Characterization of Immobilized Glucose Oxidase–Catalase and Their Deactivation in a Fluid-Bed Reactor

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ABSTRACT

2-Amino-4-chloro-*s*-triazine, a derivative of DEAE-cellulose, and acrolein/styrene copolymer were used as supports for the immobilization of glucose oxidase and catalase after being modified with diaminohexane followed by glutaraldehyde. Immobilization was carried out with optimum glucose oxidase–catalase ratios. The activity variations of the immobilized dual-enzyme systems were investigated in relation to pH and temperature. Time-dependent gluconic acid production resulting from the oxidation of glucose was monitored in a recycling fluid-bed reactor. The deactivation rates of glucose oxidase and catalase were investigated according to the first-order reaction kinetics depending on the presence of the intermediate product H_2O_2.

Index Entries: Immobilization of GOD and CAT on the support; immobilized GOD–CAT; properties of immobilized GOD–CAT; gluconic acid production with GOD–CAT; deactivation of GOD and CAT by H_2O_2.

INTRODUCTION

Multienzyme systems have drawn great interest in the industrial, medical, and analytical applications of enzymes. Removal of glucose from

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fruit juices (*1*) and liquid egg concentrates (*2*); removal of oxygen from beer (*3*); and determination of glucose in blood and serum (*4*) have been achieved by the use of glucose oxidase (GOD) and catalase (CAT) combinations. Numerous immobilizations based on physical and chemical methods have been carried out in order to expand the scope of these applications (*5–9*). Some variations may take place in the optimum pH, temperature parameters, and kinetic properties of GOD and CAT as a result of immobilization.

In the present work, activity variations of immobilized GOD and CAT systems in relation to pH and temperature, as well as the product formation rates and deactivation rates of both enzymes in fluid-bed reactor systems, have been investigated.

MATERIALS AND METHODS

Glucose oxidase (from *Aspergillus niger*) and peroxidase (from horseradish) were obtained from Sigma Chemical Co., St. Louis, MO. Catalase (from Fungal), DEAE-cellulose, acrolein, styrene, trichloro-*s*-triazine, diaminohexane, glutaraldehyde (GDA), β-D-glucose, and H_2O_2 (30%) were obtained from E. Merck, Darmstadt, FRG. All other chemical reagents used were of analytical grade.

The GOD activity was calculated from measurements carried out with an oxygenmeter of the oxygen consumption rates at 25°C in a completely filled reaction volume of 115 mL, using β-D-glucose (13.9 m*M*) prepared in a 0.1*M* citrate–phosphate buffer (pH 5.6) and oxygen at saturation concentration ($2.5 \times 10^{-4} M$). In contrast, the CAT activity was calculated from the oxygen production rate with $5 \times 10^{-4} M$ H_2O_2 in the same buffer as above, after removal of the dissolved oxygen by N_2 gas (*10*). The activities of GOD (4.1 mg/mL) and CAT were found to be 2000 μmol O_2/mL and 3600 μmol H_2O_2/mg, respectively. The activity measurements of immobilized GOD without CAT were carried out after inhibition with azide ions ($10^{-3} M$). The dimensions of the reactor used in the studies carried out in a recycling fluid-bed reactor are 1.6×6.0 cm, and the volume of the reaction chamber is about 40 mL (Fig. 1). The temperature in the reactor and in the reaction chamber was held constant at 25°C by thermostatic means. The O_2 required for the reaction was supplied by pumping air in through washing bottles and then dispersing it through a sintered glass outlet. Gluconic acid produced during the reaction was titrated with 0.5*N* NaOH, and the pH was kept constant at 5.6.

In this study the amount of glucose in the reaction medium was determined by glucose oxidase–peroxidase using *o*-dianisidine as the indicator, and the amount of H_2O_2 was determined with peroxidase (*11,12*).

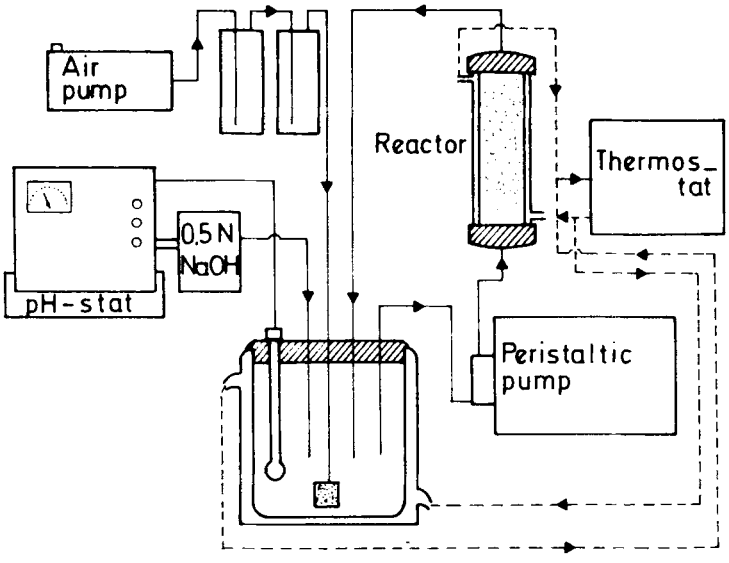

Fig. 1. Recycling fluid-bed reactor.

