Broad pore distribution
   (c) Gel structures:
      (i) Preformed gel structures
      (ii) Entrapped gel
      (iii) Copolymers

The non porous supports have limited surface area available for attachment of enzymes. However, the use of

these in the form of very fine particles or fibres overcomes this disadvantage partially. The use of supports in the form of low particle size or fine fibre form leads to problems in removal of the carrier from the reaction solution. This is overcome if the particles have special properties like magnetic properties e.g. iron filings; magnetite, Fe_3O_4; magnetic γ-Fe_2O_3.

Porous materials offer very high surface areas for immobilization. Where broad pore distribution occurs, the enzyme is excluded from such pores that are too small to accommodate macromolecules. This has been overcome by the recent introduction of the relatively cheap, controlled-pore ceramics of alumina, titania, silica and zirconia, by the Corning Glass Works, USA (Messing, 1974). These are extremely rigid particles with very high surface areas and of extremely uniform porosity of any desired range. These ceramics permit inclusion of metal ions like Mg^{2+} and Co^{2+} which may be required as cofactors or stabilizers in enzyme reactions. Such supports can be reused after pyrolysis or chemical treatment. The pores of the support also exclude microbes and protect the enzyme molecules held within from microbial attack. The major disadvantage of porous carriers is the diffusional restrictions for substrates, particularly those of high molecular weight, to enter and the product to leave.

Gel structures have been utilized for entrapment or encapsulation of enzyme. The gels also come under severe diffusional restrictions and are inaccessible to substrates

of relatively high molecular weight.

III. GLUCOAMYLASE [α,1 → 4, 1 → 6-GLUCAN GLUCOHYDROLASE (EC 3.2.1.3)]

Since glucoamylase has been used as a model system in the present study, the occurrence and general properties of the enzyme and some of the immobilized systems reported in the literature are briefly reviewed.

III.1. Amylases:

Amylases are enzymes that hydrolyse α(1 → 4) linked glucose polymers such as starch by the transfer of a glucosyl residue to a molecule of water. Amylases are either classed as α- or β-amylases or as endo- or exo-amylases.

III.2. α-Amylases:

α-Amylases [α,1 → 4 glucan 4-glucanohydrolase (EC 3.2.1.1)] are so called because the hydrolytic product possesses an α-configuration. α-Amylases hydrolyse large linear substrates, such as native starch at most internal bonds (Greenwood and Milne, 1968) and are endo-enzymes. They are widely distributed and found in mammals, plants, fungi and bacteria. The action of α-amylases is random and the products are oligosaccharide mixtures which retain the original α-configuration.

III.3. β -Amylases:

[α ,1 → 4 glucan maltohydrolase (EC 3.2.1.2)], These enzymes have been found in certain higher plants and are exoenzymes which attack alternate linkages from the non-reducing end of α(1 → 4) linked glucose polymers like starch to yield β-maltose. The nomenclature of these enzymes as α- and β- is based on the product stereochemistry and not substrate specificity.

III.4. Glucoamylases:

[α , 1 → 4; 1 → 6-glucan glucohydrolase (EC 3.2.1.3)] A third group of amylases discovered more recently than the α- and β-amylases is glucoamylase also known by the trivial name of amyloglucosidase or γ -amylase.

Glucoamylases like β-amylases are exoenzymes but remove successively a single glucose unit from the non-reducing end of its substrate with inversion of configuration. Thus maltose, maltotriose, maltotetrose, maltopentaose are completely hydrolysed (Barker and Fleetwood, 1957) to β-glucose and amylose is almost completely converted to glucose. Glucoamylase has a low degree of specificity and can also cleave α(1→ 6) and α(1 → 3) glucan linkages (Pazur, 1972). Thus isomaltose, αD(1 → 6) glucopyranosyl-D-glucose and Nigerose, α(1 → 3)D-gluco-pyranosyl-D-glucose are substrates, although hydrolysed at rates much slower than maltose (αD(1 → 4) glucopyranosyl-D-glucose). A complete degradation of amylopectin is not accomplished when α-amylase free

crystalline glucoamylase from Rhizopus niveus is used (Marshall and Whelan, 1970). Glucoamylases have also been reported to show weak transglucosylase activity. Highly purified preparations have been reported to produce traces of products like isomaltose in addition to glucose probably produced through a reversion reaction by a transfer of glucosyl units cleaved from chain ends to previously released glucose residues rather than to water as the acceptor (Kulp, 1972).

III.4.1 Occurrence and Properties:

Glucoamylases have been isolated from several species of fungi of Aspergillus and Rhizopus groups, and from some yeasts (Saccharomyces diastaticus) (Hopkins and Kulka, 1957), and bacteria (Clostridium acetobutylicum). They also occur in animal tissue mainly in the liver (Rosenfeld et al., 1959). Aspergillus niger is reported to contain atleast two extracellular isozymes separable by electrophoresis (Pazur and Knull, 1971). Pazur and Okada (1967) purified glucoamylase from R. delemar by successive chromatography and filtration on Duolite A-2 resin, Sephadex G-50 gel and Amberlite XE 64 resin. The molecular weight of the enzyme was 100 000. An ultracentrifugally and electrophoretically homogeneous enzyme preparation has been obtained from A. niger NRRL 330 (Pazur, 1972) by a procedure involving repeated steps of ethanol fractionation and DEAE-cellulose chromatography. The pure enzyme has been reported to have a molecular weight 100 000,

pH optimum of 5.0, pI value of 4.2. The glucoamylase from this source has been shown to be a glycoprotein containing residues of D-mannose, D-glucose and D-galactose (Pazur et al., 1971; Fleming and Stone, 1965). The carbohydrate residues have been shown to structurally stabilize the enzyme molecule (Pazur et al., 1970). The kinetic studies on the enzyme from R.delemar suggest that only a single active site is involved in hydrolysis of $\alpha(1 \longrightarrow 4)$ and $\alpha(1 \longrightarrow 6)$ linkages (Hiromi et al., 1966).

Kerr and Cleveland (1951) showed glucoamylase can produce glucose at the same rate in weight/unit time from equimolar solutions of amyloses altering in chain length and it was established by paper chromatography that the hydrolysate product is only glucose. Philips and Caldwell (1951) showed glucoamylase is less sensitive to high hydrogen ion activities at relatively low temperature and showed maximal enzyme activity at 40°C and pH 4.5, whereas the enzyme purified from Aspergillus phoenicis by Lineback and Baumann (1970) has an optimum temperature of 60°C and pH optimum of 4.5, while the enzyme purified from Cephalosporium charticola Lindau (Krzechowska and Urbanek, 1975) has an optimum temperature of 60°C but an optimum pH of 5.4. King (1967) observed that glucoamylase from C. cerebella is completely inhibited by Hg^{2+} but other ions at concentration of 5 mM had very little effect on enzyme activity. Ca^{2+} and Cl^{-} has no effect on enzyme activity (Smiley et al., 1971).

Krzechowska and Urbanek (1975) showed glucoamylase activity in culture fluids of Cephalosporium charticola Lindau and purified the enzyme on DEAE-cellulose to homogeneity. Radhakrishnan and Swaminathan (1970) obtained a highly purified preparation of glucoamylase from liver, spleen and intestine of monkeys. Peniksova and Ruzhakova (1977) isolated the enzyme from the filtered medium after submerged cultivation of Aspergillus awamori and showed that medium containing $NaNO_3$ and whose pH at the end of the cultivation was 7.5 - 8, α-amylase and glucoamylase activities were present. But in medium containing $(NH_4)_2SO_4$ and whose pH was maintained at 2.5 to 2.8 only glucoamylase activity was observed.

The commercial production of glucoamylase became economical mainly through strain improvements and the isolation by Underkofler (1969) of a mutant strain of Aspergillus foetidus which gave high yields of glucoamylase activity.

III.5 IMMOBILIZED GLUCOAMYLASE

Numerous examples of immobilized glucoamylase preparations have been reported, particularly in the patent literature.

The interest in this enzyme has been mainly because of its wide industrial application in hydrolysis of starch to dextrose, the largest single process in which enzymes are used industrially today. An estimated 1.3 billion lbs. of dextrose was produced in USA in 1971 (Wolnak, 1972).

Raw starch is first dextrinized either chemically by acid treatment or enzymically with α-amylase. The subsequent step currently in vogue is the hydrolysis of the dextrinized starch to dextrose with glucoamylase. Glucoamylase is an extracellular enzyme and is relatively cheap. The economic advantage of using an immobilized enzyme is therefore questionable. However, extensive studies and patents on the immobilized enzyme exist and Corning Glass Works of USA have operated a pilot plant of one cubic foot column of purified enzyme covalently attached to ZrO_2-coated porous glass for production of 1000 lbs. of dextrose daily (Weetall, 1975). The main advantages claimed for the immobilized systems are the short residence time of less than 6 min at 60°C and less than 20 to 25 min at 40 to 45°C compared to the holding time of 48 h for the "Novo" soluble enzyme process (Weetall, 1975).

The economic advantage of using an immobilized enzyme as mentioned earlier is negligible considering the low cost of the enzyme and no reports exist of production plants in which immobilized glucoamylase preparations are used (Barker, 1978).

Illustrative examples from the numerous immobilized enzyme preparations described in the literature are summarized briefly in this Section.

III.5.1. Immobilization by Adsorption:

Glucoamylase from A.niger adsorbed on molecular sieve 4A (MS) and alumina have been reported (Solomon

and Levin, 1975). These systems lost activity at alkaline pH. Leakage of activity was also reported in presence of starch substrate. Solomon and Levin (1974) have also studied the behaviour of glucoamylase conjugates adsorbed on DEAE-cellulose and other cationic resins. The native enzyme adsorbed on cationic carrier has low temperature stability, while soluble glucoamylase-ethylene maleic anhydride copolymer or glucoamylase-styrene maleic anhydride copolymer adsorbed on cationic resins were stabler and showed less leakage of activity over prolonged use.

