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Investigations of Stabilities, pH, and Temperature Profiles and Kinetic Parameters of Glucoamylase Immobilized on Plastic Supports

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ABSTRACT

The covalent immobilization of glucoamylase on new epoxide-, isocyanate-, acid chloride-, and carboxylic acid-activated plastic supports shows the viability of such supports for immobilizing enzymes (especially those reacting with 1,6-diaminohexane and glutaraldehyde) for producing side arms. The operational stability of immobilized glucoamylase could be extended by crosslinking the enzyme, by increasing the substrate concentration, or by extending the support's side arm. The pH curves for the immobilized enzyme were in general not found to be shifted from the pH optimum of the soluble enzyme. However, the immobilized enzyme's temperature activity profiles were shifted to a lower temperature range when compared to the soluble enzyme. The immobilized glucoamylase Michaelis constants increased, and the maximum rates and specific activities decreased with respect to the soluble enzyme kinetic parameters.

Index Entries: Stability; pH; temperature; activity; kinetic; enzyme; glucoamylase; immobilization; plastic supports.

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Abbreviations: k_d, k_L decay constants; K_M Michaelis constant; T_A temperature for half-residual activity in a fixed time; ½ T operational stability half-residual activity time; $t_{½}$, t_L half-lives; V_{max} maximum rate.

INTRODUCTION

Glucoamylase (α-1, 4-Glucan Glucohydrolase) (EC 3.2.1.3) is an exo-acting enzyme (1) producing β-D-glucose from the nonreducing chain ends of poly-D-glucans, such as amylopectin, amylose, and glycogen. The enzyme occurs in a wide range of fungal families (*2,3*).

Most glucoamylases have a relatively high temperature activity optimum, usually in the range 40–70°C (*4,5*). This combined with an enhanced stability and high activity at low pH 4–5 has meant that it is a convenient enzyme for industrial use, since under these conditions it can be operated without microbial contamination.

Aspergillus niger produces two isoenzymes (I and II) with slightly different amino acid composition and molecular weights (99,000 and 112,000 Dalton, respectively) (*2,6*); and both isomeric forms occur in industrial preparations of this enzyme. The stability of glucoamylase is enhanced by the covalent attachment of its carbohydrate moiety, since its removal causes the enzyme to become less stable. *Aspergillus niger* glucoamylase is thought to hydrolyze starch by a multichain attack mechanism. The active site for the hydrolysis of the α-1,4, α-1,3, and α-1,6 bonds is in the same active center of the enzyme. It is thought that the active site contains two carboxyl groups, as do other enzymes involved in carbohydrate hydrolysis (*7*). Although the amino acids have not been identified, it is possible that they are L-aspartate and L-glutamate, if a mechanism of acid-base hydrolysis is accepted, as it has been for lysozyme (*8*). An imidazole group and a tryptophan residue are thought to be involved in the binding of starch to the enzyme. There are between 4 and 7 substrate-binding subsites indicated by inhibition studies, studies that have also shown that Tris buffer inhibits glucoamylase.

Glucoamylase has been entrapped in a number of gels, e.g., polyacrylamide (*9*) and acrylamide/*N,N'*-methylene *bis*-acrylamide copolymer (*10*). Fiber entrapment of glucoamylase has produced degrees of conversion of starch to D-glucose of up to 96% (*11*). This degree of conversion has only been matched by the entrapment of glucoamylase between ultrafiltration membranes (*12*). Glucoamylase has been adsorbed onto a wide range of carriers, e.g., activated carbon (*13*), collagen, and Sepharose 6B (*14*). Solomon and Levin (*15*) have extensively studied the immobilization of glucoamylase on ion-exchange resins, achieving fair conversion rates with a very high initial activity (9000 U/g). Covalent attachment on a carrier is the chief method of glucoamylase immobilization (*16*). Lobarziewiski et al. (*17*) used keratin- and polyamide-coated inorganic matrices, such as sand, silica gel, and glass beads activated with glutaraldehyde to immo-

bilize glucoamylase. The use of transition metal-coated porous silica, further reacted with 1,6-diaminohexane and glutaraldehyde, was used to produce a hybrid method of immobilization (chelation and covalent) by Cabral et al. (*18*). Partially imidoesterized polyacrylonitrile support (*19*) has also been used with moderate success.

The aim of this research was to investigate the viability of new epoxide-, isocyanate-, acid chloride-, and carboxylic acid-activated plastic supports for immobilizing enzymes, characterizing pH and temperature profiles, kinetic properties, and stability of covalently immobilized glucoamylase as illustrative examples.

MATERIALS AND METHODS

Enzyme and Substrate

A crude industrial glucoamylase (A.M.G. 150 L) extract produced from *Aspergillus niger* was supplied by Novo Industry A/S and Potato Starch by Fisons Ltd.

Supports

All the supports used in this research were produced and provided by B.I.P. Ltd., Smethwick, and are numbered using their system. All the supports contained zinc oxide and were provided in several forms, powders < 250 and < 500 μm diameter, and as a coat on a grid.

PZ 1 was a low-molecular-weight polyester produced by condensation of the diols, propylene and neopentyl glycol with the dicarboxylic acids, terephthalic acid, isophthalic acid, and adipic acid. To provide a free carboxylic acid group along the polymer backbone, free hydroxyl groups were reacted with trimellitic anhydride. PZ 2 and 2A were the acid chloride version of PZ 1 produced by reacting PZ 1 with thionyl chloride. PZ 3, PZ 4, PZ 4A, PZ 7, PZ 8, PZ 9, and PZ 9A were isocyanate functional resins. PZ 10 was the precursor epoxy resin used to produce PZ 9, reacted with trimellitic anhydride to give a carboxylic acid functional polymer. PZ 11 was the acid chloride version of PZ 10, produced by reacting PZ 10 with thionyl chloride. PZ 12 was a carboxylic acid version of PZ 9 produced by reacting the precursor epoxy resin with succinic anhydride. PZ 13 was the acid chloride version of PZ 12, produced by reacting PZ 12 with thionyl chloride. PZ 14–16, 21–23 were all varieties of PZ 1 base polymer having different free epoxide to free acid ratios owing to the addition of an diepoxide crosslinking agent. PZ 17 was the carboxylic acid version of PZ 4 produced by reacting the base polyester (glycerol and phthalic anhydride) with succinic anhydride. PZ 18 was the acid chloride version of PZ 17, produce by reacting the polyester with thionyl chloride.

PZ 27 was produced by reacting maleic anhydride with ethylene glycol, and then further reacting with pyromellitic dianhydride to achieve a

polymer of 2000 Dalton. PZ 28 was the precursor of polyester PZ 27, reacted with trimellitic anhydride to produce free carboxylic acid groups. PZ 31A/B and PZ 32A/B were the product of crosslinking PZ 27 and PZ 28, respectively, with a diepoxide (Lopox 550). PZ 33 was the acid chloride version of PZ 31A, by reacting it with thionyl chloride. PZ 34 and PZ 35 were base polymer PZ 1 with a triethylene glycol spacer arm capped with trimellitic anhydride and with pyromellitic anhydride, respectively. PZ 36 was the acid chloride version of PZ 32A by reaction with thionyl chloride. PZ 37 and PZ 38 were fully saturated versions of PZ 31A and PZ 32A, respectively. PZ 39 and PZ 40 were the acid chloride derivatives of PZ 37 and PZ 38, respectively, by reacting with thionyl chloride. PZ 41 was a polyester sphere produced by crosslinking PZ 28 with styrene by the free radical method. PZ 42 and PZ 43 were acidified (acetic acid and sulfuric acid, respectively) versions of PZ 41. PZ 44 and PZ 45 were the acid chloride versions of PZ 42 and PZ 43, respectively, produced by reacting them with thionyl chloride.

