Simultaneous Covalent Immobilization of Glucose Oxidase and Catalase onto Chemically Modified Acrylonitrile Copolymer Membranes

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ABSTRACT: Ultrafiltration membranes from acrylonitrile copolymer were chemically modified with different concentrations of hydrogen peroxide (from 5 to 30% H_2O_2). The amount of the amide groups in the modified membranes was determined. The water flow and permeability coefficients of the initial and modified membranes were also researched. The modified membranes were used as carriers for covalent immobilization of the dual enzyme system of glucose oxidase and catalase (GOD+CAT). It was found that the best matrices for immobilization of the dual system were membranes modified with 20% H_2O_2 and the optimal activity ratio was GOD : CAT = 1 : 5. The glucose conversion efficiency with the dual enzyme system was twice as high as that of bound GOD alone. Some of the basic characteristics (optimum pH, optimum temperature, pH, temperature stability, and storage stability) of the dual enzyme system were determined and compared with characteristics of free and bound enzymes. The catalytic parameters of the enzyme reaction (K_m and V_{max}) were determined with GOD immobilized alone and with the dual system GOD+CAT. The higher rate observed with the dual enzyme system clearly showed the advantage and the efficiency of the immobilized system. Glucose oxidase without catalase was deactivated by H_2O_2 more rapidly than the immobilized dual GOD+CAT system. These experimental evidences can be explained by the protecting effect of catalase on glucose oxidase from inhibition by H_2O_2. © 2004 Wiley Periodicals, Inc. J Appl Polym Sci 91: 4057–4063, 2004

Key words: membrane; immobilization; glucose oxidase; catalase

INTRODUCTION

The early interest toward immobilized multienzyme catalysts is increasing.[1–5] One of the most widely used systems is glucose oxidase and catalase (GOD+CAT),[6–9] because of their application in food processing, fermentation areas, production of gluconic acid, desugaring of eggs in egg solids production, deoxygenating of liquid foodstuffs, chemical processing, analytical practice, and medicine. GOD catalyzes the conversion of glucose and oxygen to gluconic acid and hydrogen peroxide. During catalytic turnover, the enzyme is inactivated by H_2O_2 and by free radicals derived from oxygen. Peroxide-mediated inactivation can be reduced with catalase; prevention of substrate-mediated inactivation is, however, more difficult. Versatile methods for immobilization of the dual enzyme GOD+CAT on various carriers have been developed.[10–14] The use of matrix-bound enzymes has the two following serious problems: (1) The effectivity of the enzyme catalysts is limited by substrate mass-transfer; (2) Catalase coimmobilized to decompose H_2O_2 resulting from the first enzyme reaction is also inactivated by hydrogen peroxide. The use of porous polymer membranes as carriers for immobilization of enzymes makes the solving of these problems easier.[15–17] In this case, the substrate mass-transfer processes are improved and the inhibiting effect of H_2O_2 on both GOD and CAT is reduced because of a convective flow through the enzyme-bound membrane. The unmodified membranes of acrylonitrile (AN) copolymer are not suitable as carriers. Considerably active nitrile groups present in copolymers (AN) allow additional functional groups to be introduced by special polymer reactions.[18–20] The chemical-modified membranes of AN copolymer have good chemical and mechanical stability and are not susceptible to microbial attack. They give better performance than unmodified AN membrane and equivalency with ultrafiltration membrane from cellulose acetate.[21] A few publications deal with covalent immobilization of enzyme onto chemically modified membranes of AN copolymer, but with other modifying reagents and other enzymes.[22,23] There are different publications on the immobilization of the GOD+CAT system with glutaraldehyde but onto other carriers.[10,13,24]

The aim of the present work was to determine the optimal conditions for covalent immobilization of the GOD+CAT dual enzyme system onto chemically

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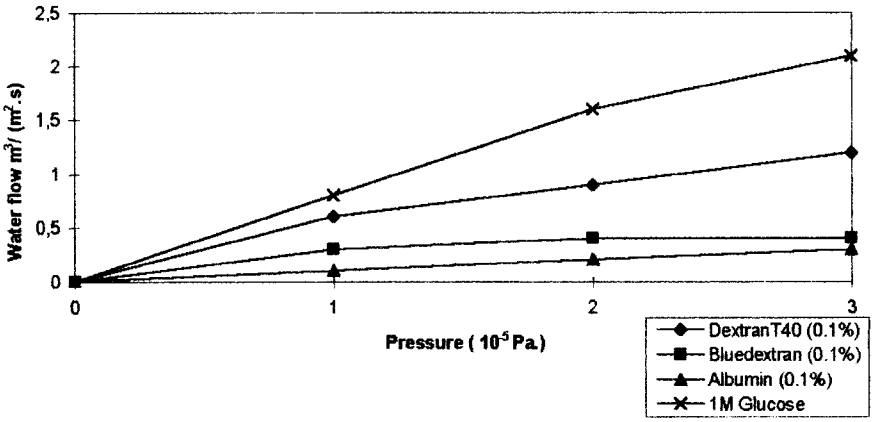

Figure 1 Water flow as a function through the membrane under varying pressures by using different celebrants.

modified acrylonitrile copolymer membranes, as well as some of the basic characteristics of the enzyme system. Because these two enzymes may be considered to be synergistic, the simultaneous immobilization is particularly interesting.