Immobilization of glucoamylase by adsorption on DEAE-cellulose and its use for continuous conversion of starch to dextrose has been reported by Bachler et al., (1970). The activity of the adsorbed preparation was only 16-55% of that of free enzyme. The temperature optimum was lowered to 40 - 50°C as compared to 60°C for the soluble enzyme. Columns retained their activity over prolonged continuous operation. Partially purified glucoamylase from A.awamori was immobilized and used in reactors continuously for 3 - 4 weeks without appreciable loss of activity. The complete hydrolysis of starch to dextrose by DEAE-adsorbed enzyme has also been described by Thompson (1976). Immobilization of enzyme from A.niger through adsorption by hydrophobic interactions with hexyl-Sepharose has been reported to give high retention of activity (Caldwell et al., 1976). Continuous operation

for 3 months reduced the conjugate activity to 40%. The adsorption of glucoamylase on stainless steel and alumina coated with TiO_2 by treating by the carriers with $TiCl_4$ under hydrolysing conditions has been mentioned, without any experimental data being presented (Hasselberger, et al. 1975).

III.5.2. Immobilization of glucoamylase by covalent attachment

(i) Polysaccharide supports

Amongst the earliest examples of glucoamylase immobilization by covalent attachment is the report by Wilson and Lilly(1969)of the enzyme attached to DEAE-cellulose, initially derivatized with 2-amino-4,6-dichloro-s-triazine (O'Neill et al, 1971; Lilly and Douglas, 1973). The immobilized enzyme has also been obtained by chemical coupling with a diazotized 3(p-aminophenoxy)-2-hydroxypropyl ether of cellulose to give a stable preparation which can be stored for 3 months without loss of activity (Barker et al. 1969). Enzyme covalently bound to CNBr-activated cellulose has also been reported and the activity of the preparation has been shown to be influenced by the nature of the cellulose surface (Maeda and Suzuki, 1972). Maeda and Suzuki (1972) have also described coupling of enzyme to halogenacetylcellulose.

(ii) Synthetic polymer supports:

Polystyrene-glucoamylase has been obtained by coupling the enzyme to diazotized polyamino styrene beads (Ledingham and Ferreira, 1973). Immobilization of glucoamylase on poly(glycidylmethacrylate) co(ethylene-dimethacrylate) has also been achieved by coupling via

glutaraldehyde to the macroporous copolymer derivative obtained from glycidylmethacrylate-ethylenedimethacrylate copolymer, the copolymer being converted to an NH_2-derivative by treatment with ammonia or 1,2-diaminomethane after oxidation with periodic acid (Svec et al., 1978).

The use of organic polyisocyanates to bind glucoamylase in the presence of an inert solid carrier like rice hulls has been described (Stanley, 1972). Enzacryl supports which consist of copolymers of acrylamide and various derrivatives of acrylamide, have been used after treatment with $TiCl_4$ to bind glucoamylase through a metal-link (Flynn and Johnson, 1978).

(iii) Inorganic supports:

A system that has been studied both in laboratory and pilot plant scale is of the enzyme coupled to silanized ZrO_2-coated porous glass carrier (Weetall, 1975; Weetall and Havewala, 1972). Two of the procedures used in case of glucoamylase involve initial silanization of support with γ-aminopropyltriethoxysilane followed by either treatment with glutaraldehyde to give a schiff's base intermediate which is then coupled to enzyme or conversion of alkylamine glass to arylamine (Weetall and Filbert, 1976) followed by diazotization and coupling to enzyme. Since silanization involves only superficial derivatization of surface hydroxyl groups, extensive leakage of activity occurs from untreated glass-carrier even at neutral pH through hydrolytic cleavage of the relatively labile

-Si-O-Si-alkylamine bonds. This has been avoided by surface-coating the glass with ZrO_2 to give a derivative via Zirconia which is comparatively resistant to cleavage at alkaline pH (Weetall and Havewala, 1972). Silanization with γ-aminopropyltriethoxysilane has been used for activation of alumina carrier followed by coupling of glucoamylase via glutaraldehyde (Allen et al., 1979).

Silicic acid grafted with $(C_2H_5O)_3$-Si$(CH_2)_2$-Si-$(OC_2H_5)_3$ has been used for immobilizing glucoamylase (Meiller and Monsan, 1977). Carrier material like brick and silicic acid have been activated with SO_2Cl_2, acetyl chloride or epoxy group containing silanes and then coupled to glucoamylase (Durang and Monsan, 1975).

The naturally occurring silicate, Hornblende, has been used for coupling glucoamylase either by the silanization - glutaraldehyde process or metal-link method in which the mineral is treated with $TiCl_4$ and then with enzyme (Flynn and Johnson, 1978).

(iv) Microbial cells:

The coupling of glucoamylase to yeast cell through a metal-link has been described by Hough and Lyons (1972). Yeast cells with no glucoamylase activity were treated first with $TiCl_4$ and then added to the enzyme solution to achieve coupling via Ti-links.

(v) Immobilized analytical systems:

CNBr-activated Sepharose has been used for immobilizing the two enzyme system of glucoamylase and

glucose oxidase (Gestrelius et al., 1972). Glucoamylase-glucose oxidase covalently attached through glutaraldehyde to the inside surface of nylon tubes has been used for automated analysis of maltose (Inman and Hornby, 1973).

III.5.3. Immobilization of glucoamylase by intermolecular cross-linking:

Coupling of glucoamylase with gelatin or other inert protein via glutaraldehyde cross-linking has been carried out in presence of fillers like bentonite (Solomon and Levin, 1974). Such procedures would result in intermolecular cross-linking between enzyme and inert protein as well as between the enzyme molecules. Cross-linking of the enzyme with glutaraldehyde in the presence of porous Titania or glass beads has also been described (Rosevear, 1976). 4,4'-Diazophenylmethane has been used in presence of silica gel for immobilization of glucoamylase (Krasnobajew, 1974).

The use of glutaraldehyde to cross-link glucoamylase in the presence of phenol formaldehyde resins has been reported by Olson and Stanley (1973). The cross-linking of glucoamylase initially adsorbed on activated carbon has been carried out with the use of water soluble carbodiimides (Cho and Bailey, 1978; Adachi et al., 1978). Glucoamylase adsorbed initially on activated alumina has been subsequently cross-linked with glutaraldehyde (Allen et al., 1979).

III.5.4. Immobilization of glucoamylase by Entrapment and Encapsulation:

In its simplest form, ultrafiltration membranes have been used for the continuous hydrolysis of starch by glucoamylase (Butterworth et al., 1970). The enzyme has been entrapped in photopolymerized acrylamide (Walton and Eastman, 1973). The immobilized enzyme has also been prepared from acylamide polymerized by γ-ray irradiation without use of cross-linking reagents like NN'-methylene-bisacrylamide (Maeda et al., 1973). γ-Ray irradiation has also been used for polymerizing vinylalcohol and N-vinylpyrrolidone to yield gel matrices with entrapped glucoamylase. The enzyme so entrapped is reported to be capable of hydrolysing dextrins of molecular weight 10 000 (Maeda et al., 1974). Transparent films with entrapped enzyme has been obtained by photopolymerization of enzyme dispersion in xylene diisocyanate, polyethylene and 2-hydroxymethyl methacrylate (Fukui et al., 1977). Wet spun cellulose fibres, which have been extensively used for encapsulating several enzymes, has also been used with glucoamylase (Dinelli, 1972).

The encapsulation of glucoamylase in liposomes has been studied by Gregoriadis et al. (1971) as a model system. The enzyme remains latent and is detected only after disruption of liposome structure with triton X-100,

The injection of liposome-entrapped glucoamylase has been used for studying the fate of liposome-associated proteins administered parentally to animals. Such administration was shown to result in targetting of enzyme mainly to lysosome fractions of tissues like liver (Gregoriadis, 1971).

PRESENT INVESTIGATION

INTRODUCTION

For commercially useful immobilized glucoamylase preparations, the main requirements would be low costs of the support and of the immobilization step(s), since the enzyme is comparatively cheap. In the systems described in the literature in which relatively pure enzyme has been used with high cost supports such as ZrO_2-coated porous glass, the half-life of the immobilized enzyme system has to be high for an economically viable process. While the usefulness of such systems has been established in pilot plant trials, no immobilized system has been used in production plants because of the difficulties of avoiding enzyme inactivation with commercial feed stocks of soluble starch (Weetall, personal communication).

The purpose of the present investigation was mainly to determine the efficiency of some cheap supports to bind glucoamylase from crude preparations and to compare the properties of these systems in which immobilization was achieved by diverse procedures.

The enzyme used in the studies was obtained from HAL, Poona and was a crude preparation obtained from Aspergillus candidus strain A-32.

A new carrier, the spinel γ-Fe_2O_3 was tested on account of its magnetic properties. The separation of magnetic particles from viscous slurries would present

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Abbreviations: DNSA - 3'5'-dinitrosalicylic acid

no problems. Such inorganic systems are also amenable for use in fluidizied bed reactors. The use of this material as support has not been reported hitherto in the literature.

Catalyst grade alumina, which is readily available in this laboratory, was also used as carrier for adsorption of glucoamylase from crude preparations.

Various procedures of carrier-pretreatment were tried out in an effort to enhance both enzyme-loading on support and enzyme-retention on reuse. A novel approach of first covalently cross-linking starch on the carrier surface through the use of cyanuric chloride followed by adsorption of glucoamylase activity by a combination probably of both affinity and ion-exchange adsorptions gave interesting results. The adsorbed activity was retained to the extent of about 40 to 90% in each reuse and the carrier could be repeatedly recycled with fresh charge of the enzyme without apparent decrease in enzyme-loading on the carrier.

The enzyme-loading obtained in these adsorbed systems has been compared with activity retention obtained by covalent attachment on a few controlled-pore ceramics which were obtained as gifts from Corning Glass Works, Corning, USA.