PZ 54 was PZ 1 stoved at 180°C for 30 min. PZ 55 was PZ 54 produced without the addition of zinc oxide. All of these preparations were stored in the appropriate buffer at 4°C until required.

Immobilization Methods

Epoxide-Activated Polyacrylamide Cellulose Graft Copolymer

To immobilize glucoamylase on epoxide-activated polyacrylamide cellulose graft copolymer, crude glucoamylase (6800 U suspended in sodium borate buffer 0.05*M*, pH 8.7, 0.5 mL) was mixed with a suspension of the support (100 mg in the same buffer, 0.5 mL). Reaction time was 16 h, room temperature (*20*). The coupled enzyme was collected by centrifugation and washed with sodium acetate buffer (0.02*M*, pH 4.5, 5 × 2.0 mL) containing sodium chloride (1.0*M*). The wash was repeated without the addition of sodium chloride.

To study the effect of hydratation on epoxide-activated polyacrylamide cellulose graft copolymer, a quantity of support was placed in a measuring cylinder (10 mL) up to the 2-mL graduation and mixed with distilled water (8 mL). The volume of the support was noted after 4 h.

Epoxide-Activated Plastic Supports

Glucoamylase was immobilized onto a range of epoxide-activated plastic supports PZ 14–16 and PZ 21–23 by Sundberg and Porath's method (*21*). To determine the effecting parameters, the assays were repeated at different crude glucoamylase solutions, different periods of time, different temperatures (in the range 35–75°C), and different pHs (pH 2.5–6.5).

Isocyanate-Activated Plastic Supports

Glucoamylase was also immobilized onto isocyanate-activated plastic support PZ 3 (>250 μm and <250 μm), and PZ 4, PZ 4A, PZ 7, PZ 8, PZ 9, and PZ 9A. The immobilization method for glucoamylase was that pro-

posed by Gemeiner et al. (22), but the washing procedures were changed to suit glucoamylase, as recommended by Cabral et al. (*18*). Crude glucoamylase was diluted to 18,000 U with sodium phosphate buffer (0.1*M*, pH 8.0) to give a total volume of 5 mL. Isocyanate-activated plastic support, PZ 3, was mixed with the glucoamylase solution. The reaction mixture was slowly stirred at 22°C for 2 h, after which the support was removed by suction filtration. The immobilized glucoamylase was then washed with sodium acetate buffer (0.02*M*, pH 4.5, 100 mL) and stored at 4°C in the same buffer (5 mL) until required.

To study the effect of crude glucoamylase concentration and coupling pH on the final activity of glucoamylase immobilized on isocyanate-activated plastic support, PZ 3 (>250 μm), the procedure was repeated using enzyme concentration in the range 500–9000 U enzyme/g support. The pH of the coupling reaction was varied by using different coupling buffers (0.1*M*) in the pH range 5–9.

Acid Chloride-Activated Plastic Support

Glucoamylase was immobilized on acid chloride-activated plastic supports, PZ 2, using the method described by Isliker (23). An alternative procedure was devised, using sodium borate buffer pH 8.0, the coupling temperature was increased to 4°C, and the coupling time was increased to 4 and 16 h. Glucoamylase (1800 U) was immobilized on two grades of the support, PZ 2 and PZ 2A, using the same method, but the coupling time was 4 h.

Glutaraldehyde-Coated Plastic Support

To immobilize glucoamylase on glutaraldehyde-coated, isocyanate-activated plastic support PZ 3 (>250 μm) and glutaraldehyde-coated, acid chloride-activated plastic support, PZ 2A (>250 μm), the supports (500 mg) were mixed with a solution of glutaraldehyde (5% [w/v], pH 8.6, 2.5 mL) for 1 h at ambient temperature. The coated support was washed with distilled water (40 mL) and then sodium acetate buffer (0.02*M*, pH 4.5, 10 mL). A solution of crude glucoamylase (10,400 U) in sodium acetate buffer (0.02*M*, pH 4.5, 4 mL) was mixed with the coated support and left for 2 h at 4°C. The immobilized glucoamylase was then washed with sodium acetate buffer (0.02*M*, pH 4.5, 150 mL) and assayed. The sample was freeze-dried, weighed, and the activity of glucoamylase per gram support calculated. The procedure was repeated using different concentrations of glucoamylase within the range 2000–25,000 U/g support and using various times within the range 0.5–5 h.

Carboxylic Acid-Activated Plastic Supports

1,6-Diaminohexane was used as a crosslinking agent to form alkylaminated carbonyl derivatives of acid chloride, PZ 2A (>250 μm) and carboxilic acid, PZ 1, activated plastic support according to Cabral et al. (*18*) and Cabral and Kennedy (*24*). Glucoamylase was coupled to PZ 1 and PZ 2A using Cabral et al.'s (*18*) method, and was crosslinked with

glutaraldehyde following immobilization in order to improve stability and to prevent loss owing to deabsorption from the support (*24*). To determine the effecting parameters, the assay was repeated at different crude glucoamylase solutions within the range 125–20,000 U, time periods within the range 45–240 min, at different temperatures within the range 4–55°C, and at pHs within the range 3.5–7.0.

Glucoamylase Activity

The assay method for the immobilized enzyme was as follows: A 1% (w/v) potato starch solution (20 mL) was placed into a thermostated water jacket at 45 °C, and allowed to reach the assay temperature. The immobilized enzyme (500 mg) was washed with sodium acetate buffer 0.02*M*, pH 4.5, into a special stainless-steel mesh (125 μm) basket reactors (*25*). At time zero, the basket reactor was placed in the starch solution, which was stirred by a magnetic stirrer at a rate of 10 rev/s. Aliquots (100 μL) were taken at 1-min intervals for 5 min, after which the basket reactor containing the immobilized enzyme was removed. A final aliquot (100 μL) was removed after a further 5 min.

The immobilized glucoamylase activity was assayed by the alkaline 3,5-dinitrosalicylic acid (D.N.S.) method (*26–28*). To study the effect of repeated assay, each support was reassayed five times, and the activity of enzyme per gram support was calculated. For each assay, a fresh quantity (500 mg) of immobilized glucoamylase was used. The assayed immobilized glucoamylase was then freeze-dried and weighed so that the activities of enzyme per gram support could be calculated. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of product (reducing sugar) in a minute under standard conditions (*29*).

Storage Stability

Immobilized glucoamylase was suspended in sodium acetate buffer (0.02*M*, pH 4.5, 10 mL) and then incubated at 4°C or at room temperature. An aliquot (1 mL) was removed at time zero, then every hour for 8 h, and assayed. The samples were frozen, dried, weighed, and the activity of glucoamylase per gram support calculated.

