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Glucoamylase Covalently Coupled to Porous Glass

LI GAOXIANG*, HUANG JIAYU, KOU XIUFEN, AND ZHANG SHUZHENG

Institute of Microbiology, Academia Sinica, Beijing, China

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Abstract

Glucoamylase (EC 3.2.1.3) was immobilized to alkylamine porous glass with glutaraldehyde. The choice and pretreatment of carrier and conditions for immobilization have been investigated. The immobilized enzyme contained about 4.0–8.0% protein and its activity was about 1000–1700 U/g. Some characteristics of the immobilized enzyme and the native enzyme have been comparatively investigated. The optimum temperature and the pH stability of the preparation were almost identical to the native one. However, the optimum pH of bound glucoamylase shifted 1.3 pH units toward the alkaline side compared to the native one. The Michaelis constant (K_m) of bound glucoamylase for soluble starch was about four times higher than that of the native enzyme, while K_m values for maltose approached those of the native material. At 45°C the half-life of IMG was 104 days under operational conditions. Alkaline protease, α-amylase, asparaginase, and penicillin acylase were also chemically coupled to porous glass by the same method and high relative activities were obtained.

Index Entries: Glucoamylase, coupling to porous glass; covalent coupling, of glucoamylase to porous glass; glass, coupling of glucoamylase to porous.

Introduction

Glucoamylase (E.C. 3,2,1,3) in soluble form is one of the most widely employed industrial enzymes. The enzyme has been immobilized to a multitude of different carriers by entrapment, adsorption, ion exchange, and covalent bonding.

The greatest number of attempts to immobilize glucoamylase have been made by covalent attachment to organic and inorganic carriers. Inorganic carriers are generally not subjected to microbial attack; they do not change configuration over

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an extensive pH range or under various solvent conditions, and with greater rigidity, they immobilize enzyme to a greater degree than do organic polymers.

Weetall et al.(*1*) first employed nitrous acid or glutaraldehyde to link glucoamylase to amine-activated porous glass. Latter, Lee et al.(*2*) found that glucoamylase bound to porous silica with glutaraldehyde was extremely stable on both laboratory and pilot plant scale.

This work deals with glucoamylase covalently coupled to alkylamine porous glass with glutaraldehyde. Condition for preparation has been optimized and the immobilized enzyme has been characterized.

Materials and Methods

Materials

Glucoamylase from *Asp. niger* (A.S.3.4309) was obtained from Wuxi Enzyme Factory in liquid form and was used after dialysis against water for 20 h. It had an activity of about 1400 U/mL, where one unit is the amount of enzyme that can liberate 1 μmol of reducing sugar (dextrose) from soluble starch per minute at pH 4.5 and 55°C.

α-Amylase from *Bacillus subtilis* was purchased from the Wuxi Enzyme Factory and had an activity of 2000 U/g, where one unit is the amount of enzyme that can liquefy 1 g of soluble starch in 1 h at pH 6.0 and 60°C.

Corn starch employed in continuous saccharification experiments was purchased from Huabei Pharmaceutical Factory.

α-Amylase-hydrolyzed dextrin solution with DE values ranging from 30 to 35 was obtained from corn starch.

Porous glass SB46-46 (250 mesh; pore diameter, 750–800 Å; silica, over 90%; pore volume, 0.7–1 cm^3/g; surface area, 65.6 m^2/g) and other kinds of porous glass were donated by Institute of Silicate Chemistry, Shanghai, China.

Corning porous glass (pore diameter, 550 Å) and Bio-glas were commercial products.

Preparation of ZrO_2-Coated Glass

ZrO_2-coated glass was prepared according to the method of Tomb and Weetall (*3*). Zirconyl chloride was used as a starting material to coat the porous glass by dissolving 3 g $ZrOCl_2$ in 25 mL water for each 10 g of porous glass. This solution was mixed with glass and then placed under a vacuum for about 15 min to remove air from the pores and allow the solution to enter the pores. The treated glass was then dried overnight at 145°C and then fired at about 375°C for 3 h.

Immobilization of Glucoamylase

Glucoamylase was attached to the alkylamine porous glass with glutaraldehyde following the procedure of Weetall (*1*).

One gram of porous glass was added to 10 mL of a 10% aqueous solution of γ-aminopropyltriethoxysilane that has been adjusted to pH 3.5 with 6*N* HCl. The

reaction mixture was heated to 75°C, refluxed for about 3 h, then washed several times with distilled water and oven dried at 120°C for 4 h. The resulting product is an alkylamine glass.