MATERIALS

All the chemicals used in the buffer preparations were of analytical grade. 3'5'-Dinitrosalicylic acid used, was from Koch-Light Laboratories Ltd. (England). Soluble starch used was from E.Merck (India) Ltd. Titanium tetrachloride used in all the experiments was prepared in the Division of Inorganic Chemistry of this Laboratory. Cyanuric chloride (trichloro-s-triazine), laboratory reagent grade, was purified by crystallization from acetone.

Glucose oxidase from *Aspergillus niger* was a commercial preparation from Koch-Light Laboratories Ltd. containing 1350 U/g. Peroxidase from horse radish was also from Koch-Light Laboratories Ltd. containing 100 U/g. o-Dianisidine was obtained from the Biochemicals Unit of CSIR, New Delhi.

Carriers:

Al_2O_3 was catalyst grade material from Chemical Engineering and Process Development Division of this Laboratory. The physical properties of the Al_2O_3 are given below:

Surface area - 150 m^2/g; Porosity - 0.6 cc/g;
Bulk density - 0.79.

γ-Fe_2O_3 used in these investigations was prepared from ferrous oxalate, according to the process described by Singh *et al.* (1975). Ferrous oxalate ($Fe_2C_2O_4.2H_2O$) was prepared by mixing stoichiometric amounts of 0.5 M oxalic

acid and 0.5 M ferrous sulfate solution. The yellow crystalline precipitate obtained was filtered, washed and dried. Dry ferrous oxalate powder was decomposed in a current of steam at 450°C to obtain ferroso-ferric oxide, Fe_3O_4. The black magnetic oxide thus obtained was slowly oxidized in the wet form at 150-200°C, in an air oven, when it gradually turned to the reddish brown γ-Fe_2O_3. Stepwise the chemical reactions involved are:

$$FeSO_4 + H_2C_2O_4 \longrightarrow Fe_2C_2O_4 + H_2SO_4$$

$$3FeC_2O_4 \longrightarrow Fe_3O_4 + 2CO_2 + 4CO$$

$$2Fe_3O_4 + \tfrac{1}{2}O_2 \longrightarrow 3Fe_2O_3$$

γ-Ferric oxide thus obtained has an inverse spinel structure of the cubic system. In this the magnetic properties originate in the vacancies in the octahedral sites. It has saturation magnetization of 71 CGS units; resonance of 900 gauss, and coercivity of 200 oesteds. It has a particle size of about 0.5 - 1.0 micron and is spherical in shape (Singh et al., 1975).

Controlled-pore ceramics:

Controlled-pore ceramics were gifts from Corning Glass Works, Corning, USA.

Al_2O_3 - Corning lot 2011 A; pore diameter - 225 Å,
Surface area - 90 m^2/g; 30/45 mesh

Al_2O_3 - Corning lot - 4711 A; pore diameter - 1000 Å; Surface area - 13 m^2/g; 30/45 mesh.

SiO_2; pore diameter - 425 Å; Surface area - 40 m^2/g; 45/80 mesh.

EXPERIMENTAL PROCEDURES

Soluble glucoamylase:

Glucoamylase prepared from *Aspergillus candidus* strain A-32, was obtained from Hindustan Antibiotics Ltd. Poona. The enzyme was a partially purified culture filtrate.

Growth of organism:

The media for growing *A. candidus* A-32 both for seed and fermentation are given below:

The composition of the seed medium was as follows:

Corn steep liquor	10 g
Glycerol	10 g
KH_2PO_4	2.5 g
K_2HPO_4	2.0 g
NH_4NO_3	2.5 g
$MgSO_4.7H_2O$	1.0 g

Water to make to 1L; pH 6.0 - 6.2;

28°C, with aeration 1 vol. per vol. media per min.

Incubation period for seed was 40 - 48 h.

The fermentation medium composition was:

Tapioca starch	25 g
Peanut meal	20 g
NH_4NO_3	5.0 g
KH_2PO_4	2.5 g
K_2HPO_4	2.0 g
$MgSO_4.7H_2O$	1.0 g

Water to make to 1L; pH 6.0 - 6.2;

28°C with aeration, 1 vol. per vol. per min.

The incubation period for fermentation was 96 h.

Crude enzyme preparation:

After fermentation the broth was filtered and adjusted to pH 6.2 - 6.5 and solid ammonium sulfate was added with stirring to a final concentration at 0.5 saturation. The precipitate which contained the glucoamylase activity was filtered off and dissolved in the minimum quantity of chilled distilled water and then dialysed against distilled water at 0 - 4°C.

The crude enzyme preparations had a pH of 7 - 7.5 and contained approximately 110 - 140 U/ml and 2 - 3 mg protein/ml.

Protein determination:

Protein was determined by the method of Lowry et al. (1951). Crystalline bovine serum albumin was used as standard and the colour was read at 500 nm on a Beckman DU or Model 26 spectrophotometer.

Enzyme assay:

Enzyme activities were determined using soluble starch as substrate and estimating glucose released. The assay was carried out at 60°C using 10 ml of test system containing 100 mg of soluble starch in 100 mM sodium acetate buffer, pH 4.5. Approximately 1 U of enzyme activity was taken for assay. Activity was assayed by determination of the glucose produced by hydrolysis of the

soluble starch. Glucose determination were routinely carried out by determination of reducing sugars by the 3',5'-dinitrosalicylic acid (DNSA) method (Bernfeld, 1948). Aliquots were withdrawn periodically, diluted to 1 ml and treated immediately with 1 ml stopping reagent containing DNSA. Parallel substrate blanks were routinely run. Values were occasionally checked using the glucose oxidase system (Bergmeyer, 1974).

1 U of enzyme is defined as the amount of enzyme required, for producing 1 µmol glucose per min under assay conditions. Specific activity of enzyme is expressed as units/mg protein.

In determination of the extent of hydrolysis of starch, allowance was made for the molecule of water added to each glucose residue.

Glucose determination by DNSA method:

Stopping reagent containing DNSA was prepared as described by Fischer and Stein (1961). 3'5'-Dinitrosalicylic acid (5 g) was dissolved in water (100 ml) and treated with an aqueous solution of sodium hydroxide (8 g/75 ml water) by dropwise addition. Sodium potassium tartrate (150 g) was added and the volume was made to 500 ml with water and the solution filtered through sintered glass to remove a slight turbidity. The reagent was stored in the dark in a flask fitted with a soda lime guard tube. Aliquots from the assay test system were treated as described earlier with 1 ml of DNSA reagent to

stop further reaction. Test tubes containing this mixturec were stoppered loosely and heated for 5 min in a boiling water bath and then cooled under running tap water. 10 ml of water were then added to the solution and the brown reduction product determined spectrophotometrically at 540 nm in cuvettes of 10 mm light path. A substrate blank was prepared under identical conditions as the assay system but without added enzyme and aliquots were taken at appropriate intervals for DNSA assay and the absorbance values were deducted from the corresponding values of the experimental.

A standard curve was obtained with glucose (0.2 to 2 mg in 1 ml water) was used for converting absorbance reading into µmol glucose (Fig. 1). CP. 39-40)

Glucose estimation with glucose oxidase and peroxidase:

Determination of glucose by DNSA method was cross-checked occasionally using the enzymic assay with glucose oxidase, peroxidase and o-dianisidine.

The reagents were made as follows:

1. Glucose oxidase, 2 mg/ml in potassium phosphate buffer 0.05 M, pH 6.
2. Peroxidase, 0.05 mg/ml in 0.05 M potassium phosphate buffer, pH 6.
3. o-Dianisidine solution was prepared by dissolving 6.6 mg base in 1 ml 0.2 M HCl, diluting to 50 ml with water and then treating with 50 ml 0.05 M potassium phosphate buffer, pH 6.

The assay was carried out by a slight modification of the method described by Wood (1969). 1 ml samples (containing 0.01 - 0.1 mg glucose) were mixed with 1.4 ml o-dianisidine, 0.1 ml peroxidase and 0.5 ml glucose oxidase solutions. The mixture was incubated at 25°C for 2 h. The reaction was stopped with addition of 4 ml of 33% v/v H_2SO_4. Samples and standard were read against blank at 530 nm using the Beckman Model 26 spectrophotometer and cuvettes of 10 mm light path. The standard curve with glucose is represented in Fig. 2. (P. 39-40)

Assay of immobilized enzyme:

Immobilized enzyme preparations were assayed using aliquots of uniform suspensions added to the test system similar to that used for assay of soluble enzyme and stirred rapidly either magnetically with a teflon coated stirring bar or with a top driven stirrer. Jacketed stirred tank reactors were used and volumes scaled up to 50 to 100 ml, without changing concentration of substrate and buffer. Samples were withdrawn periodically and either filtered rapidly with suction or clarified by leaving momentarily under a strong field of a permanant magnet in case of the magnetic support. Aliquots of the clear filtrate/or supernate were treated immediately with the DNSA stopping reagent and assayed as described for the soluble enzyme preparation.

TiO_2-coating of supports:

Supports were coated with TiO_2 by treatment with $TiCl_4$

under hydrolysing conditions essentially as described by Hasselberger *et al*. (1974). The support (50 *g*) was suspended in water (150 ml) and cooled to 5 - 6°C in an ice bath. $TiCl_4$ (25 ml) was added dropwise over a period of 5 to 10 min. The mixture was stirred in the cold for 30 min after the addition of $TiCl_4$ was completed and then stirred for another 30 min at room temperature. Excess solution was decanted off and the treated support was washed with water and then air dried. Part of the support so prepared was further activated by heating to 530°C as described by Hasselberger *et al*. (1974).

Treatment of support with cyanuric chloride:

The treatment of support with cyanuric chloride was carried out under conditions where one of the three chlorines is hydrolysed to give the monohydroxydichloro-*s*-triazine which is insoluble in both acetone and the aqueous phase. Excess trichloro-*s*-triazine was then washed off with acetone to yield support coated with the monohydroxydichloro-*s*-triazine.