Operational Stability

The determination of the operational stabilities of the preparations was carried out in a basket reactor operated on a drain/fill basis, fresh substrate being added immediately prior to each activity determination. The preparation was maintained at the required temperature (45°C) in a stirred solution of the hydrolysis products of the previous assay. Since the hydrolysis time of the substrate is short compared to that between assays, the preparations are operating in an essentially constant, very low, substrate concentration. According to Cardoso et al. (*30*), this situa-

tion is analogous to a continuous-flow stirred-tank reactor (CFSTR) operating at a constant high conversion rate.

Reducing Sugars

The method of determination for the reducing sugars produced during the hydrolysis of starch by glucoamylase was the alkaline 3,5-dinitrosalicylic acid (D.N.S.) method (*26–28*), improved by White (*31*).

Spectrophotometric Measurements

Monochromatic absorbances in the visible and UV regions were measured using an SP 550 Pye Unicam spectrophotometer (Pye Unicam Instruments Ltd.). Quartz microcuvetes of 1 cm light path and 0.5 mL were used.

Preparation of Freeze-Dried Samples

Samples were freeze-dried on an Edwards Modulyo freeze-dryer. The sample was placed in a quick-fit flask, generally a FR50/35, B24 (50 mL), and then frozen by rotating the flask in liquid air.

Procedure for the Determination of the Protein Concentration Immobilized onto a Support

Hydrolysis of Immobilized Protein

A sample of freeze-dried immobilized enzyme (200 mg to 2 g) was placed into a screw-cap hydrolysis tube together with 50 mg of phenol; 6*N* hydrochloric acid (10 mL) (which had previously been deoxygenated by bubbling oxygen-free nitrogen through it for 30 min) was pipeted into the tubes. The air in the tube was replaced with nitrogen (oxygen-free) and the tube tightly sealed. The sample was then placed in a special reflux apparatus, containing a satured sodium chloride solution at 108 °C for 23 h, after which the tube was cooled to 0°C in an ice bath. For every immobilized enzyme sample hydrolyzed, the corresponding support was also hydrolyzed to check for interference with the analysis.

Hydrolysis of Soluble Protein

The procedure was the same as above, except that 10 mg of protein were used.

Drying of Protein Hydrolysates Samples

The samples were dried using a rotary evaporating unit, modified to incorporate a cold finger cooled by liquid air.

Amino Acid Analysis of Hydrolyzed Protein Samples

The samples for analysis were thawed and thoroughly mixed before loading onto the columns of a Beckman 120C amino acid analyzer, the

acid and neutral amino acids being eluted with a stepwise gradient of 0.2*N* sodium citrate-hydrochloric acid buffer, pH 3.25, for 80 min followed by the same buffer at pH 4.25 for 100 min. The basic amino acids were eluted off a separate column using 0.2*N* sodium citrate-hydrochloric acid buffer pH 5.25 for 150 min. The protein concentration of each sample was determined as described by Kula (*32*).

Starch Solutions

Soluble starch solutions were obtained after adding boiling distilled water, cooling, and incubating with 0.02*M* sodium acetate buffer (pH 4.5).

RESULTS AND DISCUSSION

Preliminar Effecting Parameters on the Activity of Immobilized Glucoamylase

A series of experimental parameters, such as coupling temperature, concentration of 1,6-diaminohexane (during the formation of alkylaminated carbonyl derivatives of support), temperature and time of alkylamination, coating glutaraldehyde concentration, reaction time, pH and temperature, long-term use, and reassay, were checked for influence to the activity of glucoamylase immobilized on acid chloride-activated plastic support, PZ 2A (> 250 μm). Coupling temperature gave (within the range 4–55°C, at pH 8.0, 30-min reaction time) a negative effect on immobilized glucoamylase activity (from 100 up to 20% relative activity). The activity of glucoamylase immobilized on such alkylaminated support increases for increasing alkylamination temperatures (from 70 to 100% relative activity) and 1% glutaraldehyde reaction temperatures at pH 8.6 (from 57 to 100% relative activity). At pH 8.0, 45°C, 30-min reaction time, positive effects on immobilized glucoamylase activity of increasing 1,6-diaminohexane concentration from 0.25 to 5% (w/v) (from 6 up to 100% relative activity) and of changing pH of the 1% glutaraldehyde reaction at 45°C (30-min reaction time) from pH 7.0 up to 10.0 (from 4 up to 100% relative activity) were also found. Alkylamination and glutaraldehyde reaction times varying between 20 and 120 min and glutaraldehyde concentration between 0.5 and 10.0% (w/v) do not affect the activity of immobilized glucoamylase.

A slight negative effect of reassay vs activity of crude glucoamylase immobilized on alkylaminated carbonyl derivatives of acid chloride, PZ 11, PZ 13, PZ 18, and carboxylic acid, PZ 10, PZ 12, PZ 17, activated plastic supports was shown, e.g., for PZ 10, coupled activity was reduced from 4.79 to 4.36 U/g after four reassays.