EXPERIMENTAL

Materials

Poly membranes (acrylonitrile, methyl methacrylate, sodium vinyl sulfonate), with a molecular cut-off of 10,000 Da (average pore size, 0.02 μm), supplied by Spartak Co. (Bulgaria), were used. The modification of the poly(acrylonitrile) (PAN) membranes was carried out with the following chemical agents: methanol, hydrogen peroxide, sodium hydroxide, and pure dimethylsulfoxide (all supplied by Chimsnab Co., Bulgaria). The immobilization of glucose oxidase with a specific activity of 119.3 U/mg and catalase with a specific activity of 975 U/mg was carried out with pure glutaraldehyde (all supplied by Fluka Chemie AG, Buchs, Switzerland). All other reagents used for analysis of enzyme activity and bound protein were reagent grade (Fluka Chemie AG, Switzerland).

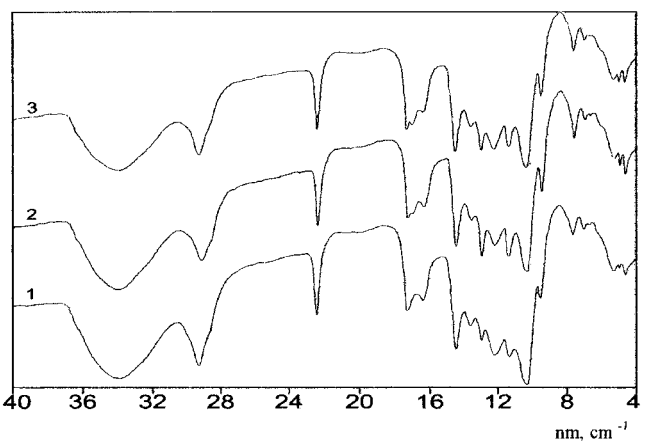

Figure 2 IR spectra of unmodified (spectrum 1) and modified with 20% H_2O_2 (spectrum 2) and with 30% H_2O_2 (spectrum 3) membranes.

Modification of PAN membranes

The membranes were immersed in a solution containing 11.5 mL methanol, 0.5 mL distilled water, 0.25 mL sodium hydroxide (2*N*), and 0.5 mL dimethylsulfoxide for 5 min at 35°C. Then, 0.5 mL hydrogen peroxide (30%) was added and the solution was stored for 3 h at room temperature. The modified membrane was washed with methanol and distilled water.

Immobilization of glucose oxidase and catalase

The modified PAN membranes were immersed in 20 mL 25% aqueous solution of glutaraldehyde for 60 min at 4°C. Then, they were washed with 1*M* sodium phosphate buffer (pH 5.8) and immersed in 20 mL mixture of 0.1% glucose oxidase and catalase in a ratio of 1 : 5 for 24 h at 4°C. Finally, the membranes were washed with 1*M* sodium phosphate buffer (pH 5.8) and distilled water.

Analysis

The amount of protein was determined by the method of Lowry.[25] The method is based on spectrophotomet-

TABLE I
The amount of Active Group in Modified Membranes from Acrylonitrile Copolymer

No.	Concentration of modifying agent (%)	Amount of amido group (meq/g)
1	5	0.990
2	10	1.071
3	20	2.080
4	30	4.920

TABLE II
Water Flow (J_v) and Permeability Coefficient (L) of Initial and Modified Membranes