Support (5 *g*) was treated with cyanuric chloride (1 *g*) dissolved in acetone (10 ml) in an ice bath at 4-5°C, along with 35 ml of 0.1 M sodium bicarbonate solution and the mixture was kept stirred for 30 min in the cold. The pH of the reaction mixture was approximately 7.5. The treated support was then washed with acetone followed by water and dried before treating with enzyme.

Treatment of cyanuric chloride-treated support with starch:

The dichloro-triazine coated support was treated with soluble starch at 60°C to cross-link starch on support surface. Support (5 g) after the treatment with cyanuric chloride as described above was added as a wet cake to a solution of 1% w/v soluble starch in 50 ml 0.1 M acetate buffer, pH 4.5 and heated for 1 h at 60°C. The support was then washed thoroughly with water.

Treatment of coated-support with enzyme:

Support (5 g) after treatment with cyanuric chloride and starch as described above was added as a wet filtered cake to 100 ml of the glucoamylase solution with agitation and stirring continued in the cold at 0-4°C for 16 h.

Adsorption of enzyme on untreated support:

Support (1 g) was stirred with the crude glucoamylase solution (15-25 ml) for 16 h at 4-5°C. The support was then filtered off and washed with water; filtrate and washings were tested for residual activity.

Covalent attachment of enzyme to controlled-pore ceramics:

The enzyme was attached to the inorganic support surface after silanization using γ-aminopropyltriethoxysilane and then coupling via the bifunctional reagent glutaraldehyde according to the procedure described by Weetall (1976).

Pretreatment of carrier:

The carrier (3 g) was initially cleaned by boiling under reflux with 5% w/v nitric acid (600 ml) for 45 min and was then washed and dried for 24 h at 120°C.

Preparation of alkylamine carrier:

The dry cleaned carrier (1 g) was added to 10% w/v γ-aminopropyltriethoxysilane dissolved in toluene (75 ml). The mixture was refluxed for 16 h on a heating mantle regulated with thermostatic control. After cooling, the carrier was filtered off and washed thoroughly first with toluene and then with acetone. The alkylamine carrier so obtained was air dried.

Preparation of active aldehyde derivative of alkylamine carrier:

The alkylamine carrier (1 g) was taken in a 25 ml capacity beaker and was treated with 2.5% w/v glutaraldehyde solution (10 ml) so as to cover the solid support. The mixture was agitated with a teflon-coated magnetic stirring bar for 60 min at room temperature. The product was then filtered on a Buchner funnel and washed with distilled water to remove excess glutaraldehyde.

Coupling of enzyme to aldehyde carrier:

The enzyme was previously dialysed for 16 h against 0.1 M phosphate buffer, pH 7.5. The aldehyde derivative of the carrier (1 g) was stirred with the dialysed enzyme solution (20 ml) at 4°C. The stirring was continued in the cold for 2 h. The strength of the buffer was adequate to maintain the initial pH value. The carrier treated with enzyme was then filtered on a Buchner funnel and washed with the same buffer as used in the coupling reaction till washings were free of protein.

RESULTS

Properties of the soluble enzyme:

The enzyme from *Aspergillus candidus* competely hydrolyses soluble starch as reported for the glucose-producing amylases of some other molds (Fleming, 1968).

The hydrolysis of soluble starch by the crude enzyme preparation followed zero order kinetics under the conditions of assay up to atleast 10% conversion of substrate during the initial h of reaction (Fig. 3).

Glucose formation in the initial 60 min of reaction under assay conditions was also proportional to the enzyme concentration in the range 0.1 to 1.0 U in 10 ml test system.

The plot of enzyme concentration *versus* glucose produced is shown in Fig. 4.

Substrate level in the assay system was also optimal and identical values were obtained with initial soluble starch concentration of 2%.

The progress curves for the hydrolysis of soluble starch with two different levels of enzyme concentration 11 U and 33 U respectively are shown in Fig. 5. With the lower level of enzyme, approximately 50% substrate hydrolysis occurred during the first h of hydrolysis. With the higher activity of enzyme, complete hydrolysis of substrate was obtained in 6 h.

The extent of hydrolysis was 100% by the DNSA method and approximately 92% by enzymic glucose oxidase assay