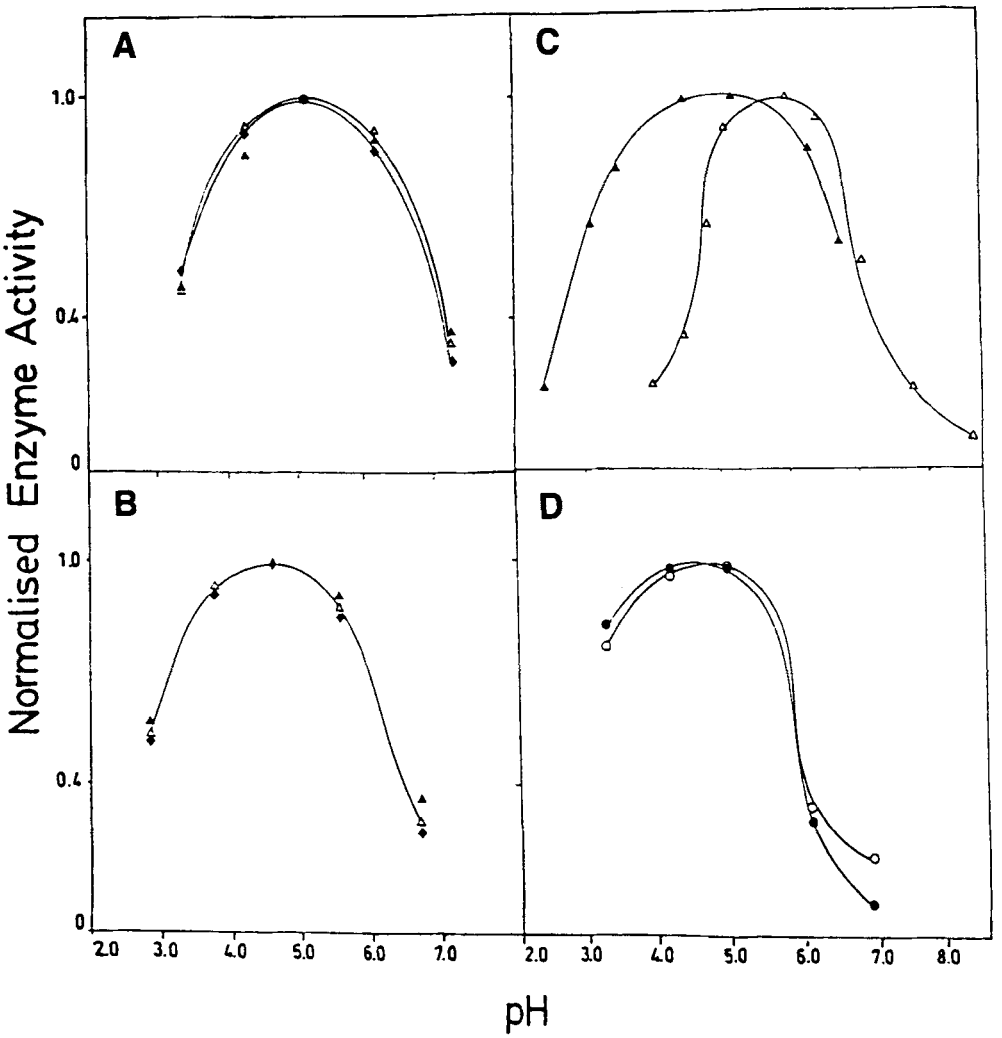

Fig. 1. Activity vs pH profiles of soluble and immobilized glucoamylase. (**A**) immobilized on epoxide-activated PZ 14 (▲), PZ 15 (△), and PZ 16 (◆) plastic supports; (**B**) immobilized on PZ 1 (AG) n = 1 (▲), 2 (△), 3 (◆) support; (**C**) soluble (▲) and indirectly immobilized on PZ 2A support (△); (**D**) immobilized on PZ 2A (AG) n = 2 (○), 3 (●).

The pH activity profiles of crude glucoamylase directly immobilized on epoxide-activated plastic supports PZ 14, 15, and 16 increased by 0.45 pH unit compared to that of the soluble enzyme (Fig. 1A and C) because of the presence of free carboxyl groups on the surface of the support partitioning protons. The pH profiles of glucoamylase immobilized onto different side arm length alkylaminated carbonyl derivatives of carboxylic acid-activated plastic support PZ 1 showed pH activity profiles that did not vary with side arm length (Fig. 1B). The pH optima decreased 0.1–0.2

Table 1
Optimum Temperature and Relative Activation Energies for Soluble and Immobilized Glucoamylase

Support	Method	Optimum temperature °C	Relative activation energy %
—	—	65.0	100
PZ 1	$(AG)_1$	53.5	49
PZ 1	$(AG)_2$	53.5	52
PZ 1	$(AG)_3$	53.5	51
PZ 2A	$(AG)_1$	46.0	53
PZ 2A	$(AG)_2$	55.0	51
PZ 2A	$(AG)_3$	55.0	51
PZ 14	Direct	56.0	46
PZ 16	Direct	55.5	47

pH unit when compared to the soluble enzymes. This small difference may be the result of protons partitioning owing to unreacted 1,6-diaminohexane amino groups, conferring a slight positive charge on the supports.

Glucoamylase immobilized onto acid chloride-activated plastic support PZ 2A (>250 μm) showed a large pH shift compared to the soluble enzyme, increasing the pH optimum by 0.85 pH unit (Fig. 1C). This may be caused by the lower support microenvironmental pH produced by the hydrolysis of acid chloride groups. The effect was mitigated by the rereaction of the support with 1,6-diaminohexane and glutaraldehyde before coupling glucoamylase (Fig. 1D).

Temperature vs activity dependence for soluble crude glucoamylase and crude glucoamylase immobilized on alkylaminated carbonyl derivatives of acid chloride-activated plastic support, PZ 2A (>250 μm), on longer side arm alkylaminated carbonyl derivatives of acid chloride, PZ 2A (>250 μm), and carboxylic acid, PZ 1, and on epoxide, PZ 14 and 16, activated plastic supports was measured (Table 1). The optimum temperature for activity of immobilized glucoamylase was, in every case, lower than that found for the soluble enzyme. For PZ 1 support, the side arm length did not affect the temperature optimum for glucoamylase. Immobilization onto PZ 2A support caused a decrease in temperature optimum for glucoamylase that was recuperated by increasing the side arm length. The Arrhenius plots of the temperature profiles showed that a decreased apparent activation energy was required for the immobilized enzymes compared to the soluble enzymes (Table 1). This may indicate that external diffusion plays a part in the process of substrate conversion at the polymer surface face (33).

Table 2
Kinetic Parameters
for Soluble and Immobilized Glucoamylase

Support	Method	K_M g/L	V_{max} U/mg
—	—	2.03	107
PZ 1	$(AG)_1$	10.6	21
PZ 1	$(AG)_2$	11.3	18
PZ 2A	$(AG)_1$	17.2	23
PZ 2A	$(AG)_2$	11.9	19
PZ 34	$(AG)_1$	10.2	3
PZ 35	$(AG)_1$	12.3	1
PZ 42	$(AG)_1$	12.6	11
PZ 44	$(AG)_1$	13.1	11

The kinetic behavior for soluble glucoamylase and glucoamylase immobilized on alkylaminated carbonyl derivatives of acid chloride-activated plastic support, PZ 2A (>250 μm), on longer side arm alkylaminated carbonyl derivatives of acid chloride PZ 2A (>250 μm), PZ 34 and PZ 35, and carboxylic acid PZ 1, PZ 42, and PZ 44, activated plastic supports was studied (Table 2). A range of soluble starch solutions was produced at various concentrations (0.25–10 g/L, pH 4.5) and used in the assay of diluted (1:200) soluble and immobilized glucoamylase. For the kinetic determinations, care was taken to remove the effects of external diffusion (to assay within the limits of linearity for the enzyme loading at constant temperature and pH). The results were processed using a nonlinear regression program (*34*) to obtain the kinetic parameters K_M and V_{max}. The kinetic constants of crude *Aspergillus niger* glucoamylase were identical to those found by Cabral et al. (*18*). The K_M values for glucoamylase immobilized by the indirect method are within the range 10.2–13.1 g/L, except for glucoamylase immobilized on PZ 2A, which has a larger K_M of 17.2 g/L (Table 2), potentially indicating exceptional diffusional resistance in using PZ 2A support or some change to the enzyme's substrate-binding site. The immobilized glucoamylase V_{max}'s were in the range 0.7–21% of those for the soluble enzyme because of either increased enzyme rigidity (*35*) or diffusional effects. The Arrhenius data suggest the latter may be the case, although an increase in the enzyme's rigidity would explain the decreasing V_{max} with increasing side arm length. The low V_{max} for glucoamylase immobilized on PZ 34 and PZ 35 corresponds to long half-lives (data not shown), as would be expected if increased rigidity were the cause.

Table 3
Storage Stability at 4°C
of Glucoamylase Immobilized
on PZ 1 and PZ 2A Plastic Supports

Support	Side arm length, AG_n	$t_{1/2}$ d
PZ 1	1	17.6
PZ 1	2	19.6
PZ 1	3	20.3
PZ 2A	1	15.7

Stability Profiles

Storage Stability

Glucoamylase immobilized on various side arm length derivatives of PZ 1 and PZ 2A showed half-lives of >15 d when stored in buffer at 4°C. Increasing the side arm length had a pronounced effect on the storage stability of glucoamylase immobilized on the different derivatives of PZ 1 (Table 3), the half-life of the enzyme being extended by 3 d on PZ 1 $(AG)_3$ when compared to PZ 1 $(AG)_1$. Table 3 summarizes the results of the $t_{1/2}$ studies.