Modification of the Supports and Immobilization of Enzymes

Initial modification of DEAE-cellulose was effected by 2-amino-4, 6-dichloro-*s*-triazine (AsT) obtained according to the method of Kay and Lilly (*13*). DEAE-cellulose (7 g) preswollen in the same solvent mixture was suspended in a solution of AsT (2.4 g) in 70 mL of water/acetone (1:1). A 17% Na_2CO_3 solution containing 0.6 vol 1*M* (HCl was added to the reaction medium at 50°C through a side inlet for a period of 5 min. The pH was adjusted to less than 7.0 by addition of about 4 mL concentrated HCl over 10 min, and the reaction mixture was washed several times with aqueous acetone solutions of decreasing acetone concentration.

Aminochloro-*s*-triazine, a derivative of DEAE-cellulose, was suspended in 100 mL of 0.1*M* borate buffer (pH 8.7), and 100 mL of 2*M* diaminohexane prepared in the same buffer was added to the reaction medium. During the final modification stage, the support (4 g) was reacted with 4% GDA prepared in 60 mL 0.1*M* borate buffer (pH 8.7) at 25°C for a period of 21 h.

The acrolein/styrene copolymer that was the second support (II) used in the immobilization studies was obtained by radical copolymerization of the appropriate monomers in a 1:1 molar ratio in the presence of dibenzoyl peroxide under a nitrogen atmosphere at 45°C for a period of 48 h. The copolymer, which was of a nonporous structure, was milled and fractionated to obtain particles in the 0.1–0.2-mm size range. The modification of the copolymer (0.5 g) via the carbonyl groups was achieved by reacting it overnight at 70°C with a 5 mL solution of diaminohexane (0.75 g) having a pH of 8.0. The resulting imine bonds were reduced with 50 mL sodium

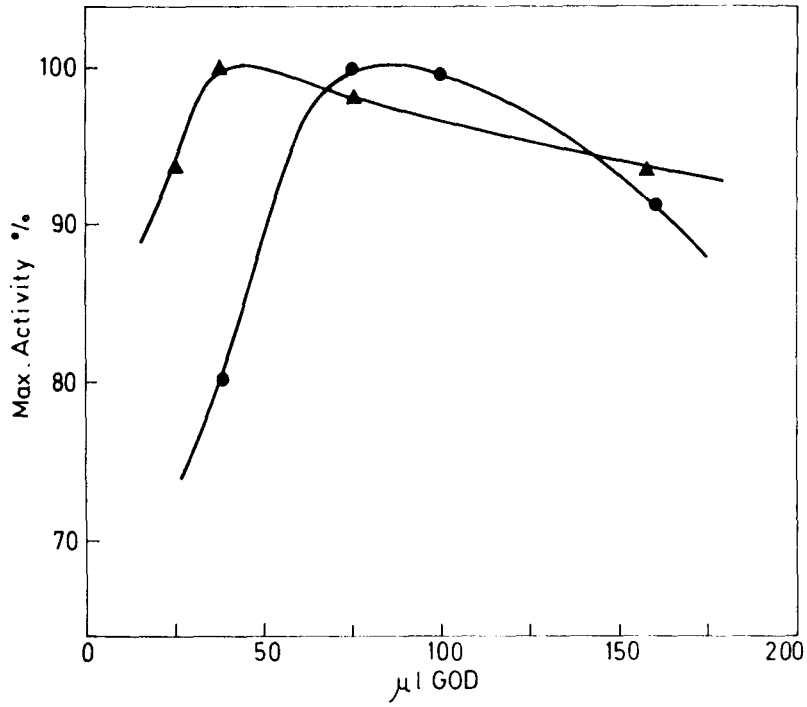

Fig. 2. Variation of GOD activity in relation to the immobilizations effected with varying amounts of GOD/CAT, having an activity ratio of 1/7 in the immobilization medium. The GOD activities were measured with $[G]=13.9\times10^{-3}M$ and $[O_2]=2.5\times10^{-4}M$, at pH 5.6 and 25°C. Preparations I (●) and II (▲).

borohydride (2 mg/mL) at 50°C for a period of 4 h. Further modification after reduction via the amine bonds was carried out with 50 mL of 1.5% GDA solution prepared in 0.1*M* sodium carbonate (pH 9.0) at room temperature for 150 min.