One gram of alkylamine derivative was added to a 20 mL of 2.5% glutaraldehyde solution (25% commercial glutaraldehyde solution diluted with 0.1*M* pH 7.2–7.5 phosphate buffer). The reaction was performed at room temperature for 1 h that included 30–45 min in a desiccator and attached to an aspirator to remove any air trapped in the particles. The carrier was then washed several times with distilled water.

For immobilization, about 100 mg glucoamylase was offered per gram of derivatized support material. The enzyme solution was diluted in cold 0.04 *M* phosphate buffer at pH 7.2–7.5 and added to the support material in an ice bath with occasional stirring. The solution was maintained at pH 7.2–7.5 and held for 15 h at 4°C. The immobilized enzyme (IME) was washed several times with about 500 mL of distilled water and stored at 4–8°C in distilled water.

Enzyme Assay

Native Glucoamylase. The enzyme was assayed at 55°C for 10 min at pH 4.5 using 1% soluble starch as substrate. The reaction was stopped in the boiling water bath for 5 min. Reducing sugars were determined by hypoiodite method according to Willstätter and Schudel (*4*). One unit of activity represents the production of 1 μmol of reducing sugar (dextrose) from soluble starch per minute at 55°C.

Immobilized Glucoamylase (IMG). IMG activity was assayed in 200 or 300 mL 1% soluble starch at 55°C for 10 min at pH 4.5 with stirring. The sugar produced was assayed as described above.

Other Enzymes. Alkaline protease was assayed by Folin's method. One unit of activity represents the production of 1 μmol of tyrosine from 1% casein/min. at 40°C.

The activity of asparaginase was determined according to the method of Peterson (*5*). A unit of activity is defined as the amount of enzyme that produces 1 μmol of ammonia from 0.3% asparagine/min at 37°C.

The activity of penicillin acylase was determined according to the method of Kutzbach (*6*). A unit of activity is defined as the amount of enzyme that produces 1 μmol of 2-nitro-5-aminobenzoic acid from 0.06% 2-nitro-5-phenylacetamido benzoic acid (NIPAB) per min at 37°C.

Protein Determination

Protein was determined by Lowry's method (*7*), using crystalline bovine serum albumin (BSA) as a standard. The amount of protein immobilized on the porous glass is the difference between the amount of protein before coupling and that left in liquid and washing solution after coupling.

Determination of DE Values

Values of DE (dextrose equivalent), a measure of the reducing power of the solution based on pure glucose as 100 and pure starch as near 0 were calculated. Solids content was determined by pycnometer.

Results

Choice and Treatment of Porous Glass

Pore Size and Surface Area. The effects of pore size and surface area on the activity of glucoamylase are shown in Tables 1 and 2. The observed activity increased with decreasing pore diameter with a maximum at 500 Å. Below this pore diameter, a drop in activity was observed (Table 1). The activity of IMG increased with increasing surface area of the porous glass (Table 2) with a maximum at 123 m^2/g. This may be related to the ability of either the enzyme or the substrate molecules to enter the pore, where the majority of the surface area is located.

Particle Size. The effect of particle size of porous glass on the activity of glucoamylase was studied. As shown in Table 3, the IMG activity increased with decreasing glass particle size.

TABLE 1
Effect of Pore Size on the Activity of Immobilized Glucoamylase[a]

Avg. pore diameter, Å	Enzyme coupled, U/g	Protein coupled, mg/g	Activity, U/g	Relative activity, %
200	2285	45	787	34
500	2992	46	1251	42
1000	1011	18	512	51
1500	1080	20	411	38

[a]The carrier was Bio-glas.

TABLE 2
Effect of Surface Area on the Activity of Immobilized Glucoamylase[a]

Porous glass No.	Surface area, m^2/g	Enzyme coupled, U/g	Protein coupled, mg/g	Activity, U/g	Relative activity, %
SB 126	123	7500	95.5	1175	15
SB 128	69	4500	45.5	954	21
SB 127	21	2500	9.5	650	26

[a]The carrier was Shanghai-porous glass.

TABLE 3
Effect of Particle Size on Activity[a]

Particle size, mesh	Enzyme coupled, U/g	Protein coupled, mg/g	Activity, U/g	Relative activity, %
100	2500	34.0	300	12.0
100–200	3750	40.0	657	17.5
200–250	3250	42.5	832	25.6
250–300	4250	56.0	950	22.3

[a]The porous glass was Shanghai SB 46-46.