The effect of storage pH on soluble crude glucoamylase and crude glucoamylase immobilized to alkylaminated carbonyl derivatives of acid chloride-activated plastic support, PZ 2A (>250 μm), was measured as follows: Samples of crude glucoamylase (50 μL) were diluted with a range of buffers (0.02*M*, pH 1.5–9.5, 4.95 mL) and then assayed. After being incubated at 4°C for 24 h they were reassayed, and the percentage of remaining activity calculated and plotted (Fig. 2B). Samples of immobilized glucoamylase (500 mg) were assayed, stored separately in a range of buffers (0.02*M*, pH 1.5–9.5, 5 mL) at 4°C for 24 h before being reassayed, and the percentage of remaining activity calculated and plotted (Fig. 2B). The pH stability curve for glucoamylase immobilized on alkylaminated carbonyl derivatives of PZ 2A (Fig. 2B) shows that immobilized glucoamylase is very sensitive to low pHs (<4.0). This supports the hypotheses that a proportion of the enzyme has been either stabilized or removed from the denaturant, or that external influences (e.g., low pH) cause the enzyme to decay rapidly into a more stable form with a reduced specific activity.

For measuring the temperature storage stability, soluble crude glucoamylase and crude glucoamylase immobilized on epoxide-activated PZ 14 and PZ 16, on alkylaminated carbonyl derivatives of acid chloride-activated plastic support, PZ 2A (>250 μm), on longer side arm alkylaminated carbonyl derivatives of acid chloride-activated plastic support, PZ

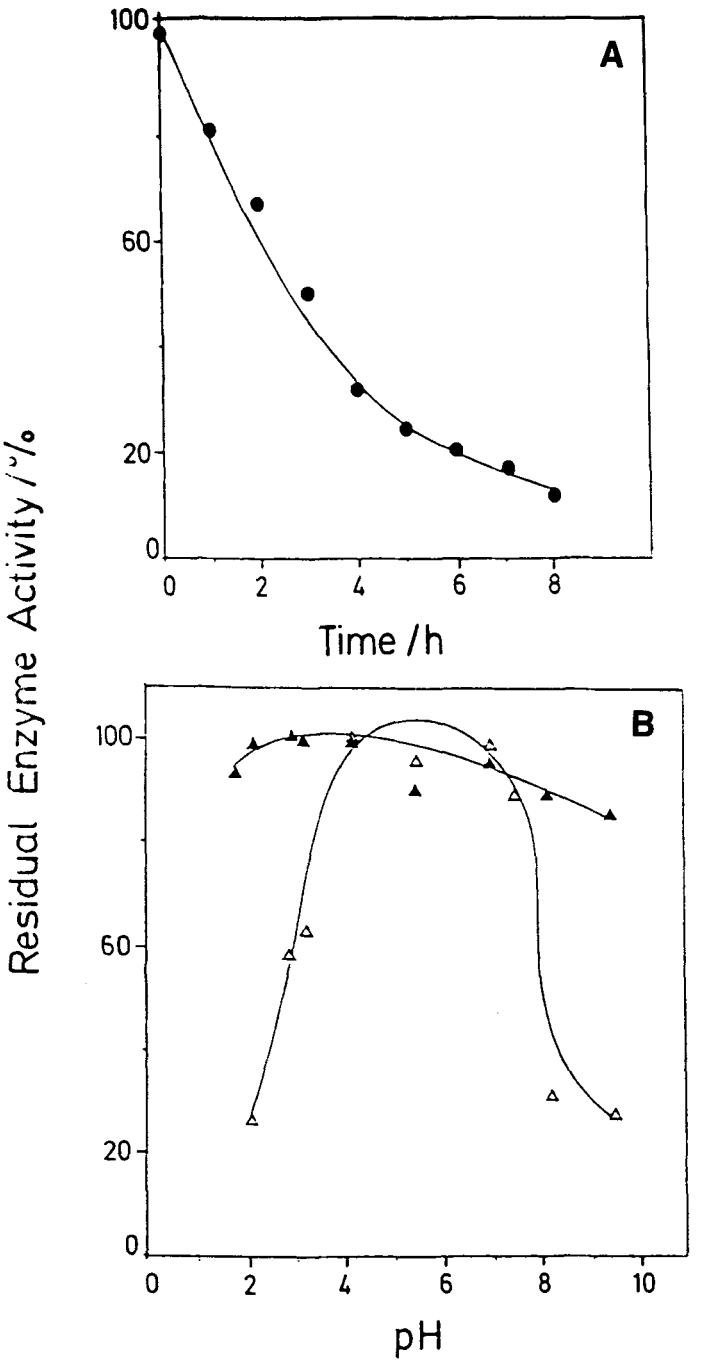

Fig. 2. Storage stability at 25°C (**A**) and the effect of storage pH (**B**) of soluble (▲) and immobilized (△) glucoamylase on acid chloride-activated plastic support PZ 2 and PZ 2A, respectively.

2A (>250 μm) and carboxylic acid PZ 1 (PZ 34, PZ 35, PZ 42, PZ 44), activated plastic supports were assayed, suspended in sodium acetate buffer (0.02*M*, pH 4.5, 5 mL) and incubated at a specific temperature in the range 35–75°C for 2 h. The enzymes were then reassayed, and the percentage of remaining activity calculated and plotted.

The enzyme was very unstable when immobilized onto PZ 14 to PZ 16 ($t_{1/2}$ = 10, 9, and 10 h; k_d = 0.069, 0.077, and 0.069 h^{-1}, respectively), and because of the low initial activities, only rough estimates of half-lives could be produced. The thermostability of glucoamylase directly immobilized onto PZ 14 and PZ 16 was the lowest achieved using the plastic supports (T_A of 45°C, all other preparations showed almost 100% activity at this temperature) (Fig. 3A). Glucoamylase immobilized onto PZ 1 $(AG)_n$ and PZ 2A $(AG)_n$ (n = 1, 2, 3) showed decreased thermostability when compared to the soluble enzyme. The thermostability of immobilized glucoamylase on poly (ethyleneamide)-coated glass beads using glutaraldehyde was reduced to a fraction of its soluble counterpart (*36*). It has been proposed by Klyosov et al. (*37*) that there are limits to the thermostability of glucoamylase immobilized on inorganic supports. This hypothesis has not been tested for glucoamylase immobilized on organic supports. Increases on immobilization of thermostability have been found (*15*), although the method used was adsorption on resins, not covalent attachment.

Figure 3B and C shows that both immobilized and soluble glucoamylase decay with the same reverse sigmoidal curve. Increasing side arm length using PZ 1 and PZ 34 leads has no effect on the enzyme's thermostability, possibly indicating that multipoint binding to the support with increasing side arm length is unlikely (this should cause an increase in the enzyme's T_A) unless separate parts of the enzyme were involved in the processes of operational and temperature activity decay, when an increase in the stability of the enzyme to one form of deactivation would not increase its stability to the other. Glucoamylase immobilized onto PZ 35 showed a slight decrease in T_A of 4°C when compared to that coupled on PZ 34, which could account for its lower operational stability. This effect could be the result of the effects of aggregation (*see above*). The thermostability of glucoamylase immobilized onto various derivatives of PZ 2A was lower than the soluble enzymes by 6.3°C, a decrease that could result in the enzyme's poor operational stabilities on this support. The T_A values were determined in the absence of substrate, indicating that glucoamylase immobilized on plastic supports would have a short operational stability at the temperatures used at present in industry (>65°C).