In order to obtain a good efficiency and fast destruction of the H_2O_2 produced by GOD, the activity ratios of the immobilized GOD/CAT system should not be above 1/1 (*14*). Therefore, in the series of immobilization reactions carried out with varying amounts of GOD(2000 μmol O_2/mL) and CAT (3600 μmol H_2O_2/mg), the GOD/CAT activity ratio in the reaction medium was kept at 1/7. Immobilization of GOD and CAT prepared in 50 m*M* phosphate buffer at pH 7.0 on the modified supports I (15 mg) and II (30 mg) was effected in a total volume of 3 mL, at +4°C, overnight.

The GOD and CAT activities of the preparations were measured at 25°C in 0.1*M* citrate-phosphate buffer (pH 5.6) on an oxygen meter.

RESULTS

The immobilization reactions carried out with varying amounts of GOD and CAT show that the activity of GOD bound to the support increases up to a certain point, and then drops slightly (Fig. 2). From Figure 2, the

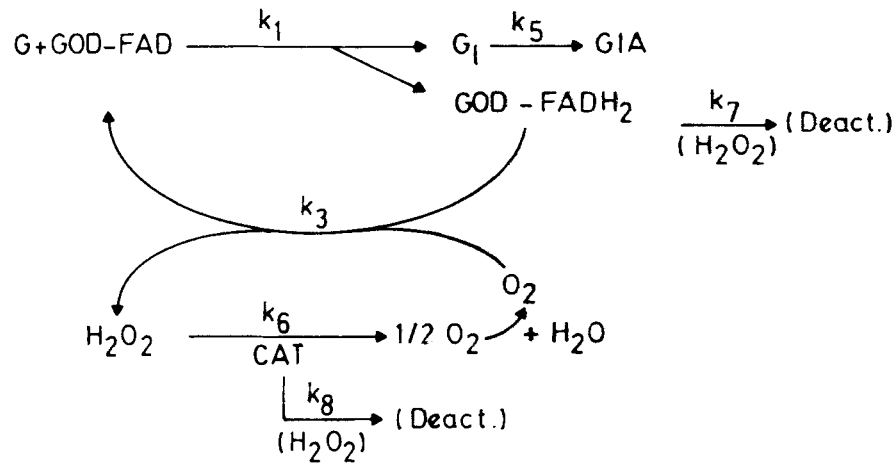

Fig. 3. The GOD–CAT catalyzed reaction scheme.

optimum activity ratios of GOD and CAT in the immobilization medium were found to be 150/1050 and 80/560 for the modified supports I (15 mg) and II (30 mg). The GOD/CAT activity ratios for the immobilized preparations I and II under optimum conditions were found to be 44.28/96.12 and 5.87/53.58, respectively.

The reaction scheme for the oxidation of glucose (G) by a GOD and CAT combination, according to the simplified pseudohomogenous model, is shown in Figure 3. Gloconic acid (GlA) is produced as a result of the first-order hydrolysis reaction of gluconolactone (G_1), which is an intermediate product in the oxidation of glucose.

The reaction rate for the combined reaction steps of glucose (G) and oxygen (O_2) is given by

$$1 / v = (1 / k_1[G]) + (1 / k_3[O_2]) \quad (1)$$

The equation gives the exact rates for rate-limiting glucose or oxygen concentration levels, respectively ($v = k_1[G]$ or $v = k_3[O_2]$) (*15*).

In a batch-type reactor, the variation of glucose concentration in the medium in relation to time was monitored using preparation I (15 mg) with $[G] = 2 \times 10 - 4M$ and $[O_2] = 2.5 \times 10^{-4}M$ (air saturation at pH 5.6 and 25°C; (Fig. 4). The observed rate constant in accordance with pseudo-first-order reaction kinetics is given by

$$k_1 = 3.04 \times 10^4 \text{ 1/mol-s}$$

The pseudo-first-order reaction rate with respect to the oxygen is valued at high glucose concentrations. The rate constant obtained by following the time-dependent variation of oxygen under the same condition with $[O_2] = 2.5 \times 10^{-4}M$ and $[G] = 13.9 \times 10^{-3}M$ is given by