KOH Treatment. Porous glass was placed in 0.5*N* KOH at 30°C for 1 h, filtered, washed with distilled water, and oven dried at 120°C. We found that pretreatment of glass could greatly increase the surface area, the amount of enzyme coupled, and the activity of IMG (Table 4).

Kinds of Carriers. The comparisons of IMG on various porous glasses with other carriers are given in Table 5. The results indicate that the efficiencies of the Shanghai glasses SB46-46, SB43-43, and SB43-48 were almost identical to those of the Corning glasses and Bio-glas. The activities ranged from 1037-1450 IU/g IMG on dry weight basis. γ-Porous alumina had the lowest activity. The activity of the zirconia-coated SB46-46 was lower than that of the uncoated SB46-46.

Choice of Optimal Conditions for Immobilization of Glucoamylase

In order to reduce the preparative cost of IMG, the preparative conditions for the immobilized enzyme have been investigated.

Silanization. Porous glass was silanized with γ-aminopropyltriethoxysilane in toluene or in distilled water following the procedure of Weetall (*8*).

Alkylamine glasses prepared in aqueous or organic solvent were used to bind the enzyme. As shown in Table 6, the quantity of enzyme coupled and the activity of IMG obtained by silanization in distilled water were higher than in toluene. Subsequently, the alkylamine glass was prepared by using water as a solvent. The influence of the amount and concentration of γ-aminopropyltriethoxysilane on the activity of enzyme was investigated. The data in Table 7 indicate that the quantity of enzyme bound and the activity of IMG were slightly influenced by the concentration of silane coupling agent (2–10%). However, maximum enzyme activity was obtained in the case of 10% of the agent.

The results of the silanization at varying pH values are listed in Table 8. It is observed that the enzyme bound and the enzyme activity were slightly increased by decreasing the pH values of silanization, and high activity was observed at pH 3.5.

Glutaraldehyde Activation. The alkylamine glass was activated with glutaraldehyde of various concentration. The results indicated that, between 0.5 and 2.5% of glutaraldehyde, the enzyme bound and IMG activity were similar,

TABLE 4
The Treatment of Porous Glass

Glass No.	Treatment	Surface area, m^2/g	Enzyme coupled, U/g	Protein coupled, mg/g	Activity, U/g	Relative activity, %
SB 46-46	None	20/7	560	14	213	38
SB 46-46	0.5*N* KOH 30° C,1 h.	89.5	3549	43	960	27

TABLE 5
Comparison of Immobilized Glucoamylase on Various Porous Glass and Alumina

Support material	Avg. pore diameter, Å	Surface area, m^2/g	Enzyme coupled, U/g	Protein coupled, mg/g	IMG activity, U/g	Relative activity, %
Corning glass	550	60–80	4360	56	967	15.7
Bio-glas	500	60–80	2990	46	1250	41.8
SB 46-46-Zr[a]	750	65	4808	46	1052	21.9
SB 46-46	750	65	5500	56	1458	26.5
SB 43-43	750	84	7170	64	1037	14.4
SB 43-48	750	45	4540	68	1171	25.7
Porous alumina	200	120	1987	—	375	18.9

[a]SB 46-46-Zr represents porous glass, ZrO_2-coated.

TABLE 6
Comparison of Silanization in Toluene and in Distilled Water

Silanization solvent	Enzyme coupled, U/g	Protein coupled, mg/g	Activity, U/g	Relative activity, %
Toluene	1915	23.8	733	38.2
Water	2992	42.4	1252	41.8

TABLE 7
Influence of the Amount and Concentration of Silane Coupling Agent on the Activity

Agent					
Concentration, %	Added, mL/g glass	Enzyme coupled, U/g	Protein coupled, mg/g	Activity, U/g	Relative activity, %
10	10	2700	35.5	1125	41.6
10	5	2800	35.5	960	34.2
4	5	2500	29.5	875	35.0
2	5	2300	21.5	865	37.6

TABLE 8
Effect of Silanization at Varying pH Values on the Activity

Silanization, pH	Enzyme coupled, U/g	Protein coupled, mg/g	Activity, U/g	Relative activity, %
3.5	4125	43	1000	24.2
5.5	3500	38	940	26.8
7.5	2875	33	885	30.8

with only slight differences (Table 9). Therefore the quantity of glutaraldehyde offered could be decreased to 0.5%.