The same pattern of decay was followed by the immobilized glucoamylase on PZ 42 and PZ 44 as for the other immobilized enzyme preparations and the soluble enzyme. The T_A values of the enzyme coupled on the $(AG)_1$ versions of the supports were nearly identical to those found using the PZ 1 support (55.8°C), because the same coupling mechanism was used. PZ 44 does not decrease the thermostability of glucoamylase

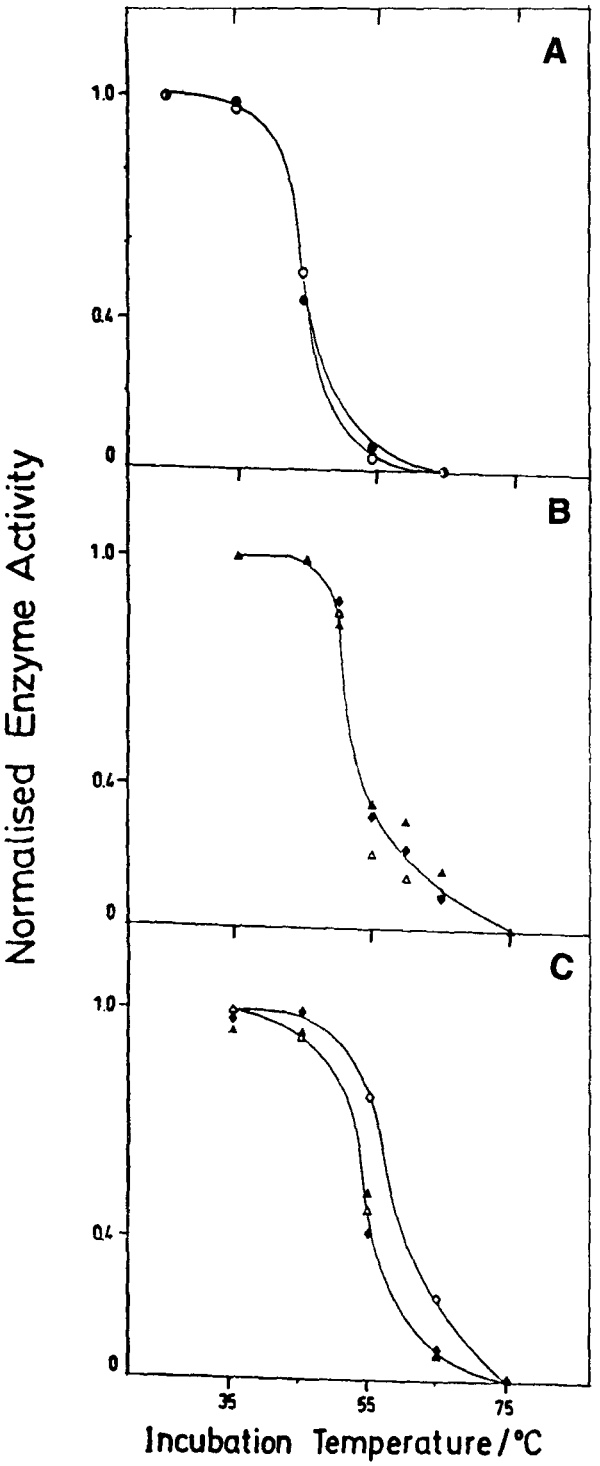

Fig. 3. Thermostability of soluble and immobilized glucoamylase. (**A**) immobilized on epoxide-activated PZ 14 (○) and PZ 16 (●) supports; (**B**) immobilized on various side arm derivatives of PZ 1, PZ 1 (AG) n = 1 (▲), 2 (△), 3 (◆); (**C**) soluble (◇) and immobilized on various side arm derivatives of PZ 2A, PZ 2A (AG) n = 1 (▲), 2(△), 3 (◆).

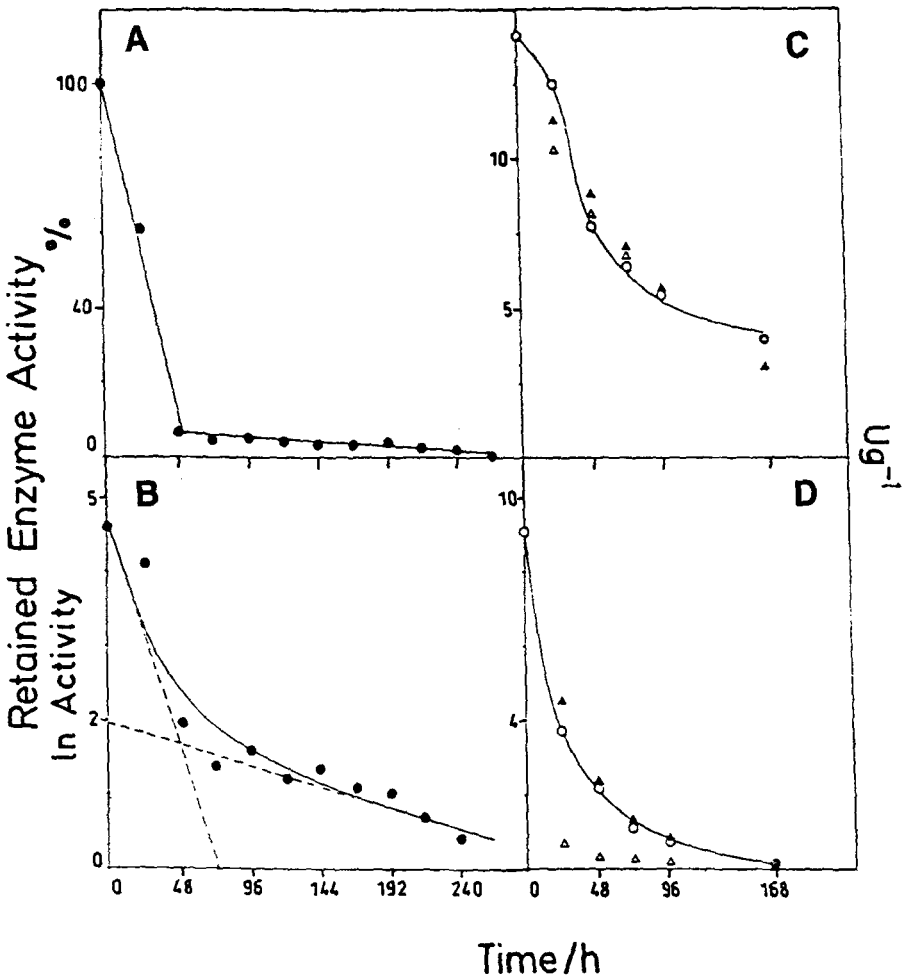

Fig. 4. Deactivation curves for immobilized glucoamylase. (**A**) On glutaraldehyde-coated PZ 2A support; (**B**) multistage exponential decay of (A); (**C**) on PZ 1 support, data (○), exponential decay model (▲), inverse linear decay model (△); (**D**) on PZ 2A support, data (○), exponential decay model (▲), inverse linear decay model (△).

immobilized on it, and increasing the side arm length has no effect either. Increasing the side arm length for PZ 42 and PZ 44 decreases the T_A of attached glucoamylase by 1.5°C because of the blockage of the pores by the extra side arms, preventing some of the enzyme population being protected in this way.