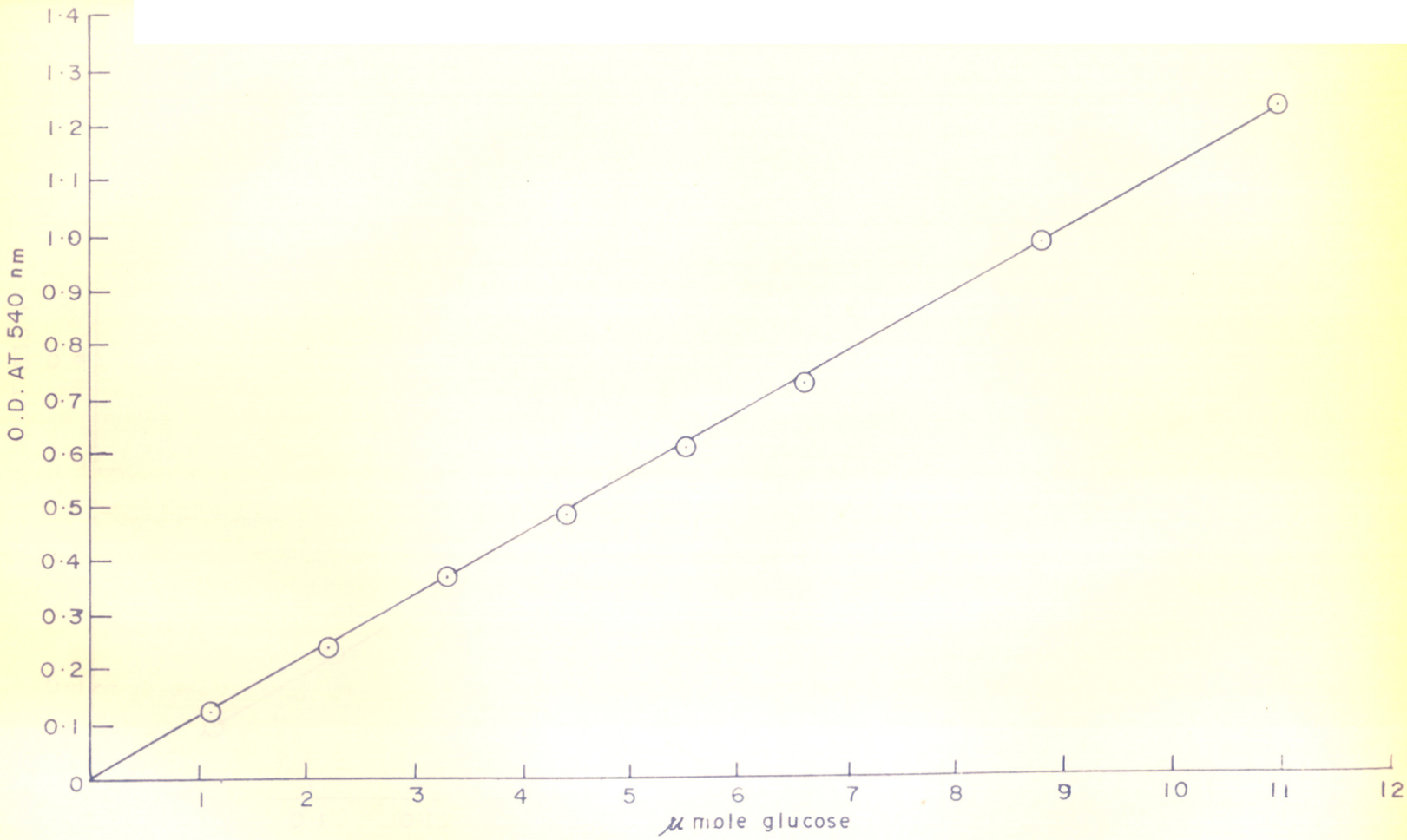

FIG. 1. STANDARD CURVE OF GLUCOSE BY DNSA ASSAY
Conditions are described in text

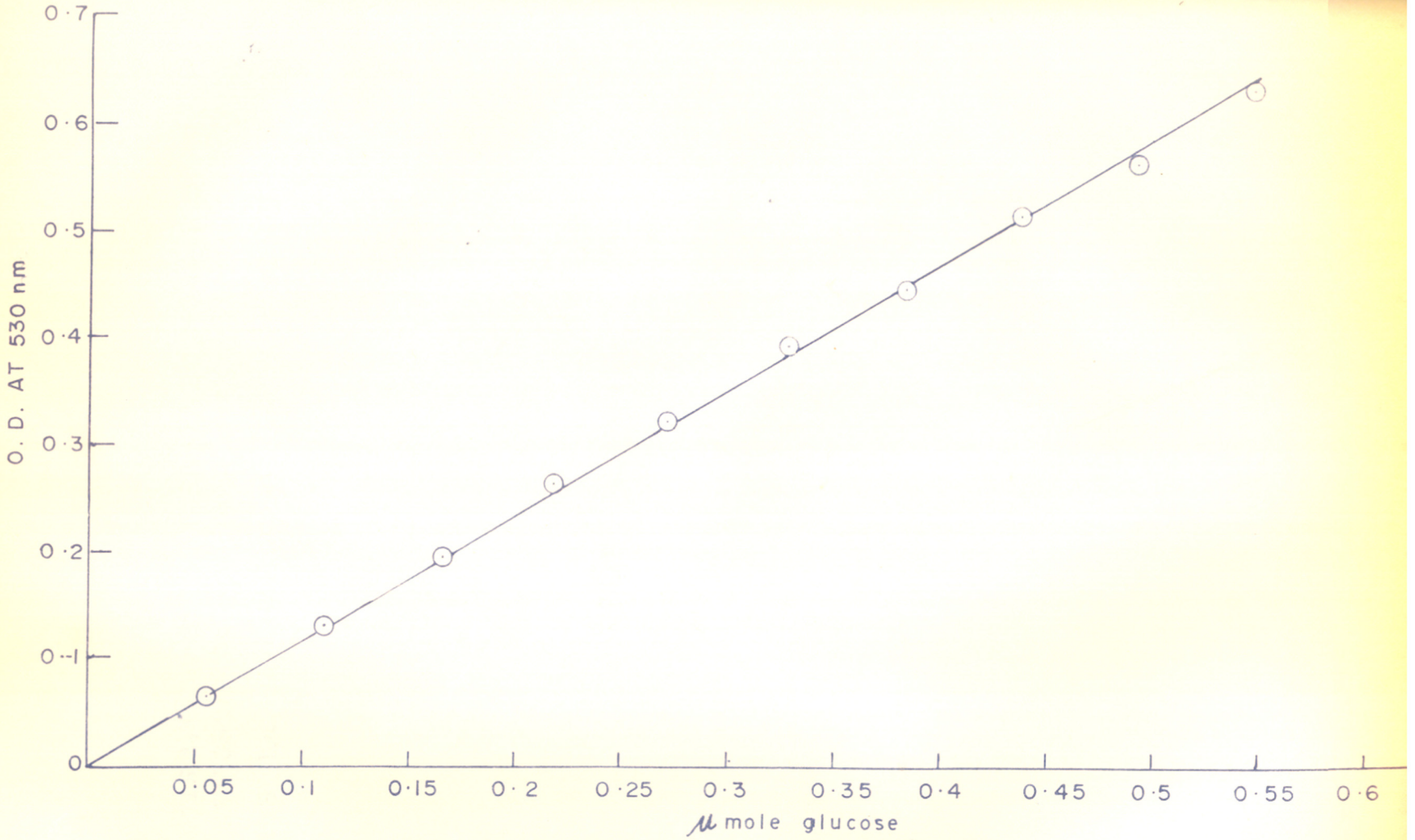

FIG. 2. STANDARD CURVE OF D-GLUCOSE BY ENZYME ASSAY
Conditions are described in text

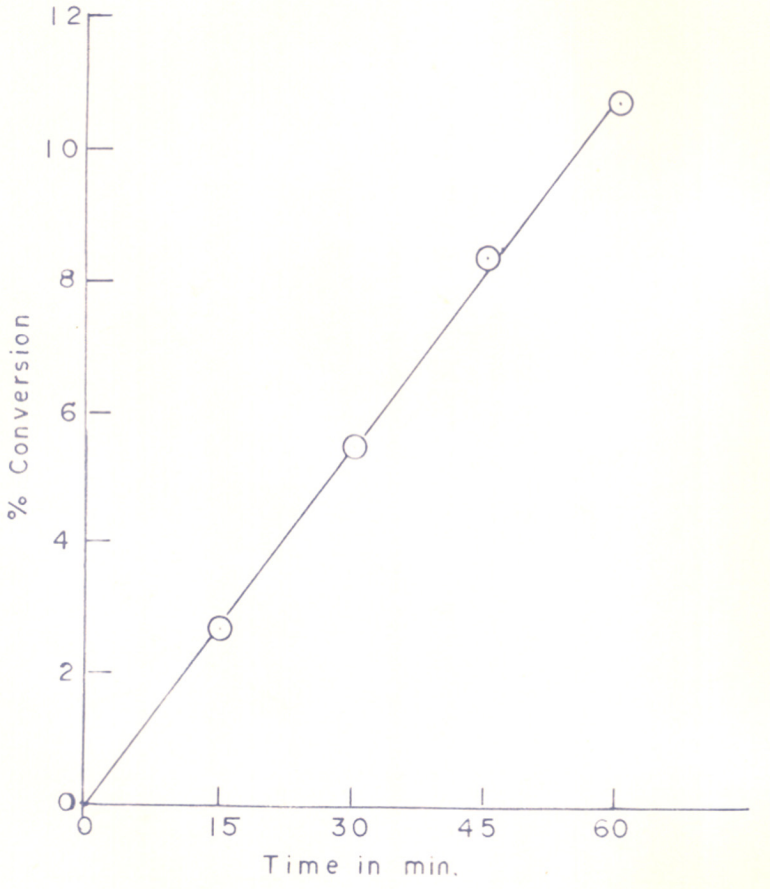

FIG. 3. PLOT OF TIME VERSUS ACTIVITY OF SOLUBLE GLUCOAMYLASE DURING INITIAL HYDROLYSIS OF STARCH. ENZYME, 1·1 U; 10 ml. 1% STARCH; pH 4·5; 60°C

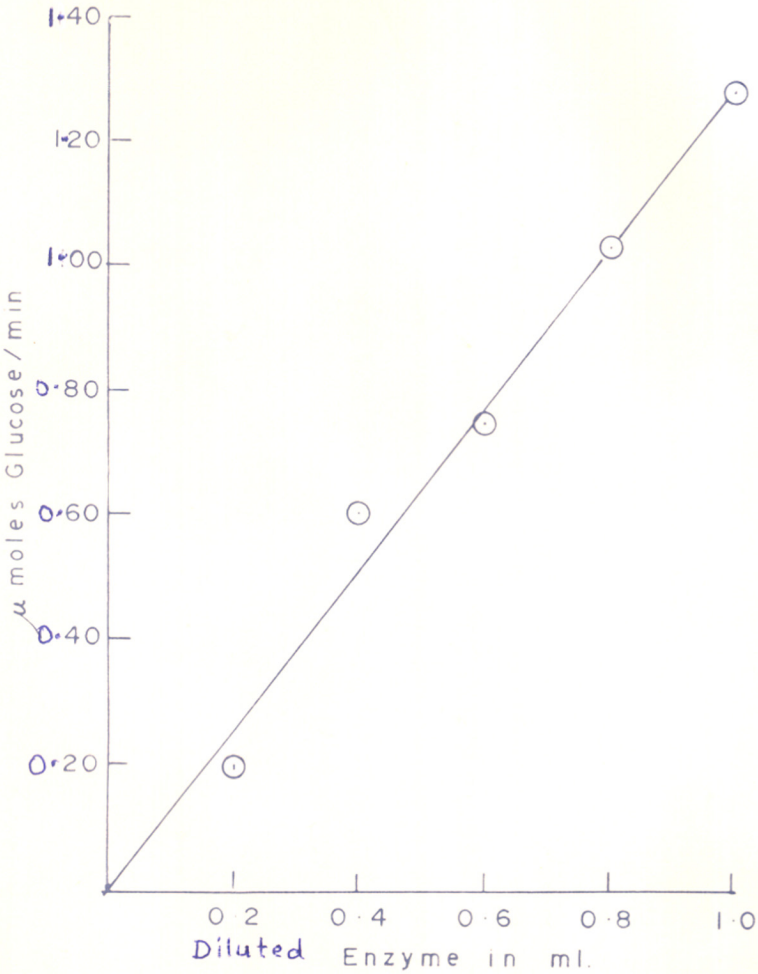

FIG. 4 RELATION BETWEEN AMOUNT OF SOLUBLE ENZYME TAKEN & INITIAL RATES OF GLUCOSE RELEASE. 1% SOLUBLE STARCH IN 0·1 M SODIUM ACETATE BUFFER, pH 4·5; 60°C, ENZYME SOLUTION 130 U/ml PROTEIN 2 mg/ml. DILUTED 1:100

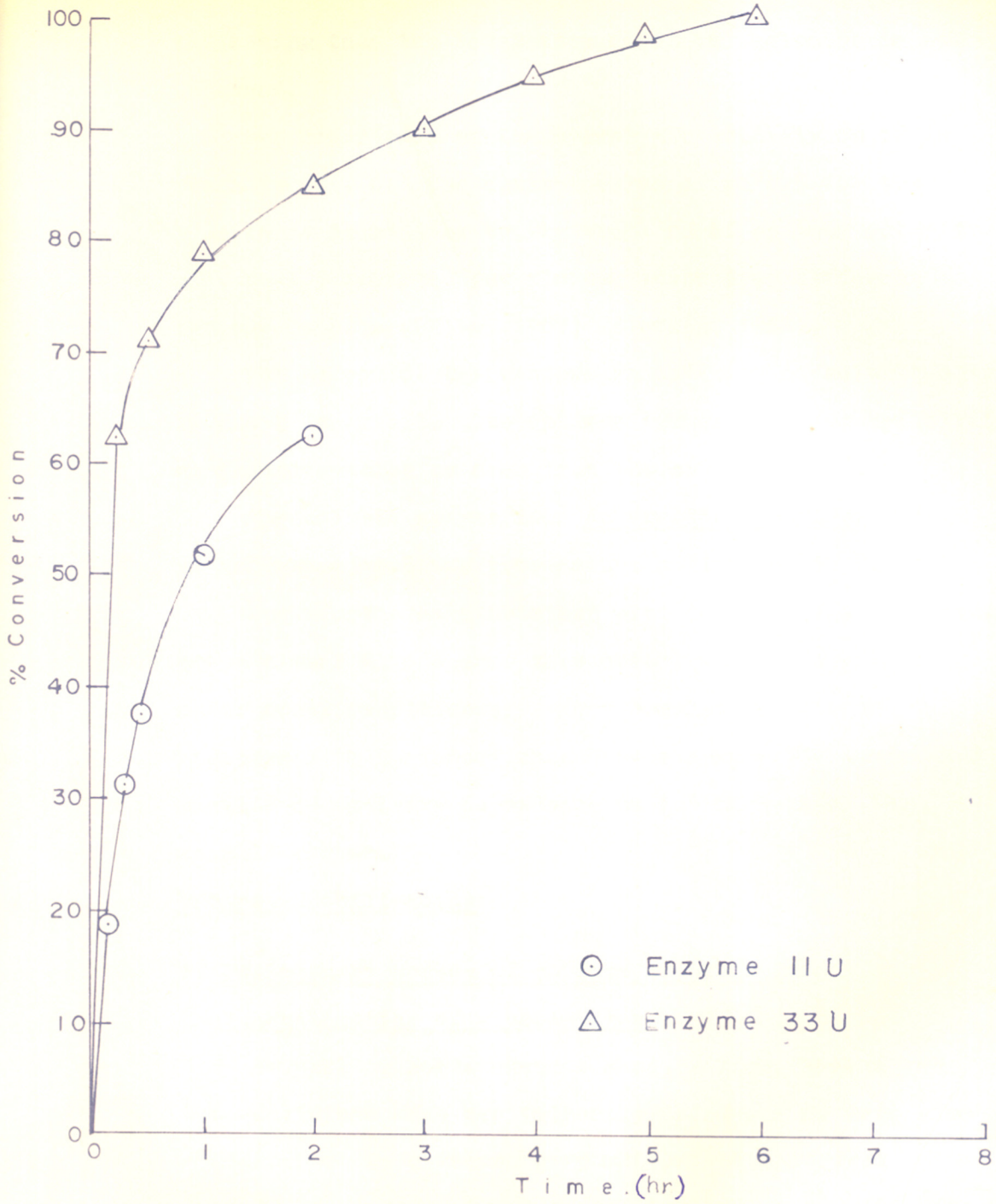

FIG. 5. PROGRESS CURVE OF HYDROLYSIS OF STARCH BY SOLUBLE GLUCOAMYLASE. 1% STARCH IN 0·1 ACETATE, PH 4·5; 60°C

indicating that the total conversion to D-glucose is not obtained.