$$k_3 = 8.86 \times 10^5 \text{ 1/mol-s}$$

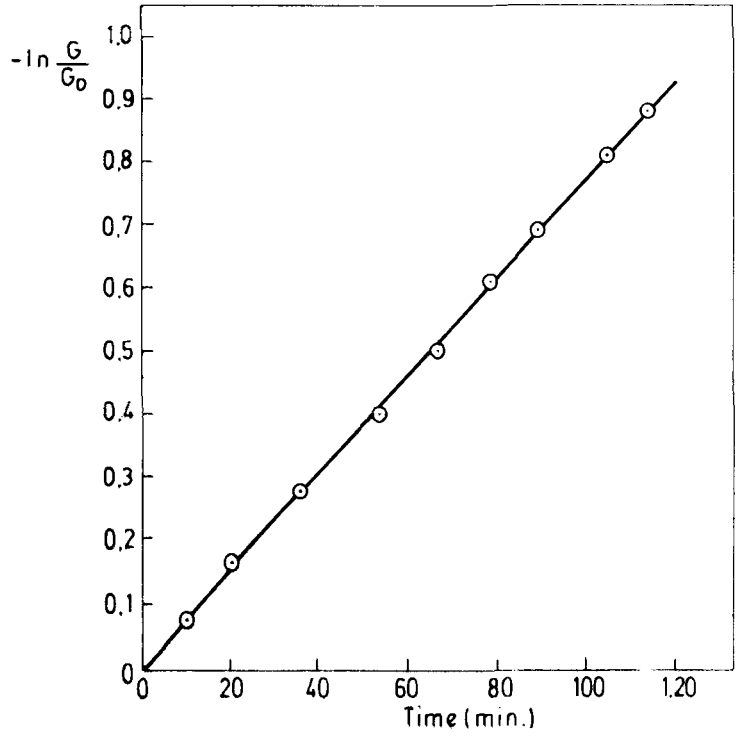

Fig. 4. Conversion of glucose as a function of time. Immobilized preparation I (15 mg); $[G]=2\times10^{-4}M$ and $[O_2]=2.5\times10^{-4}M$, ph=5.6; 25°C.

pH-Dependent Activity and Stability Relationships

Activity variations of immobilized GOD–CAT preparations in citrate–phosphate buffers of different pH values were investigated using O_2 at a concentration of $2.5\times10^{-4}M$ and glucose at a concentration of $13.9\times10^{-3}M$ at 25°C. As can be seen from Fig. 6, the pH-activity variation of preparation I is quite similar to that of the native GOD, with the exception that the curve has a more spread-out bell shape. A slight shift to the alkaline region was observed for the optimum pH value in the case of preparation II.

The retained activity values of the native and immobilized preparations were measured under the same conditions as was the activity, after storing in buffers of different pH values at 30°C for a period of 24 h (Fig. 7). Preparation I was seen to gain better stability properties than native GOD. When the same procedure was repeated for CAT, practically no activity loss was observed in the pH range of 5.0–7.5.

Temperature-Dependent Activity and Stability Relationships

In the case of preparation I, activity variations measured at pH 5.6 and different temperatures were quite similar to those of native GOD,