The alkylamine glass was activated with glutaraldehyde at different pH values from pH 6 to pH 9, and then glucoamylase was attached to the activated glass at pH 7. The results obtained are shown in Table 10. The values of protein bound and IMG activity were slightly different, although a slightly higher activity was exhibited in the range of pH 6–7.

Enzyme Coupling. The pH optimum for the coupling of glucoamylase to activated glass was found to be in the range of pH 6–7 (Table 11). The enzyme bound and IMG activity were decreased with increasing pH values.

In order to determine the minimum quantities of coupling reagents suitable for immobilization of enzyme, 1 g of IMG was prepared with 5 mL of 2%

TABLE 9
Effect of Concentration of Glutaraldehyde on the Activity

Glutaraldehyde			Enzyme coupled, U/g	Protein coupled, mg/g	Activity, U/g	Relative activity, %
Concentration, %	Added, mL/g	pH				
2.5	20	7	7250	77.5	1010	13.9
1.5	20	7	8250	77.5	975	11.8
1.0	20	7	8550	81.5	920	10.7
0.5	20	7	7650	77.5	925	12.1
0.5	10	7	9050	81.5	930	10.2
2.5	20	9	6050	61.5	832	13.7

TABLE 10
The Effect of Glutaraldehyde Activation on the Activity at Difference pH Values

Activated pH	Enzyme coupled, U/g	Protein coupled, mg/g	Activity, U/g	Relative activity, %
6	5375	65	965	17.9
7	6375	61	900	14.1
8	5375	57	865	16.1
9	5375	49	895	16.6

TABLE 11
The pH Dependence for the Enzyme Coupled Reaction

Coupled pH	Enzyme coupled, U/g	Protein coupled, mg/g	Activity, U/g	Relative activity, %
6	3396	44.8	900	26.5
7	4000	40.8	910	22.7
8	2600	34.8	825	31.7
9	2400	30.8	750	31.2

γ-aminopropytriethoxysilane and 10 mL of 0.5% glutaraldehyde in an aqueous solution, as previously described. As shown in Table 12, the values for enzyme bound and IMG activity were slightly lower than those of the IMG prepared under usual conditions.

Comparison of Native and Bound Enzyme

Effect of pH on Activity. The relative activities of IMG and native enzyme as a function of pH are given in Fig. 1. The pH profiles for IMG and for the native enzyme are quite different, with the native enzyme showing an optimum at pH 4.7. The IMG was found to peak at pH 6.0, representing an 1.3 pH unit shift from the pH optimum for the native enzyme.

Temperature. The results are given in Fig. 2. Detectable deterioration of IMG activity and native glucoamylase activity began at temperature above 65°C. At temperatures higher than 65°C, both curves had the same slope; for lower temperatures, IMG activity decreased more slowly than native enzyme when temperature decreased. At 45°C the IMG still shows 87% of its maximum activity.

Effect of pH on Stability. The IMG was stable at pH 4, which was the same as the native glucoamylase (Fig. 3).

TABLE 12
Comparison of Two Preparations of Glucoamylase Attached to Porous Glass

Quantity of coupling reagents				Enzyme coupled, U/g	Protein coupled, mg/g	Activity, U/g	Relative activity, %
Amino-silane		Glutaraldehyde					
Conc., %	Added, mL/g	Conc., %	Added, mL/g				
10	10	2.5	20	3950	31.5	1173	29.6
2	5	0.5	10	2850	27.5	936	32.8

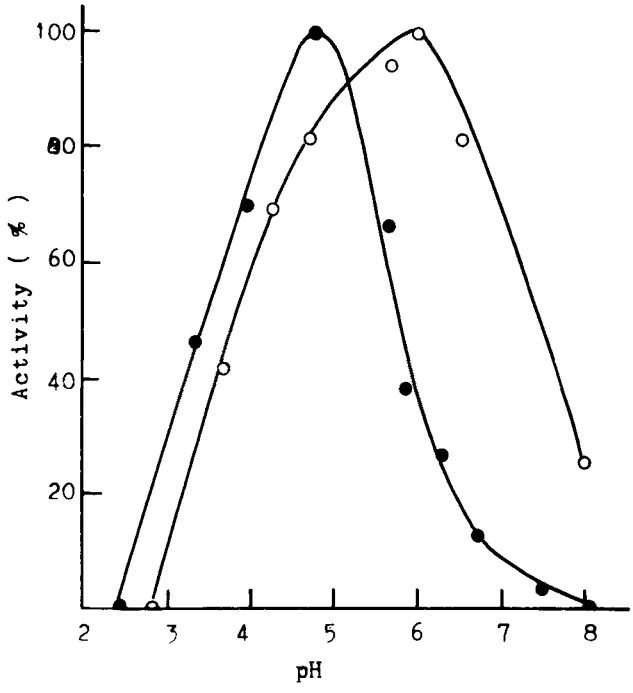

Fig. 1. pH profiles of both native and IMG. 100 μg of the native enzyme and 230 mg dry weight of IMG were used for each assay: ○, IMG; ●, native glucoamylase.