Operational Stability

The operational stability of crude glucoamylase immobilized on glutaraldehyde-coated acid chloride-activated plastic support, PZ 2A (>250 μm) (Fig. 4A and B), crude glucoamylase coupled to alkylaminated carbonyl derivatives of acid chloride, PZ 2A (>250 μm) (PZ 11, PZ 13, PZ 18, PZ 33, PZ 36, PZ 39, PZ 40), and carboxylic acid PZ 1 (PZ 10, PZ 12, PZ

17, PZ 31A, PZ 32A, PZ 34, PZ 35, PZ 37, PZ 38, PZ 41, PZ 42, PZ 44, PZ 54, PZ 55) activated plastic supports (Fig. 4C and D), chemically modified crude glucoamylase immobilized on alkylaminated carbonyl derivatives of acid chloride, PZ 2A (>250 μm), and the effect of:

1. Ammonium sulfate;
2. Side arm length; and
3. Starch concentration on the operational stability of crude and modified glucoamylase immobilized on alkylaminated carbonyl derivatives of acid chloride, PZ 2A (>250 μm), and carboxylic acid, PZ 1, activated plastic supports were measured.

The operational stability decay of glucoamylase coupled to glutaraldehyde-coated, acid chloride-activated support, PZ 2A, showed a particular pattern (Fig. 4A and B). Wada et al. (*38*) have identified three categories of denaturation pattern:

1. One-step transition between two stages, the native and the denatured. Exponential decay describes this activity loss.
2. One-step transition in which some transitions begin in advance of others. Protein molecules exist in various reversible equilibrium conformations and will therefore start to decay from different positions (*39*). There is evidence that glucoamylase exists in these resonance forms, so this pattern of decay may be attractive (*40*); and
3. The transition in which both backbone and the side chains undergo several discrete changes from the native to a completely denatured state.

This pattern of decay is generally termed series deactivation (*41–43*). The only clear evidence of series decay is usually when activation of the enzyme occurs at the start of a decay curve. Fig. 4A and B indicates that glucoamylase immobilized to glutaraldehyde-coated PZ 2A support goes through initial fast decay followed by a much slower deactivation. The initial decay has a $t_{1/2}$ of 12.1 h, much slower than that of glucoamylase directly bound to PZ 2A support ($t_{1/2}$ = 2.8 h). The reason for this initial quick decay (k_1 = 0.057 h^{-1}), involving 93% of the immobilized glucoamylase, may still be because of the destabilizing influence of the acid chloride groups, although the effect is much reduced because of further washing procedures and the presence of substrate. The second stage of the decay involves only 7% of the initial active enzyme population, which deactivates with a $t_{1/2}$ of 168.0 h and with k_2 = 0.004 h^{-1}. The difference in stability of the two fractions could be the result of several factors:

1. The two isoenzymes of glucoamylase, I and II, have different stabilities. It is unlikely that the effect would be so marked and a stability difference has not been noted in the literature.
2. Seven percent of the glucoamylase coupled to the support does so by multipoint attachment (fractional decay);

3. Seven percent of the glucoamylase is immobilized in an area of the support that does not have a significant acid chloride concentration (fractional decay); and
4. The initial glucoamylase decays into a more stable preparation because this is its innate pattern of decay or because of an external influence (series decay).

It is known from other researchers (*18,25,37*), all working on inorganic supports, that immobilized glucoamylase decays exponentially or by inverted linear decay. No other worker has previously reported a two-stage decay pattern for crude immobilized glucoamylase. This evidence discounts hypotheses 1 and to a certain extent 4.

Another decay model was also considered, that of "inverted linear decay." This model was first described by Cardoso (*25*) and Cardoso et al. (*30*). Dagys et al. (*44*) suggest that the model can more correctly be described by a fractional decay equation series. This being stated, the model is simple and does describe the decay of glucoamylase immobilized on inorganic supports (*18,25,30*).

The deactivation curve for glucoamylase immobilized on alkylaminated carbonyl derivatives of PZ 1 is shown in Fig. 4C. The inverted linear decay model fitted the deactivation curve better giving a t_L of 65.4 h compared to a time taken to reach half activity of 50.4 h. The exponential decay model leads to an estimation of $t_{1/2}$ = 74.4 h. What emerges from the literature is a wide range of half-life data for immobilized glucoamylase, extending from an almost infinite half-life at low temperature (35°C) (*45*) to 1 h at 45°C (*46*).

Glucoamylase immobilized on PZ 2A by the indirect method showed an exponential pattern of decay (Fig. 4D), with a "1/2 *T*" of 17 h and a $t_{1/2}$ of 24 h, the inverted linear decay model predicted at t_L = 1.9 h. The 1/2 *T* of glucoamylase immobilized onto PZ 2A was only 33% of glucoamylase immobilized onto PZ 1 by the same method, the difference between the supports being that PZ 2A surface carboxylic acid groups have been converted in acid chloride groups, which can release hydrochloric acid. The PZ 2A support surface must have a low pH during or even after coupling, which makes the enzyme more labile, decreasing its half-life.

The deactivation pattern of glucoamylase immobilized on PZ 2A support was altered from the exponential decay shown in Fig. 4D to inverted linear decay or two-stage exponential decay. The 1/2 *T* was altered from 17 to 18 h on the addition of 1*M* ammonium sulfate to the incubation substrate, the salt changing the decay pattern of the immobilized enzyme, but not significantly increasing stability.

Postimmobilization crosslinked glucoamylase coupled on PZ 2A was also incubated with salt. The pattern of decay was altered, the activation step was removed, and the deactivation of the enzyme proceeded faster with 1/2 *T* = 85 h as opposed to 95 h previously attained. The presence of salt appears to prevent the attainment of the higher specific activity state. Thus, the enzyme shows a shorter 1/2 *T* (data not shown).

The pattern of deactivation of glucoamylase immobilized on PZ 1 by the optimized indirect method showed greater exponential decay with a longer half-life (112 h, as opposed to 74 h) and 1/2 *T* extended from 50 to 115 h. The increase in stability could be the result of several factors:

1. Increase in protein concentration;
2. Change in support morphology;
3. Increased multipoint binding; and
4. Increased external diffusion effects.

When immobilized on PZ 2A, glucoamylase showed a faster deactivation than when immobilized on PZ 1. Again this is the result of the effects of the acid chloride groups. The decay pattern was gentler than that produced by either the best-fit exponential decay curve or inverse linear decay. The 1/2 *T* was extended from 17 to 38 h when compared to that achieved for the original indirect method. The increase may be the result of the factors outlined above, or the increased prehydrolysis and reaction of acid chloride groups before enzyme coupling. Measurement of the protein concentration on the support after the operational stability revealed a loss of only an 8% of the original amount coupled (28.6 mg/g at 0 time, 26.3 mg/g at 168 h), so the deactivation curves could not be produced by loss of active enzyme. Silman and Katchalski (*47*) and Barker (*48*) noted that hydrophobic forces had a destabilizing effect on bioactive molecules and enzymes. The internal hydrophobic interactions are thought to play a large part in conformation of proteins (*49*) and any external hydrophobic interactions would destabilize a protein.