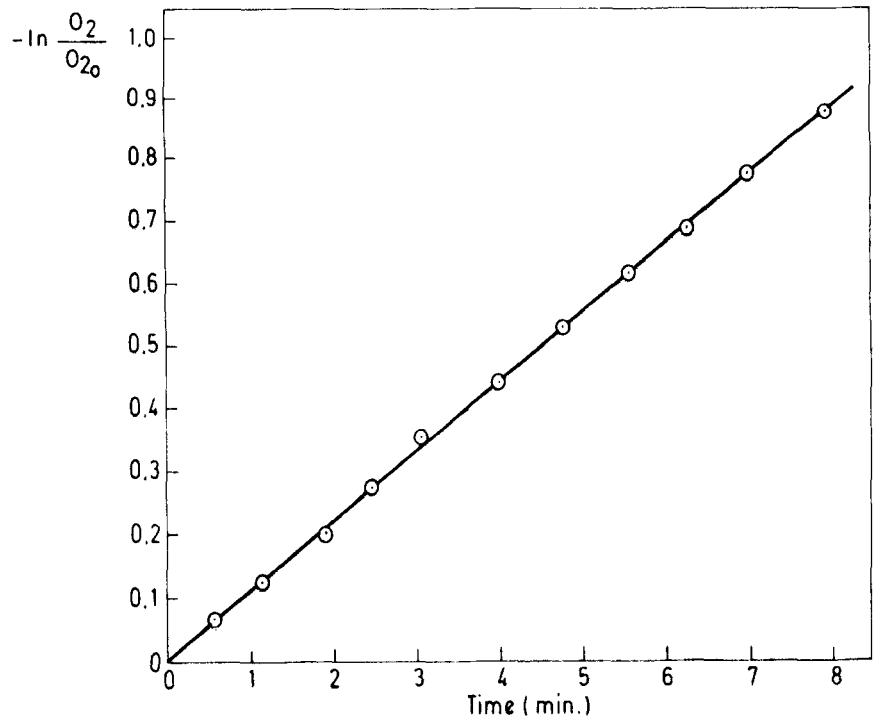

Fig. 5. Conversion of oxygen as a function of time. Immobilized preparation I (15 mg); $[O_2]=2.5\times10^{-4}M$; $[G]=13.9\times10^{-3}M$; pH=5.6; 25°C.

whereas the temperature dependency of activity was found to be somewhat reduced in preparation II (Fig. 8). The retained activity values, obtained after incubation in citrate–phosphate buffer of pH 5.6 at the same temperatures for a period of 6 h, showed that preparation II behaved in a manner quite similar to that of native GOD, whereas preparation I behaved in a more stable manner (Fig. 9). When the same procedure was repeated for CAT, no activity loss was observed in the temperature range of 20–50°C.

Application in a Fluid-Bed Reactor

The reaction rates within the reactor system were monitored, using 100 mg each of preparation I and II with 0.3*M* glucose and $2.5\times10^{-4}M$ O_2 concentrations at pH 5.6 and a flow rate of 8.7 mL/min, by measuring the amount of NaOH spent for neutralization of the gluconic acid produced with time. The amount of gluconic acid formed as a result of oxidation in the first 20 min was taken as the initial rate (v_0). The time-dependent variation of rate was monitored as v/v_0 (Fig. 10).

The values of v/v_0 for preparations I and II become approximately constant after a certain increase. The maximum value of v/v_0 is attained sooner for preparation II, in which the immobilization has taken place on the surface.

The hydrolysis step of gluconolactone to gluconic acid was followed by automatic titration in a recycling fluid-bed reactor. The approximately

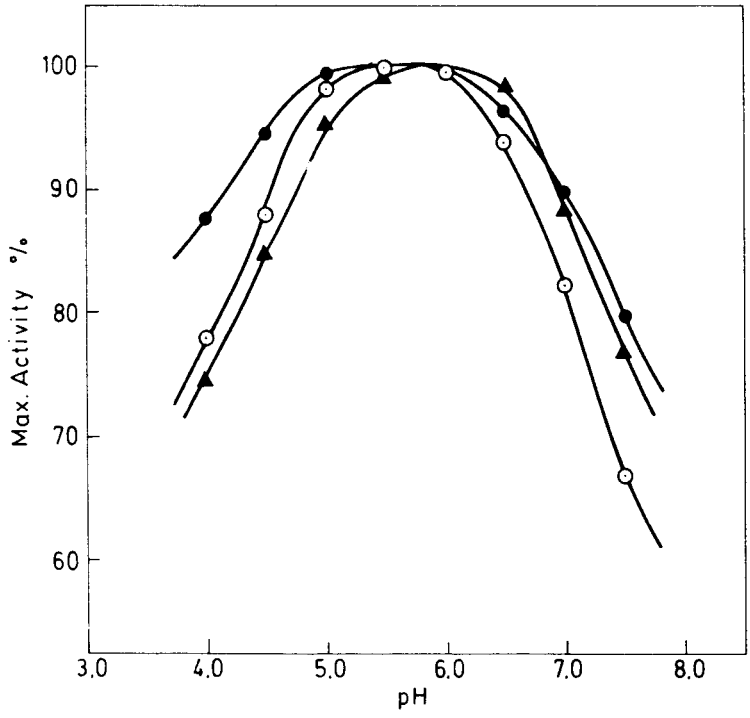

Fig. 6. The pH-activity profiles for the native and immobilized GOD. $[O_2]=2.5\times10^{-4}M$; $[G]=13.9\times10^{-3}M$; 25°C; native GOD (○); preparations I (●) and II (▲).

constant rate for the first-order reaction, obtained with 0.1*M* glucose and 100 mg of immobilized preparation I under the same conditions, was found to be

$$k_5 = 0.3 \times 10^{-4}\,s^{-1}$$

The effect of the quasistationary H_2O_2 concentration obtained experimentally on the deactivation of both enzymes was monitored with respect to time by continually repeated oxidation of 0.1*M* glucose in the reactor (Fig. 11). GOD and CAT are deactivated by H_2O_2, according to first-order reaction kinetics (*16,17*).