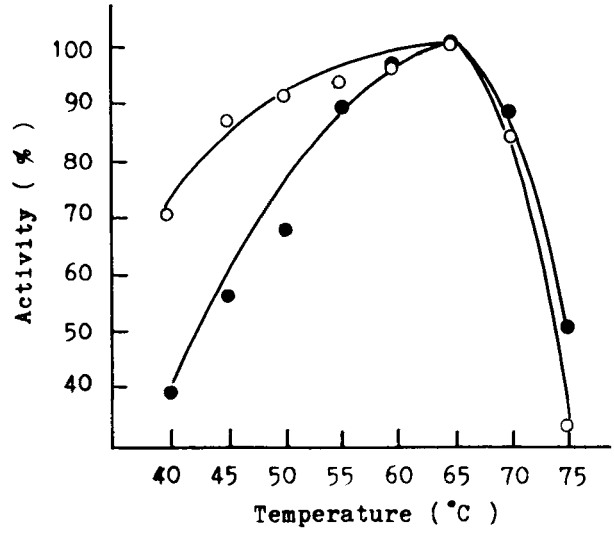

Fig. 2. Effect of temperature on activity of IMG and native glucoamylase. 70 μg of the native enzyme and 230 mg dry weight of IMG were used for each assay: ○, IMG; ●, native glucoamylase.

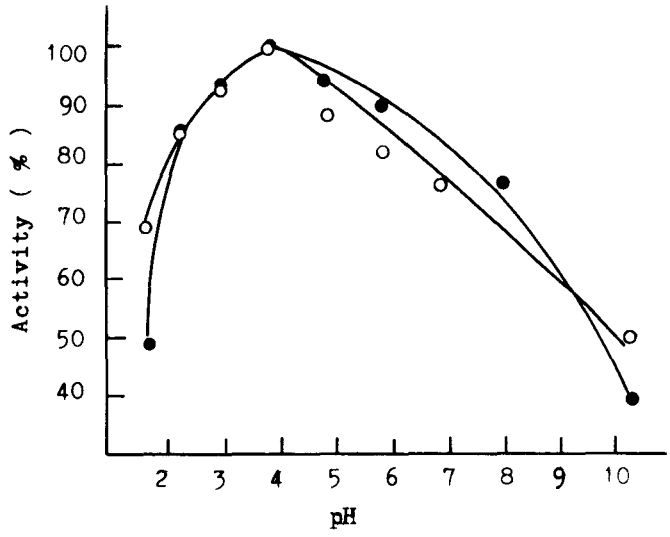

Fig. 3. Stability of native glucoamylase and IMG at various pH values: ○, IMG; ●, native glucoamylase. Samples of enzyme were incubated at various pHs at 28°C for 50 h and after adjusting to pH 4.5 portions of each sample were assayed as previously described. At pH 1.7, pH 2.3–7.9 and pH 10.3, 0.05*M* McIlvaine's buffer-HCl, 0.05*M* McIlvaine's buffer, and 0.1*M* borate buffer were used, respectively. Here, 56 μg of native glucoamylase and 230 mg of IMG were used for the enzyme assay.

Enzyme Kinetics. The K_m values of IMG and native glucoamylase were determined and the results are shown in Figs. 4 and 5. The K_m for maltose of IMG and of native enzyme were 3.84×10^{-3} and 3.3×10^{-3} g/mL, respectively. The apparent K_m IMG is a little larger than that of the native enzyme. The K_m values for soluable starch of IMG and native enzyme were 8.3×10^{-3} and 1.96×10^{-3} g/mL, respectively. The apparent K_m value of IMG is larger than that of the native enzyme.

Continuous Operation

The design of the operational studies is illustrated in Fig. 6. Operational data on columns are shown in Fig. 7. The glucoamylase columns were operated continuously for 64 days. During this period there was no decrease of DE values, and product DEs ranged from 90 to 99. The average DE was about 96. Half-life data are summarized in Table 13. The total activity of columns showed only a 34.7% loss over the 64-day period. The total volume of thinned cornstarch passed though the columns was 31.2 L. Half-life was determined by applying a least-squares regression to plots of ln (activity) vs time. The apparent half-life, calculated after 840 and 1536 h of operation at 45°C, was 104 days.