		Initial membrane		Modified Membrane	
No.	Concentration of modifying agent (%)	$J_v \times 10^5$ [$m^3/(m^2 s)$]	$L \times 10^{14}$ [$m^2/(Pas)$]	$J_v \times 10^5$ [$m^3/(m^2 s)$]	$L \times 10^{14}$ [$m^2/(Pas)$]
1	5	5.446	4.90	6.22	5.598
2	10	—	—	6.44	5.796
3	20	—	—	6.69	6.021
4	30	—	—	3.06	2.754

ric measurement at 750 nm of the intensity of the blue color resulting from the interaction between cupric ions and the peptide bonds in alkali medium (biuretic reaction) and from the reaction of the amino acid residue of the proteins from when treated with Folin reactant (Specol 11, Carl Zeiss Jena, Germany). The amino groups were proven by residual potentiometric titration (Radelkis pH-meter, Hungary) with sodium hydroxide (0.05*N*) in heterogeneous medium.[26] The free and immobilized enzyme activities of glucose oxidase and catalase were determined by an indicator reaction with peroxidase and *o*-dianizidin, giving a dye as product, each with the corresponding substrate glucose or hydrogen peroxide with Specol 11, at 460 nm.[27] The degree of glucose oxidation was measured with Sartorius laboratory cell, containing immobilized GOD+CAT membrane where the glucose (0.1*M*) was passed through the membrane with a flow rate of 0.5 mL/min for operation time of 7 h. The glucose conversion was calculated on the basis of glucose oxidation. The concentration of glucose was determined by a spectrophotometric measurement at 625 nm of the red color intensity resulting from the interaction between the hydrolyzed product of glucose and anthron (9-ketho-10, 10-dihydroantracen).[28] The flux (the volume of water passed through unit area of the membrane for unit time) was determined with Lab Unit-20 supplied by DDS (Denmark).[29] The membrane permeability coefficient was calculated as the ratio of the flux to the differences of pressure (ΔP) on both sides of the membrane. The rejection coefficient (the rejection degree of flow solution component from the membrane) was measured with a Sartorius laboratory cell. IR spectra were obtained from a KBr tablet on Specord IR75 (Germany).

RESULTS AND DISCUSSION

Ultrafiltration membranes from AN copolymer, retaining substances with a molecular weight higher than 10,000 Da, were used. The water flow through the membrane under varying pressures was determined with the following celebrants: 0.1% solutions of albumin (65,000 Da); blue dextran (50,000 Da); dextran T40 (40,000 Da); glucose 1*M* (180 Da). As can be seen from Figure 1, water flow increased with the increase of pressure showing good transport properties of ultrafiltration membranes.[21] The rejection of the membranes for 0.1% solutions of albumin and blue dextran were also determined. The results (albumin $R_0 = 100\%$ and blue dextran $R_0 = 99\%$) revealed good rejection properties of the membranes.

The initial membranes were chemically modified with different solutions of hydrogen peroxide (from 5 to 30%).[19] Thus, the nitrile groups were oxidized to obtain amide groups, which were proven by comparing the IR spectra of modified and unmodified membranes as presented in Figure 2. A new band at 1670 cm^{-1} was observed which was attributed to deformation vibrations of N—H bond in the amide group as shown in Figure 2, spectra 2 and 3. The intensity of new band was higher at modification with 30% H_2O_2 than with 20%. The change in the IR spectra of modified membrane with 5 and 10% was negligible. The amide groups introduced in the membranes improved their hydrophilicity as shown in Table I. The amount

TABLE III
The Amount of Bound Protein and the Relative Activity of Immobilized GOD+CAT

		Glucose oxidase		Catalase	
No.	Concentration of modifying agent (%)	Relative activity (%)	Bound protein (mg/cm^2)	Relative activity (%)	Bound protein (mg/cm^2)
1	5	55.27	0.020	55.6	0.02
2	10	73.59	0.025	66.6	0.03
3	20	78.50	0.030	73.5	0.04
4	30	59.31	0.030	77.1	0.04

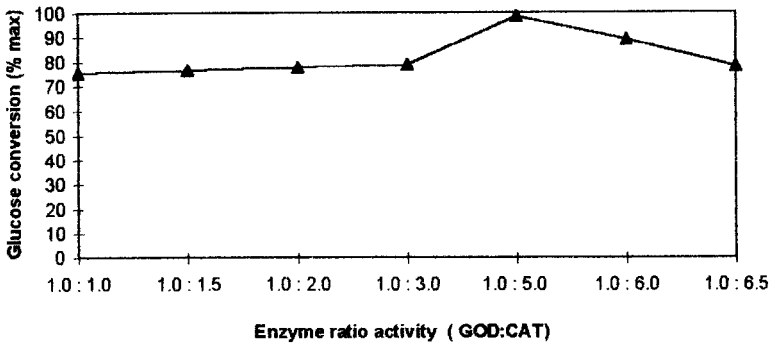

Figure 3 The effect of ratio enzyme activities (GOD : CAT) on the conversion of 0.1*M* glucose by dual enzyme system. Glucose flow rate, 0.5 mL/min; time, 7 h; pH 5.8, 28°C.

of these functional groups in the modified membranes was determined as presented in Table I. The increase of modifying agent concentration was found to increase the amount of introduced groups.[30]

The water flow and permeability coefficient of the modified membranes were determined under a pressure of 2×10^5 Pa and the results were compared to those of the initial membranes as presented in Table II. It can be seen that the transport characteristics of the modified membranes slightly improved after treatment with 5, 10, and 20% H_2O_2 but were much worse with 30% H_2O_2. The improvement in the first three cases was due to the increase of membrane hydrophilicity resulting from the modification, while in the last case (treatment with 30% H_2O_2), the pores of the modified membranes were probably contracted as a result of the high concentration of the modifying agent.