$$-(\text{d GOD} / dt) = k_7 \cdot H_2O_2 \cdot \text{GOD} \quad (2)$$

$$-(\text{d CAT} / dt) = k_8 \cdot H_2O_2 \cdot \text{CAT} \quad (3)$$

The deactivation rate constants calculated from the activity half-lives of GOD and CAT, respectively, were found to be

$$k_7 = 2.95 \times 10^{-3}\ 1/\text{mol-s}$$

$$k_8 = 2.23 \times 10^{-3}\ 1/\text{mol-s}$$

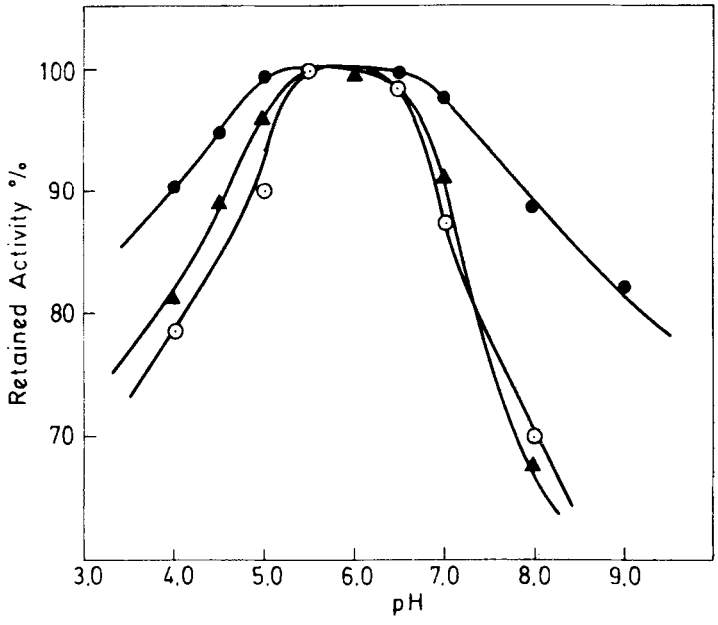

Fig. 7. The pH-stability of native and immobilized GOD at 30°C and for 24 h. The GOD activities were measured with $[G] = 13.9 \times 10^{-3} M$; $[O_2] = 2.5 \times 10^{-4} M$; at pH 5.6 and 25°C. Native GOD (○); preparations I (●) and II (▲).

The decomposition of H_2O_2 that is formed during the reaction by CAT that is immobilized in the same matrix as GOD appears to have the effect of increasing the activity half-life of GOD.

DISCUSSION

The enzymes GOD and CAT, immobilized on modified DEAE-cellulose and acrolein/styrene copolymer in optimum ratios, showed some variations in their pH- and temperature-related properties. The bonding of GOD and CAT to the support by glutaraldehyde takes place via the ω-NH_2 and α-NH_2 groups present in the enzyme molecules. Hence, there may be some differences in the total charges of the particles after immobilization. This situation causes the enzyme to exhibit activity at a different pH value in the macroenvironment than it does in the microenvironment (*18,19*). No significant change takes place in the total charge as a result of immobilization on the modified DEAE-cellulose; hence, the optimum pH value is the same as for the native GOD. However, immobilization on a support with a higher hydrophilic character causes the pH-dependent activity variation curve to become more spread-out. The total negative charge increases as a result of immobilization on the acrolein/styrene-