Immobilization of Some Other Enzymes

Alkaline protease, α-amylase, asparaginase, and penicillin acylase were also chemically coupled to porous glass SB 46-46 by the same method. As shown in

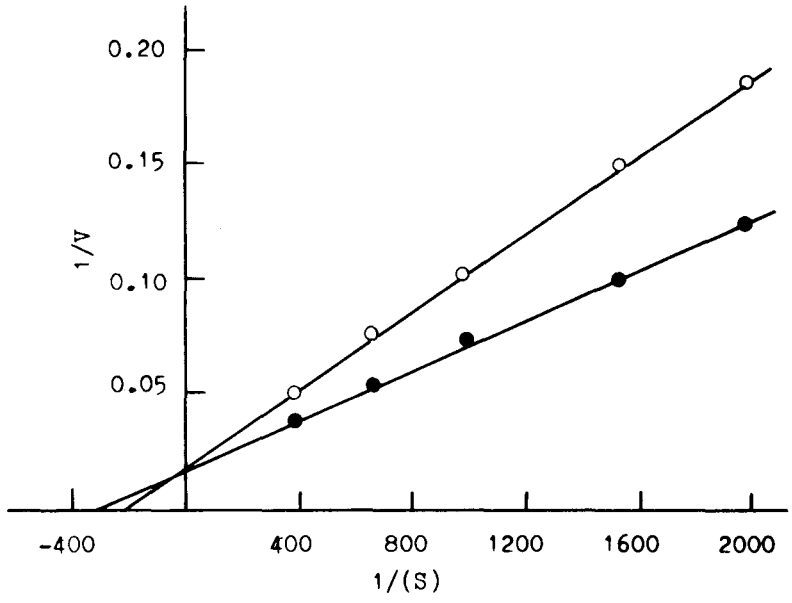

Fig. 4. Lineweaver-Burk plot of IMG and native enzyme substrate, maltose: ○, IMG, ●, native enzyme.

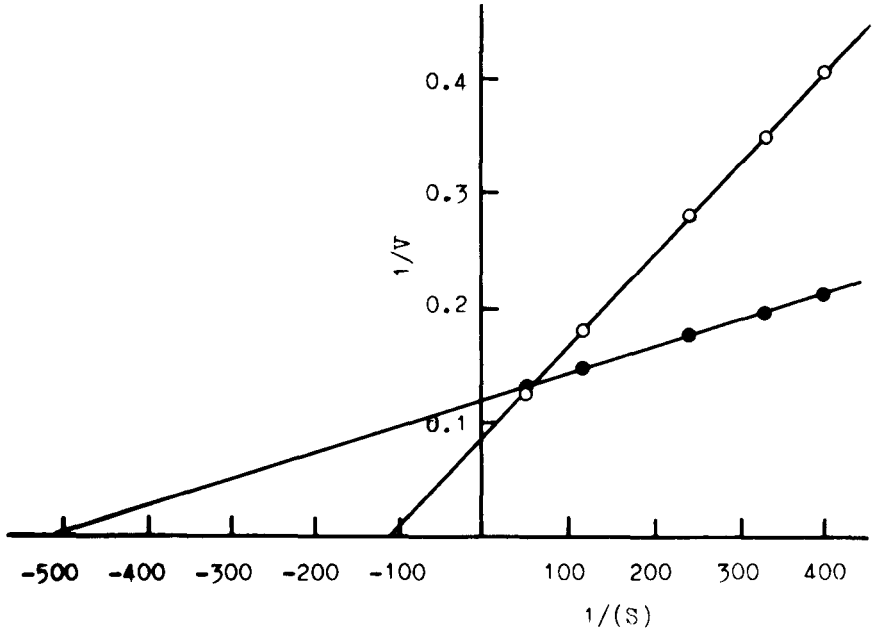

Fig. 5. Lineweaver-Burk plot of IMG and native enzyme substrate, soluble starch: ○, IMG; ●, native enzyme.