The dependence of the hydrolytic activity on pH is shown in Fig. 6. The optimum value of pH 4.5 for the hydrolysis is similar to the usual pH of optimum activity of 4 to 4.5 reported for glucoamylases from different sources by others (King, 1967; Fleming, 1968).

The effect of temperature on activity of soluble enzyme is shown in Fig. 7. The optimum temperature for hydrolysis of soluble starch as seen from the curve is 60°C.

The soluble enzyme from Aspergillus candidus has a pH optimum of 4.5 and temperature optimum of 60°C.

The kinetic data obtained with the immobilized enzyme preparations also showed zero order reaction kinetics under assay conditions; almost complete substrate hydrolysis to D-glucose when excess enzyme was used; and similar pH activity dependence as in the case of the soluble enzyme.

Enzyme immobilization:

i) Preliminary survey of various supports for immobilization of glucoamylase:

Several supports such as sand, silica, carbon and DEAE-cellulose were preliminarily tried out to determine whether the crude enzyme preparation obtained from A.candidus could be bound to the carrier, used either as such or after preliminary treatment. Chromatography grade

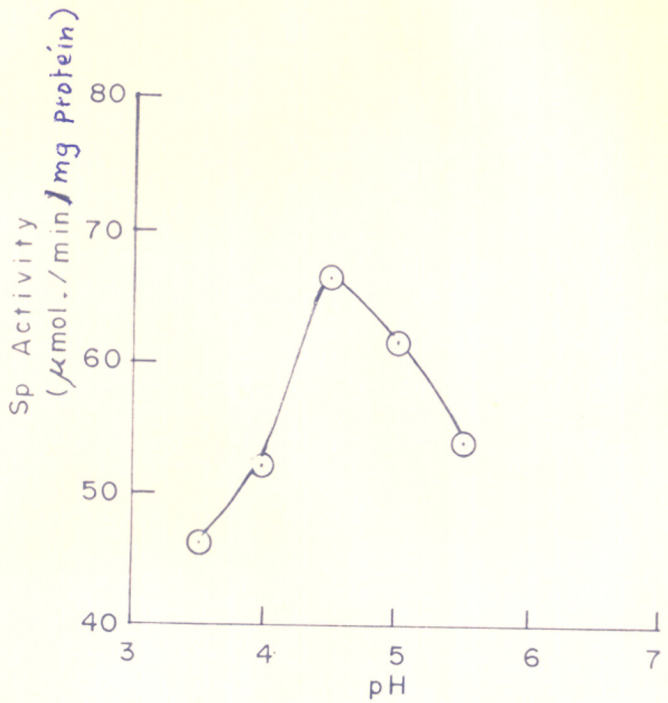

FIG. 6 EFFECT OF pH ON ACTIVITY OF NATIVE GLUCO-
-AMYLASE. 1% SOLUBLE STARCH IN 0·1M ACETATE
BUFFER OF VARIOUS pH VALUES; 60°C

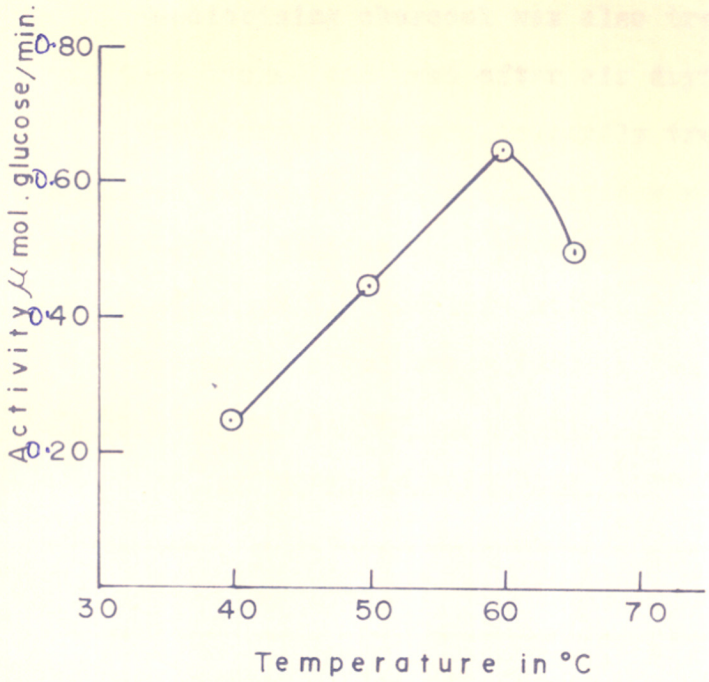

FIG. 7. EFFECT OF TEMPERATURE ON ACTIVITY OF SOLUBLE GLUCOAMYLASE, ENZYME 0·65 U 1% STARCH IN 0·1M ACETATE BUFFER 4·5.

silica gel was coated with TiO_2 by treatment with $TiCl_4$ under hydrolysing conditions as described under Experimental procedures. The support was washed and used after drying in air or after heating for an hour at 530°C followed by cooling.

Decolorizing charcoal was also treated with $TiCl_4$ and then washed and used after air drying.

Nylon powder was preliminarily treated with cyanuric chloride in acetone at pH 7.5 then washed and treated with enzyme with adjustment of pH using 10% Na_2CO_3 solution to maintain pH 7.5 and left stirring overnight at 4-5°C.

The results are summarised in Table 1.

Table 1: Preliminary survey of effectiveness of diverse supports in adsorbing glucoamylase from solution

Support	% activity bound*
1. TiO_2-coated silica, air dried	8
2. TiO_2-coated silica, heated at 530°C	8
3. TiO_2-coated carbon	0
4. Cyanuric chloride treated Nylon Powder	0
5. DEAE-cellulose, pH 7.5	0

*Calculated from the difference between activity taken and activity recovered in filtrate and washings.

It will be evident from Table 1 that none of the supports bound enzyme to any appreciable extent. Although DEAE-cellulose has been used by others for binding glucoamylase activity, with the crude enzyme preparation from A. candidus activity remained totally in the supernatant solution.

Adsorption of glucoamylase on γ-Fe_2O_3:

Data on retention of activity and on reuse is shown in Table 2.

Table 2: Adsorption of glucoamylase activity by γ-Fe_2O_3. Assayed at 60°C, pH 4.5.

Support	Activity bound U/g (a)	% Binding	No. of use	U/g (b)
Untreated γ-Fe_2O_3	1233	70	1st	675
			2nd	67.5

a - Calculated from the difference between activity taken and activity recovered in filtrate and washings.

b - Value obtained on assay.

γ-Fe_2O_3 effectively picks up activity from solution. Approximately 1200 U are picked up per g support as calculated from the difference between the activity in soluble enzyme before treatment with support and the activity recovered in supernate and washings.

When enzyme was taken in limiting amounts, adsorption was virtually quantitative.

The adsorbed activity when assayed gave a value of approximately 54% of that picked up from solution. A single reuse however resulted in leakage of 90% of the activity of the immobilized enzyme.

Adsorption of glucoamylase on $TiCl_4$ treated γ-Fe_2O_3:

In the preliminary studies with γ-Fe_2O_3 treatment of the support with $TiCl_4$ (1.25 ml) in CCl_4 (10 ml) was attempted to determine whether the metal-link method could be applied for attaching enzyme to magnetic support.

1 g support was treated with 10 ml CCl_4 containing 1.25 ml $TiCl_4$ with stirring for 20 min at 0°C and then washed with cold 0.1 M acetate buffer, pH 4.5, followed by treatment with 50 ml of enzyme solution. Support was stirred for 16 h with enzyme at 4°C; then filtered and washed with buffer. No activity was bound to the support under these conditions.

An attempt to cross-link enzyme with epichlorohydrin in presence of magnetic supports was also attempted.

Two separate lots of 1 g each of γ-Fe_2O_3 were separately treated with 50 ml crude enzyme solution followed by 1 ml epichlorohydrin added to the enzyme adjusted to either pH 4.5 or 7.5. The samples were stirred overnight filtered and tested for activity on testing the carrier treated at pH 4.5 and 7.5 activities bound were 9 U/g and 5 U/g, respectively.

γ-Fe_2O_3 treated with $TiCl_4$ under hydrolysing conditions as described earlier was tested for enzyme adsorption under a variety of conditions. Treatment with cyanuric chloride in acetone was carried out as described in detail under Experimental procedures. Treatment with thionyl chloride was by addition of 0.5 g of the reagent under stirring to 1 g support suspended in 10 ml acetone, removal of support after 1 h by filtration, followed by washing with acetone and then addition of 20 ml enzyme solution at 0-4 °C. Treatment with cyanuric chloride/dioxane was carried out with 1 g support. The support was first washed with dioxane and then treated with 0.5 g cyanuric chloride in 20 ml dioxane. After stirring for 2 h at room temperature, the support was washed first with dioxane and then with acetone and dried in vacuo. The dry support was stirred with 25 ml enzyme solution at 0-4 °C.