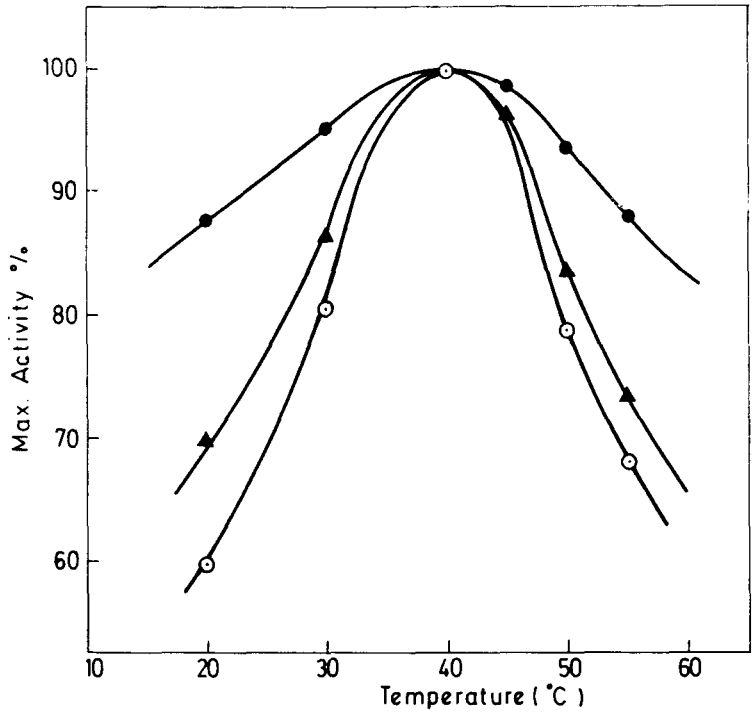

Fig. 8. Temperature-activity profiles for the native and immobilized GOD. $[O_2]=2.5\times10^{-4}M$; $[G]=13.9\times10^{-3}M$; pH=5.6; native GOD (○); preparations I (●) and II (▲).

based support, but the shift of the optimum value to an associated position is reduced considerably because of diffusion being at a minimum level as a result of immobilization on the surface. hence, following immobilization, GOD gains more stable characteristics with respect to pH and temperature.

One of the important factors affecting the efficiency of the immobilized-enzyme systems is the limitation of the substrate diffusion. Although the value of k_3/k_1, which gives the relative rates for oxygen- and glucose-coupled reaction steps, is 140 for the native enzyme, this value was found to be as low as 29.14 for the immobilized GOD (preparation I). This situation is explained by the fact that the k_3 value of the much faster reaction step is much higher than that of the reaction step in which the limitation of oxygen diffusion in the matrix is dependent on the presence of glucose. In a recycling fluid-bed reactor system, maximum reaction rates are attained after a certain period of time. This situation may be caused by the hydrolysis rate of gluconolactone at pH 5.6 as well as the limitation of diffusion of the substrate and product in the microenvironment (*20*). In the case of acrolein/styrene-based support, in which the immobilization has been effected on the surface, the fact that maximum value is attained in a shorter time at lower v/v_0 values reveals the effect of the limited diffusion.

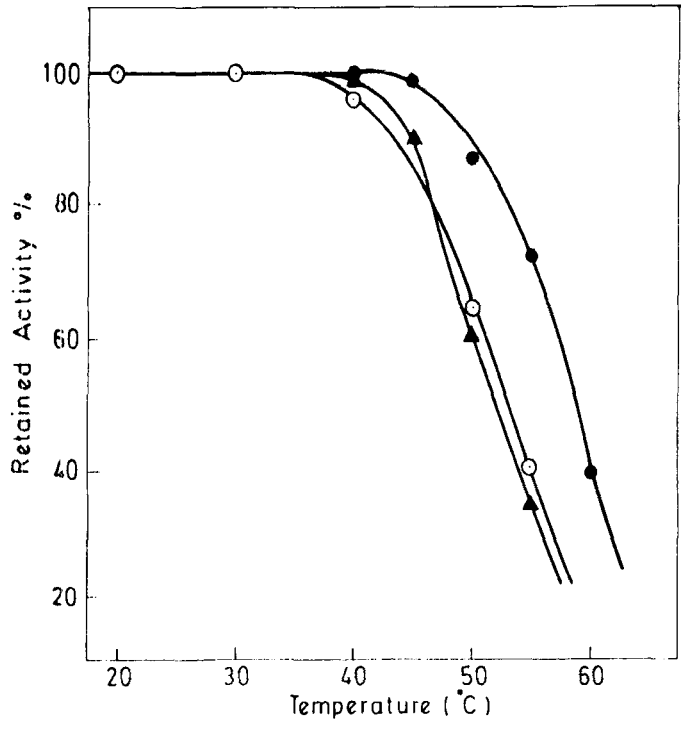

Fig. 9. Thermal stabilities of the native and immobilized GOD at pH = 5.7 for 6 h. The GOD activities were measured with $[G] = 13.9 \times 10^{-3} M$ and $[O_2] = 2.5 \times 10^{-4} M$, at pH 5.6 and 25°C. Native GOD (○); preparations I (●) and II (▲).

The ability of the intermediate product H_2O_2 to deactivate both GOD and CAT is the cause of yet another important problem encountered in the GOD–CAT systems. However, when both these enzymes are bound to the same support, the deactivation rates, which could be high because of the limited diffusion of H_2O_2, are in fact reduced to the utmost. The deactivation rate of the immobilized GOD–CAT system is the main factor determining the economics of its applications.