Hydrophobic interactions are distance-dependent, so it may be possible to extend the half-life of an immobilized enzyme by removing it from the support. This can be achieved by extending the length of the side arms, in this case by reacting alkylaminated carbonyl plastic support derivatives with 1,6-diaminohexane and glutaraldehyde. Given the complex nature of undistilled glutaraldehyde, the side arms produced would not be straight or of a uniform length. Many glutaraldehyde mers and polymers would react with each other and with several free amino groups. This would explain the decrease in the activity per gram noted for glucoamylase immobilized on these support derivatives. Glucoamylase had a decreased deactivation rate when immobilized on these supports. When immobilized on derivatives of PZ 1, glucoamylase deactivation did not approximate very well to either decay model used. Exponential decay gave the better fit, but this model did not show a consistent rise in half-life with side arm length as did the inverted linear decay model. The decrease in deactivation was very marked with side arm length with glucoamylase immobilized on derivatives of PZ 2A support. The 1/2 *T* of glucoamylase was increased by 34.5 h from PZ 2A (AG) to PZ 2A $(AG)_2$, and 35.0 h from PZ 2A $(AG)_2$ to PZ 2A $(AG)_3$. The increase with side arm length was surprisingly constant, which could indicate that the increase was not the result of a lessening of any unfavorable support interaction.

The removal of acid chloride groups by rereaction with 1,6-diaminohexane or the removal of hydrochloric acid because of the extra incubation and washings prior to coupling was probably more significant together with the increased potential for multipoint binding of the enzyme to the support. The decay pattern of glucoamylase immobilized onto PZ 2 $(AG)_2$ did not show either exponential or inverted linear decay. As for glucoamylase immobilized onto PZ 1 $(AG)_3$, the preparation would have been better described by a two-stage exponential decay model.

Glucoamylase indirectly bound to PZ 34 showed a dramatic increase in operational stability compared to glucoamylase immobilized by the same method onto PZ 1. The pattern of decay was more complex than either of the models used, and hence, the discrepancy between the half-life data. This decay pattern must be the result of the supports activation by pyromellitic dianhydride.

Glucoamylase immobilized indirectly to PZ 1 and PZ 2A showed an increase in stability when incubated between assays with a high concentration of maltodextrins, showing exponential decay ($t_{1/2}$ = 199 h, a 453% increase on that achieved at the lower substrate concentration) (PZ 1), a large increase in stability of 240% and a more complex decay pattern (PZ 2A). Klyosov et al. (37) found that an increase in maltodextrin concentration did indeed stabilize immobilized glucoamylase against increasing temperature, proposing that glucoamylase has the ability to be stabilized by its substrate, the enzyme substrate complex being stabilized by its substrate, so it decays more slowly into an deactivated state than the free enzyme.

Glucoamylase immobilized on PZ 55 (PZ 54 produced without zinc oxide) by the indirect method had a half-life of 174 h, close to that achieved for glucoamylase coupled to washed PZ 2A support. Although it is clear that the presence of zinc oxide has a slight destabilizing effect, the mechanism remains unclear.

Glucoamylase bound to PZ 10 showed exponential decay with a long half-life when compared to other preparations ($t_{1/2}$ = 170 h). Glucoamylase immobilized on PZ 11 showed a very fast exponential decay having a $t_{1/2}$ of only 13 h. Glucoamylase immobilized on PZ 12 displayed a very short half-life of 21.6 h, having a 1/2 T of 32.4 h. The pattern of deactivation was only approximated by first-order decay, and the inverted linear decay model did not fit the data at all. Glucoamylase immobilized on PZ 13 showed the usual quicker decay for an acid chloride support approximating inverted linear decay having a t_L of 2.6 h and a 1/2 T of 3.0 h. PZ 11, PZ 12, and PZ 13 fit into the pattern of fast enzyme decays when the enzyme was immobilized near the support surface. Since PZ 9 base polymer contains a large number of aromatic groups, a fast enzyme deactivation would be expected owing to unfavorable hydrophobic interactions. Glucoamylase bound to PZ 11 and PZ 13 showed the usual quicker decay because of the presence of acid chloride groups on these supports. A major difference of PZ 10 when compared to PZ 11 and PZ 12 is the low

surface free carboxylic acid concentration of only 2 mg KOH/g. It is unclear how this may affect the stability of glucoamylase, since a pH optimum shift was not detected with any carboxylic acid support. There is evidence (*50*) that ionic supports decrease the stability of enzymes owing to disruption of internal salt linkages. Glucoamylase immobilized on PZ 17 shows an intermediate decay between exponential and inverted linear decay, having a 1/2 T of 28.0 h, a $t_{1/2}$ of 48.0 h, and a t_L of 1.4 h. Glucoamylase immobilized on PZ 18 (with a large amount of potential free acid chloride groups) showed the familiar quick decay, which tends to be better described using the inverted linear decay model.

Glucoamylase in the supports would suffer from internal diffusion and, therefore, may produce the low specific activities found, also playing a part in the deactivation of the enzyme coupled to the support. As one enzyme decayed, a new underused enzyme molecule would take over. This would produce a plateau followed by a sudden decay. The decay curve pattern of glucoamylase immobilized on the longer side arm $(AG)_2$ version of PZ 42 can be explained by the fact that the support's pores would be blocked by the chains of 1,6-diaminohexane and glutaraldehyde. This decay curve falls in between exponential and inverted linear decay, having a 1/2 T of 190.0 h, a $t_{1/2}$ of 216 h, and a t_L of 145.4 h. The increase in 1/2 T from 171 to 190 h must be related to the increase in distance the enzyme is from the support or multipoint binding. Glucoamylase immobilized on PZ 44 showed the usual quick decay of the enzyme immobilized onto an acid chloride support. PZ 44 was also rereacted with 1,6-diaminohexane and glutaraldehyde to produce an $(AG)_2$ support. The effect of the low concentration of acid chloride groups was almost mitigated, and the operation stability approached a $t_{1/2}$ of 150 h.

CONCLUSIONS

In this work, the viability of new epoxide-, isocyanate-, acid chloride-, and carboxylic acid-activated plastic supports for immobilizing glucoamylase, especially those reacting with 1,6-diaminohexane and glutaraldehyde for producing side arms, was shown. The best conditions (crosslinking, longer support's side arm, high substrate concentration) for extending operational stability, and the nonshifted pH and shifted temperatures profiles for soluble and immobilized glucoamylase were established. With respect to the kinetic parameters, the immobilized enzyme showed decreased k_{cat}/K_M ratio and specific activities when compared to the soluble enzyme. These results, disappointing from the point of view of functionality, will be confirmed or disproved by additional information forthcoming from alternative enzymes immobilized in a similar way. This will lead to conclusions as to their suitability for immobilizing enzymes.

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