The results are summarized in Table 3.

It will be evident from Table 3 that the highest binding of activity is obtained with TiO_2-coated γ-Fe_2O_3 pretreated with cyanuric chloride in acetone. Experiments were therefore carried out with the objective of enhancing levels of activity that could be bound to the support.

Table 3: Data on adsorption of glucoamylase by γ-Fe_2O_3 pretreated as described, assayed at 60°C, pH 4.5

Support and conditions of pretreatment	% Activity bound	U/g support
1. TiO_2-coated γ-Fe_2O_3	0	0
2. TiO_2-coated γ-Fe_2O_3 treated with cyanuric chloride in acetone at pH 7.5 and treated with enzyme at pH 7.5	17.8	46.8
3. TiO_2-coated γ-Fe_2O_3 treated with cyanuric chloride in acetone at pH 7.5 and treated with enzyme at pH 4.2	0	0
4. TiO_2-coated γ-Fe_2O_3 treated with thionyl chloride	0	0
5. TiO_2-coated γ-Fe_2O_3 treated with enzyme + 2% glutaraldehyde	N.D.*	27.7
6. TiO_2-coated γ-Fe_2O_3 treated with cyanuric chloride in dioxane	0	0

*N.D. - not determined

Adsorption of enzyme to TiO_2-coated γ-Fe_2O_3 pretreated with cyanuric chloride:

It was observed that TiO_2-coated γ-Fe_2O_3 pretreated with cyanuric chloride in acetone followed by treatment with enzyme at pH 7.5, after its first use for the hydrolysis of starch, picked up much higher levels of activity when put back with fresh enzyme than in the first treatment.

The results obtained with γ-Fe_2O_3 and TiO_2-coated γ-Fe_2O_3 supports so treated are shown in Table 4.

Table 4: Enzyme adsorption on γ-Fe_2O_3 treated first with cyanuric chloride followed by heating with 1% starch at 60°C.

Support	% Activity bound
1. TiO_2-coated γ-Fe_2O_3 treated with cyanuric chloride at 0°C and at pH 7.5	26.5
2. TiO_2-coated γ-Fe_2O_3 treated with cyanuric chloride at 0°C and at pH 7.5 followed by heating at 60°C with 1% starch at pH 4.5	87.5
3. γ-Fe_2O_3 treated with cyanuric chloride followed by heating at 60°C with 1% starch at pH 4.5	89.5

As seen from Table 4 the cyanuric chloride treated support after heating with 1% starch at 60°C and pH 4.5

effectively adsorbs glucoamylase activity. With cyanuric chloride treatment it was not necessary to coat the support with TiO_2. The behaviour is in marked contrast to adsorption of glucoamylase activity by support which had not been treated with cyanuric chloride.

It was of interest to determine whether the cyanuric chloride treated support bound starch when heated at 60°C for 1 h.

4 g γ-Fe_2O_3 was treated with 0.8 g cyanuric chloride in 7.5 ml acetone, in presence of 28 ml 0.1 N sodium bicarbonate for 1/2 an hour in ice bath. The support was washed with acetone and air dried. The dried powder was divided into two equal lots one of the lots was heated with 50 ml of 1% starch with stirring at 60°C and the other lot with 50 ml 1 % glucose solution under similar conditions. Both lots of support were separately filtered off and washed with water and the filtrates and washings from each lot pooled and separately made up to 100 ml. Control without supports were run under identical conditions. Aliquots of the glucose solutions were taken directly for assay by the dinitrosalicylic acid method. Aliquots of the starch solutions were preliminarily treated with soluble glucoamylase till complete hydrolysis and then assayed for glucose released. Amount of starch bound to support was calculated from the difference in readings obtained with control and sample treated with support, allowance being made for the molecule of water

added to the glucose residue on hydrolysis.

Results are summarised in Table 5.

Table 5: Binding of starch to cyanuric chloride treated γ-Fe_2O_3

Support	Starch (Glucose) Bound (mg/g support)
1. γ-Fe_2O_3 treated with cyanuric chloride and heated with starch	67
2. γ-Fe_2O_3 treated with cyanuric chloride and heated with glucose	0

As seen from Table 5, heating of the support after cyanuric chloride treatment in presence of starch apparently results in the covalent attachment of starch to the support. Reaction between starch and cyanuric chloride is catalysed at alkaline pH value, however in the present experiment probably reaction takes place even at the unfavoured pH of 4.5. Heating of treated support at alkaline pH was found to abolish subsequent enzyme binding, probably through removal of monohydroxy-dichlorotriazine from support surface as the soluble sodium salt.

Reuse of enzyme adsorbed on γ-Fe_2O_3 treated with cyanuric chloride and starch:

The operational stability data on glucoamylase

adsorbed on γ-Fe_2O_3 which had been pretreated with cyanuric chloride and starch, was determined in repeated cycles of use at 60°C and substrate concentration of 1% soluble starch. Activity retention was determined as described earlier with aliquots of the immobilized enzyme preparations after every cycle of use in which the immobilized enzyme was taken in excess for complete hydrolysis of substrate, usually 100 ml of 1% soluble starch for lots of 2 g support.

Table 6 summarises the data from a typical experiment. Reproducible values were obtained repeatedly. Initial binding of activity varied between 80-90% calculated on basis of units taken and units recovered in filtrate and washing. The actual activity obtained on assay was about 25% of that bound. This is evidently due to diffusional restrictions, steric hindrances and other factors when immobilized enzymes are used, particularly with macromolecular substrates. The adsorbed enzyme apparently leaks out gradually in use and lower activities are observed with repeated cycles of use. An interesting feature is the ability of the support to pick up fresh enzyme after the initial activity has dropped, on treatment with a fresh charge of soluble enzyme. The fact that starch is covalently bound to the cyanuric chloride treated support might indicate that the glucoamylase is taken up atleast partly by adsorption

through bio-specific affinity. This was borne out by the data obtained with the starch-treated support treated with crude glucoamylase preparation in excess. Determinations of both activity and protein in the soluble enzyme used in the binding experiment and that recovered in filtrate and washings after addition of support, indicated that while about 60% of the activity was bound, only about 15% of the protein was taken up.

Table 6: Reuse and recycle data on enzyme adsorbed on γ-Fe_2O_3 treated with cyanuric chloride and starch. γ-Fe_2O_3 (5 g) treated first with cyanuric chloride and then heated with starch at 60°C, pH 4.5. Used till 20% level of initial activity and charged with fresh enzyme. Hydrolysis at pH 4.5; 60°C.

Treatment with enzyme	Units activity taken	Units Bound (a)	% binding	No. of use	U/g support (b)
1st	11700	9866	84	1	450
				2	378
				3	315
				4	270
				5	234
				6	90
2nd	11700	6427	55	1	450
				2	297
				3	207
				4	81

a - Calculated from the difference between activity taken and activity recovered in filtrate and washings.

b - Value obtained on assay.

Adsorption of glucoamylase on catalyst grade Al_2O_3:

Data on retention of activity and leakage on reuse is shown in the Table 7.

Table 7: Adsorption of glucoamylase on catalyst grade Al_2O_3. Assayed at 60°C, pH 4.5.

Support	Activity bound (U/g) (a)	% binding	No. of Use	Determined activity (U/g support) (b)
Untreated Al_2O_3	315	19.5	1st	150
			2nd	7.5

a - Calculated from the difference between activity taken and activity recovered in filtrate and washings.

b - Value obtained on assay.

The support picks up approximately 315 U/g from solution which in use has an effective equivalent of 47% of the activity taken up from solution. A single reuse results in leakage of 95% of activity of the immobilized enzyme.

To achieve higher adsorbed activity and greater retention in cycles of use, diverse conditions of treatment of the support were tried out both with TiO_2-coated Al_2O_3 and the support untreated with $TiCl_4$ under hydrolysing conditions. The results obtained with the TiO_2-coated alumina ~~and those on uncoated supports~~ are summarised in Table 8.

Table 8. Adsorption of glucoamylase on catalyst grade Al_2O_3 pretreated under varying conditions. Assayed at 60°C, pH 4.5.

Support	Units bound/g support
TiO_2-coated alumina, air dried	250
TiO_2-coated alumina, heated 1h at 530°C.	250

Coating of the support with TiO_2 reduces its capacity to bind enzyme, untreated Al_2O_3 binds, 315 U/g while the TiO_2-coated material both air dried and activated by heating at 530°C, binds only 250 units probably through clogging of pores and consequent reduction in available surface area.

Adsorption of glucoamylase on Al_2O_3 treated with cyanuric chloride and starch:

Since experiments with γ-Fe_2O_3 indicated a marked increase in enzyme binding as well as enzyme retention on reuse and repeated recharge on leaving reaction-deactivated support with fresh enzyme, similar studies were carried out with Al_2O_3.

Table 9 summarises the effect of the starch treatment of Al_2O_3 on enzyme binding.

Table 9: Enzyme adsorption on catalyst grade Al_2O_3 treated with cyanuric chloride and heated with starch

Support	Units bound/g support (a)
Al_2O_3 treated with cyanuric chloride at pH 7.5 washed and treated with 1% starch at 60°C for 1 h, pH 4.5.	725

a - Calculated from the difference between activity taken and activity recovered in filtrate and washings.

As seen from Table 9, a marked enhancement of enzyme binding from 315 to 725 U/g is achieved as in the case of γ-Fe_2O_3 by treatment of the support first with cyanuric chloride at pH 7.5 and 0°C followed by heating for 1 h at 60°C with soluble starch at pH 4.5.

The data on binding of starch to support was obtained as described earlier for γ-Fe_2O_3 and this is summarised in Table 10.