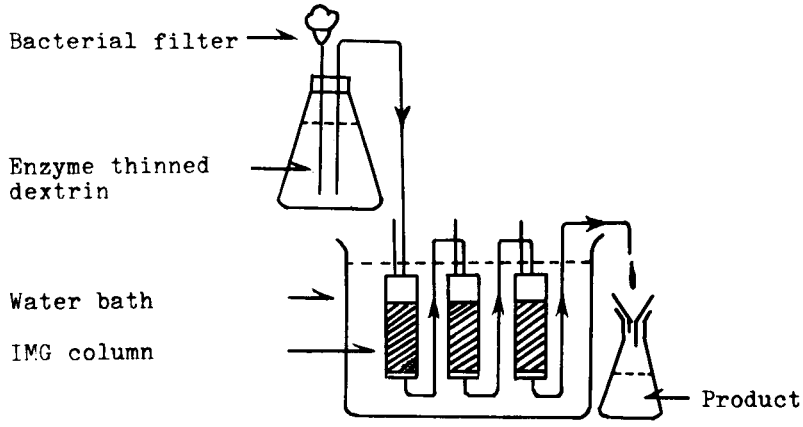

Fig. 6. Schematic diagram of packed-bed reactor used for the operational studies. Twelve grams (dry weight) of IMG was divided into three parts and packed in a series of three glass columns (1.9 cm id) with a bed volume of 10.7 cm^3, and a height of 3.8 cm.

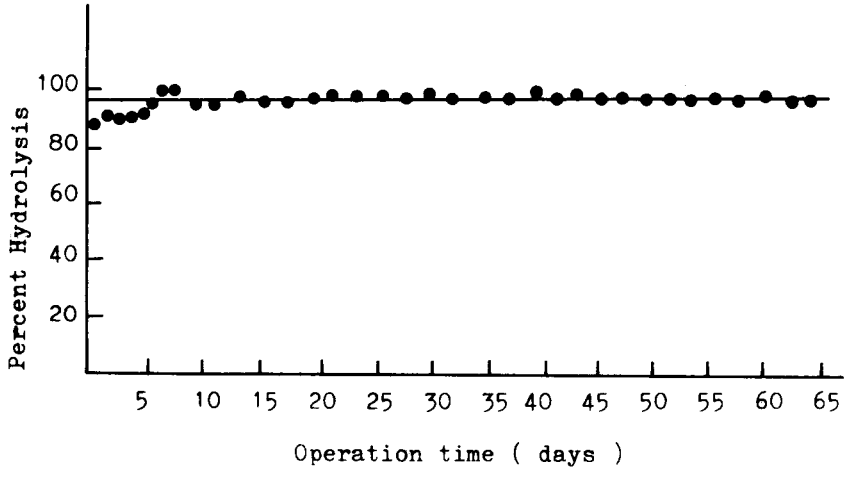

Fig. 7. Continuous operation of immobilized glucoamylase columns. The enzyme columns were continously operated for 64 days at 45°C and pH 4.5 using 28–33 DE enzyme-thinned dextrin (21–22 wt%) containing 0.1% of sodium benzoate as substrate feed, at flow rates of 20.3 mL/h.

Table 14, the relative activities of these immobilized enzymes were rather high. Specifically, immobilized penicillin acylase had 94.5% activity compared with that of the native enzyme toward NIPAB of low molecular weight.

Discussion

The work reported here has yielded several noteworthy results.

Greater increases in activity may be achieved by alkali treatment of the porous glass. One possible explanation for this is that alkali-treated glass appears to have

TABLE 13
Column Half-Life Results

Carrier experiment	Operation time, h	Initial activity, U/g	Remaining activity		Decay constant, h^{-1}	$t^{1/2}$, days, at 45°C
			U/g	%		
Porous glass	840	1704	1352	79.3	2.75×10^{-4}	104.8
SB 46-46	1536	1704	1113	65.3	2.77×10^{-4}	104.2

TABLE 14
Enzymes Covalently Coupled to Porous Glass SB 46-46

Enzymes	Assay of enzyme activity			Enzyme added, U/g	Enzyme coupled, U/g	Protein coupled, mg/g	Activity. U/g	Relative[a] activity, %
	Substrate	pH	Temp., °C					
Alkaline protease	Casein	10.0	40	29400	24360	45.0	12141	49.8
α-Amylase	Starch	6.0	60	4266	927	53.3	295	31.8
Asparaginase	L-Asn.	8.4	37	13040	9840	93.6	5440	55.3
Penicillin acylase	NIPAB[b]	7.7	37	62.6	51.3	53.6	48.5	94.5

[a]The activity of native enzyme was taken as 100.
[b]NIPAB, 2-nitro-5-phenylactamidobenzoic acid.

greater surface area and the glass surface thus activated permits an increase in the quantity of enzyme coupled.