SUMMARY

2-Amino-4-chloro-s-triazine, a derivative of DEAE-cellulose, and acrolein/styrene copolymer were used as supports for the immobilization of glucose oxidase and catalase after being modified with diaminohexane followed by glutaralhedyde. Immobilization was carried out with optimum GOD/CAT ratios. The activity variations of the immobilized dual-enzyme systems were investigated in relation to pH and temperature. Time-dependent gluconic acid production resulting from the oxidation of glucose was monitored in a recycling fluid-bed reactor. The deactivation rates of

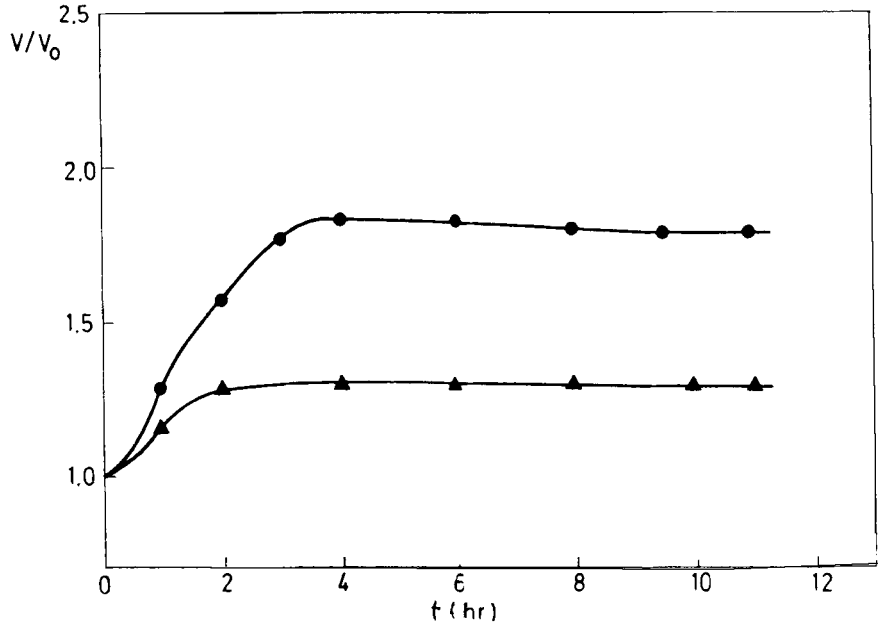

Fig. 10. The time-dependent variation of rate in a recycling fluid-bed reactor. The reaction rates were measured with 100 mg each of preparations I (●) and II (▲); [G] = 0.3*M*; [O_2] = 2.5×10^{-4}*M*; pH 5.6; 25°C; flow rate, 8.7 mL/min.

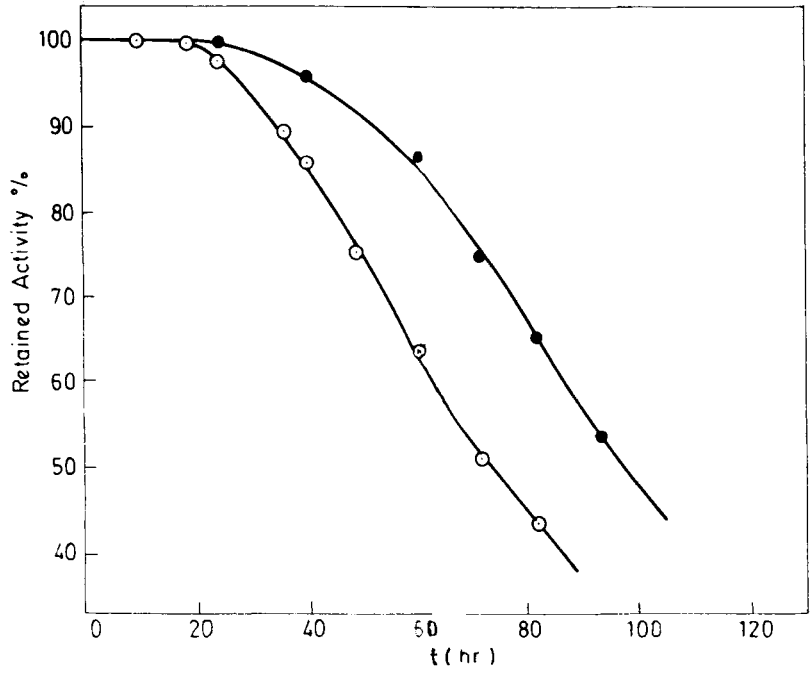

Fig. 11. Deactivation of immobilized GOD-CAT preparation I in recycling fluid-bed reactor at 25°C, pH = 5.6; [G] = 0.1*M*; [O_2] = 2.5×10^{-4}*M*; flow rate, 8.7 mL/min. GOD (○), CAT (●).

GOD and CAT were investigated according to the first-order reaction kinetics depending on the presence of the intermediate product H_2O_2.

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