Table 10: Binding of starch to cyanuric chloride treated Al_2O_3

Support	Starch bound (mg/g support)
Al_2O_3 treated with cyanuric chloride at pH 7.5 and heated with starch at 60°C for 1 h at pH 4.5	12

The binding of starch is 12 mg/g Al_2O_3. This value is considerably lower than the value 67 mg/g obtained in the case of γ-Fe_2O_3.

Reuse data on enzyme adsorbed on Al_2O_3 treated with cyanuric chloride and starch:

Reuse data on Al_2O_3 support were obtained as earlier for γ-Fe_2O_3, using 1% soluble starch and 60°C in repeated cycles of use. Activity retention was determined on aliquots of the immobilized enzyme preparation after every cycle of use at 60°C in which 2 g support was added to 100 ml 1% soluble starch, pH 4.5 and the reaction continued till complete hydrolysis.

The results are summarised in Table 11.

As seen from Table 11 the starch treated support retains markedly higher proportion of its initial activity on reuse. Such material which has been used is recharged on treatment with fresh lot of enzyme. The ability for carrier regeneration has obvious economic advantages, since the overall cost of enzyme immobilization is substantially reduced.

Table 11: Reuse and recycle data on enzyme adsorbed on Al_2O_3 treated with cyanuric chloride and starch

Treatment with enzyme	Activity bound U/g (a)	No. of use	U/g (b)
1st	725	1	405
		2	259
2nd		1	472
		2	180
3rd		1	675
		2	360
4th		1	720
		2	304
5th		1	686
		2	659
		3	281
		4	144
		5	144

a - Calculated from the difference between activity taken and activity recovered in filtrate and washings.

b - Value obtained on assay.

Covalent attachment to inorganic supports:

The crude enzyme was used for coupling to controlled pore ceramics for the sake of comparison of binding efficiency, loading and enzyme efficiency in hydrolysis of starch.

Supports were silanized with γ-aminopropyltriethoxy silane and coupled through glutarldehyde as described under experimental procedures.

The data is summarised in Table 12.

Table 12: Immobilization of glucoamylase on controlled pore ceramics after silanization with γ-aminopropyltriethoxy silane followed by treatment with glutarldehyde

Support	Average pore diameter of support (Å)	Activity bound (U/g of support)	Activity determined (U/g)
Al_2O_3	225	2100	413
Al_2O_3	1000	747	57
SiO_2	425	1200	-

It can be seen from Table 12 that the maximum activity is bound on covalent attachment to controlled-pore Al_2O_3, 225 Å pore diameter, ceramic. The determination of protein in the crude enzyme solution and in the filtrate and washings indicated that the % binding of activity and protein were similar indicating

nonspecific coupling of protein to support. The activity of the bound enzyme was approximately 20% of that of the native soluble enzyme. Weetall and Havewala (1972) have reported an efficiency of 25% in case of the enzyme immobilized on controlled-pore alumina.

On reuse of the bound enzyme, a loss of about 30% of the activity occurred in the first cycle after which no measurable drop could be observed in subsequent cycles. The initial loss probably is of enzyme adsorbed and not covalently attached to the support, Since in the present study the immobilized enzyme preparation had not been preliminarily washed either with high ionic strength buffer or of acidic and alkaline pH values. Markedly lower activity was bound to Al_2O_3 1000 Å pore diameter. The studies carried out by Weetall (1976) with porous glass and glucoamylase (presumably from A. niger) indicated ~~that~~ an optimum pore diameter of 300 Å for enzyme loading with marked drop in activity with lower pore size. Higher pore diameters were also shown to result in lowered binding of activity. Support of 1000 Å pore diameter bound approximately 10% of the activity of the 300 Å pore diameter.

With the enzyme from A. candidus used in the present study and Al_2O_3 as support, high binding was obtained with the 225 Å diameter material. The activity bound with

1000 Å pore diameter material was 14% of that of support of 225 Å pore diameter. Materials of intermediate pore diameters were not available for study of effect of this parameter.

The binding of activity to controlled-pore SiO_2 (425 Å) was less than that to Al_2O_3 of 225 Å pore size. The immobilized SiO_2 was not tested for activity since such supports uncoated with ZrO_2 are known to loose activity through leaching (Weetall and Havewala, 1972).

DISCUSSION

In the present study, the comparative merits of immobilizing glucoamylase from crude enzyme solutions on inorganic supports by adsorption processes and by covalent attachment have been studied.

Data on the glucoamylase activities bound to the inorganic supports after some of the diverse conditions of pretreatment tried out in the present investigations are summarized in Table 13. While relatively high activity can be picked up on untreated γ-Fe_2O_3, such activity is leached out to the extent of about 90% in a single use. Untreated catalyst grade Al_2O_3 picks up significantly lower amounts of activity. γ-Fe_2O_3-coated with TiO_2 adsorbs no activity while TiO_2-coated Al_2O_3 picks up some activity but less than the plain support.

Treatment of supports with cyanuric chloride in acetone followed by heating with 1% starch solution at pH 4.5 and at 60°C for 1 h results in the support picking up starch probably through intermolecular cross-linking. Initial treatment of support with cyanuric chloride at pH 7.5 and 0°C, could be expected to result in the hydrolytic cleavage of one of the three reactive -Cl of the reagent. Heating at 60°C in presence of starch would result in the reaction of the remaining Cl atom(s), either with substrate or with water. The estimation of starch indicates that some of the polysaccharide is

Table 13: Immobilization of glucoamylase on inorganic support

Support	Method	Support pretreatment	Activity bound U/g (a)	Activity determined U/g (b)	Approximate loss in single use %
γ-Fe_2O_3	Adsorption	Untreated	1233	675	90
	Adsorption	TiO_2-coated	0	0	0
	Adsorption	Cyanuric chloride-starch	2000	450	20
Catalyst grade Al_2O_3	Adsorption	Untreated	315	150	95
	Adsorption	TiO_2-coated	250	-	-
	Adsorption	Cyanuric chloride-starch	725	405	40
Controlled pore Al_2O_3, 225 Å	Covalent binding	γ-aminopropyltriethoxy silane-glutaraldehyde	2100	413	30 (in 1st use) negligible later
Controlled pore Al_2O_3 1000 Å	Covalent binding	γ-aminopropyltriethoxy silane-glutaraldehyde	747	57.0	-
Controlled pore SiO_2 425 Å	Covalent binding	γ-aminopropyltriethoxy silane-glutaraldehyde	1200	-	-

a - Calculated from the difference between activity taken and activity recovered in filtrate and washings.
b - Value obtained on assay.

picked up on the support. The starch (or its degradation product) attached to the support results in binding with enzyme by a bio-specific interaction. This is indicated in the preferential removal from solution of glucoamylase activity from the crude soluble enzyme preparation. The -Cl atom(s) removed by hydrolysis with water would result in the formation of insoluble cyanuric acid or its derivatives which would confer a negative charge to the support. The starch-coated support could thus also bind proteins non specifically through its ion exchange behaviour. The starch-coated support treated with crude enzyme picks up approximately 2000 U/g support in case of γ-Fe_2O_3 and 725 U/g support in case of catalyst grade Al_2O_3. The efficiency of the bound enzyme has been shown to be 23% in case of γ-Fe_2O_3 and 56% in case of Al_2O_3. The supports retain approximately 40 to 90% of its activity in each reuse in the first few cycles of use. An added advantage is the ability of support from which more than 80% activity has been leached out in repeated use to be almost completely regenerated on treatment with fresh enzyme. The Al_2O_3 support recharged even after the fifth cycle of enzyme treatment retained or improved in its original capacity to adsorb enzyme from the crude soluble enzyme preparation.

Comparison with the enzyme covalently attached from crude preparations to silanized controlled-pore ceramics showed that preparations of comparable activity results

from the adsorption process using the starch treated supports. The use of immobilized glucoamylase preparations industrially has not come about yet on account of the low cost of the enzyme. However, the use of cheap inorganic supports such as Al_2O_3 and spinel γ-Fe_2O_3 (approximate cost Rs. 10/- Kg.) as adjunct to the soluble enzyme might reduce further the requirements of soluble enzyme in batch operations. An advantage with γ-Fe_2O_3 is its magnetic property and the possibility of its use in fluidized bed reactors with easy removal even from viscous media through its magnetic property. To our knowledge spinels like γ-Fe_2O_3 have not been used hitherto as carrier for enzyme immobilization. Nor has a process based on biospecific affinity adsorption been applied in the case of glucoamylase.

SUMMARY

Immobilization of glucoamylase activity from a crude enzyme preparation isolated from *A. candidus* A-32 on the inorganic supports: magnetic γ-Fe_2O_3, catalyst grade Al_2O_3 and controlled-pore ceramics has been investigated.

Immobilization on γ-Fe_2O_3 and catalyst grade Al_2O_3 was obtained by adsorption under a variety of conditions. The former support adsorbs about 1200 U/g support even without pretreatment. Coating of the support with TiO_2 abolishes adsorption. Activity adsorbed on the untreated support is almost completely leached out in a single reuse. Untreated as well as TiO_2-coated Al_2O_3 adsorbs low levels of about 250 to 300 U/g support. Marked enhancement of adsorption was achieved in case of both supports by the novel procedure of treating the support preliminarily under mild hydrolysing conditions with cyanuric chloride in aqueous acetone followed by heating of the treated supports with soluble starch at pH 4.5. Under these conditions the activities picked up by γ-Fe_2O_3 and Al_2O_3 are approximately 2000 and 725 U/g, respectively. The adsorbed activity is relatively stable under conditions of use for the hydrolysis of starch and the leakage is only 10 to 60% in every cycle of use. A further advantage is the property of regeneration, on treatment with fresh enzyme, of the supports in which activity has been leached out

in repeated use. Evidence is presented for the possibility of an affinity process being involved in the adsorption of activity under these conditions.

The more expensive process of covalent binding of glucoamylase activity from crude enzyme preparations to controlled-pore ceramics such as Al_2O_3 and SiO_2 results in preparations with only comparable or lower efficiency of binding than that achieved by adsorption on cyanuric chloride-starch treated γ-Fe_2O_3 and catalyst grade Al_2O_3.

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