Zirconia coating is known to increase the alkaline durability of porous glass and to increase substantially the half-life of the immobilized enzyme. In our laboratory, the IMG, when operated at 50°C, give a half-life on the ZrO_2-Bio-glas 500 of 54.4 days longer than that observed on the uncoated Bio-glas 500 ($t_{1/2}$, 46.8 days). However, the observed specific activities of coated glasses were lower. It is probable that zirconia coating causes a decrease in the number of available reactive groups on glass. These results are similar to those of Weetall et al.(*1*).

The data shown in Table 2 indicate that the enzyme activity increases with an increase in the surface area. However, for glass with an average pore diameter of 200 Å, the activity level dropped to lower value (Table 1). The reduction in the activity of IMG is caused by steric hindrance between the carrier and substrate or by the inability of the enzyme molecules to enter the pore, where the majority of surface area is located.

The lower activity for large particle size glass may in fact be indicative of internal diffusion control resulting from the large particle size.

Most of the IMG cost will be attributable to carrier and immobilization costs. For industrial applications, it is necessary to decrease the quantity of reagents in the immobilization process. From the results it can be seen that the quantity of reagents could indeed be decreased to one-fifth or one-tenth the original quantity.

The pH profile of the IMG yielded some interesting data. The glutaraldehyde derivative showed optimum activity at a higher pH than the native enzyme, which is different from the result of Weetall et al.(*1*). If we assume that the pH optimum of the enzyme itself does not change, this indicates that the pH at the surface of the glass carrier was more acidic, either because of the silanols or the added enzyme. That is, the microenvironment of IMG becomes more acidic than that of the external solution. Accordingly, the enzyme reaction effectively proceeds on the acidic side of the external buffer pH, and the optimum pH apparently shifts to the alkaline side. However, the effect of pH on the stability of the IMG (Fig. 3) is almost the same as that of the native one. The optimum pH is approximately 4.

The results show that no change of optimum temperature occurs on immobilization; however, for lower temperatures, the bound enzyme activity decreased more slowly than the native enzyme when temperature decreased. The IMG still shows 87% of maximum activity at 45°C. Thus, it is of great advantage to lower operational temperature by using this IMG. It is probable that an increase in total product could be obtained by operating at lower temperature.

It is intersting to note that the K_m values of IMG for starch are about four times higher than those of the native enzyme. The change in K_m values upon immobilization was considered to be caused by diffusion limitation of the substrate and product in the carrier and resulted in an apparent increase in K_m. In the case of maltose, the effect of internal diffusion of the substrate and product on the reaction rate became less and the K_m value approached that of the native enzyme.

Glucoamylase bound to porous glass with glutaraldehyde was very stable; no decrease in the product DE of about 96 was observed after operation for 64 days at

45°C and the half-life was calculated to be about 104 days. This enzyme preparation, we feel, might be adequate for certain industrial saccharification purposes.

References

1. Weetall, H. H., and Havawala, N. B., in *Enzyme Engineering,* Wingard, Jr., L. B., ed. Wiley, New York, 1972, pp. 241–266.
2. Lee, D. D., Lee, Y. Y., Reilly, P. J., Collins, Jr., E. V., and Tsao, G. T., *Biotechnol. Bioeng.* **18,** 253 (1976).
3. Tomb, W. H., and Weetall, H. H., US Patent 3,783,101, January 1 (1974); Immobilized Enzymes: Preparation and Engineering Techniques, *Chemistry Technology Review* No. 39, Gutcho, S. J. ed., Noyes Data Corporation, Park Ridge, New Jersey, pp. 92–98.
4. Willstätter, R., and Schuded, G., *Ber.* **51,** 780 (1918); Browne, C. A., and Zerban, F. W., *Physical and Chemical Methods of Sugar Analysis,* third edition, 1948, pp. 895–896.
5. Peterson, R. E., and Ciegler, A. *Appl. Microbiol.* **17**(6), 929 (1967).
6. Kutzbach, C., and Rauonburth, E., *Hoppe-Seylers Z. Phys. Chem., Bd* **354,** S 45, (1974).
7. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Ranadall, R. J., *J. Biol. Chem.* **193,** 265 (1951).
8. Weetall, H. H., in *Enzymology,* vol. 1, *Immobilized Enzymes Antigens, Antibodies and Peptides, Preparation and Characterization,* edited by H. H. Weetall, Dekker, New York (1975).