The modified membranes were used as carriers for covalent immobilization of two enzymes, glucose oxidase and catalase, forming an enzyme system. The next aim of the experiments was the determination of the optimal conditions for covalent immobilization of both enzymes onto modified PAN membrane and the estimation the efficiency of the system. The choice of the matrix to which enzyme is ultimately attached can have an important influence on the activity and properties of that enzyme. To select the most suitable modified membrane for immobilization, both enzymes were first immobilized separately. The amounts of bound protein and relative activities of each enzyme were determined as shown in Table III. It was found that the best matrices for immobilization of GOD were the membranes modified with 10 and 20% H_2O_2, while for CAT the membranes modified with 20 and 30% H_2O_2, because the relative activities measured with these matrices were the highest. Based on these results, the membrane modified with 20% H_2O_2 was selected for further experiments.

Another important factor for the efficiency of the system GOD+CAT is the absolute activity of the enzymes and their ratio. Therefore, the activity ratio of the two enzymes was varied in the immobilization solution and the degree of conversion of glucose by the dual bound systems was measured. Figure 3 shows that the best results were obtained for the activity ratio GOD : CAT = 1 : 5. Thus, results from other authors were confirmed stating that for effective enzyme reaction the activity of catalase should be higher.[17,24] When the optimal conditions for immobilization were established, the coupled enzymes with activity ratio GOD : CAT = 1 : 5 were covalently immobilized onto membranes modified with 20% solution of H_2O_2. The water permeability of the membranes was conserved. The total amount of bound

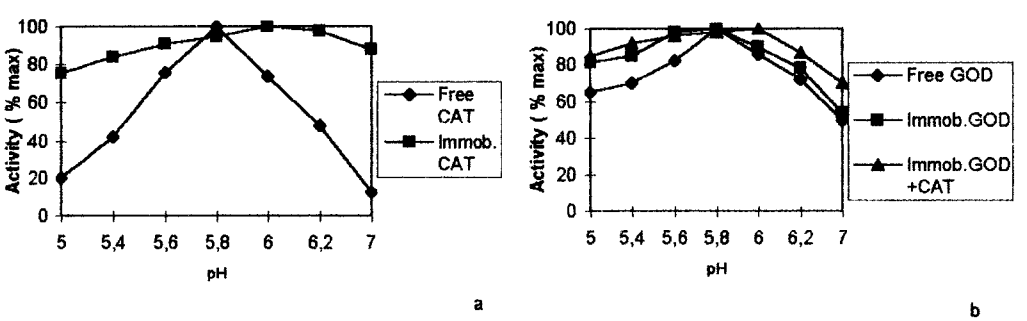

Figure 4 The effect of solution pH on the activity of (a) free and immobilized CAT; (b) free GOD and immobilized GOD, GOD+CAT at different pH values. Activity was measured in a pH-stat at 28°C, 18% glucose in 0.1*M*-phosphate buffer.

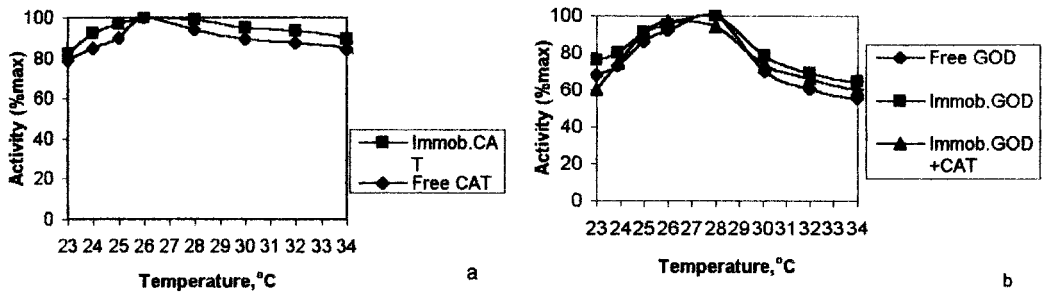

Figure 5 The effect of temperature on the activity of (a) free and immobilized CAT; (b) free GOD and immobilized GOD, GOD+CAT at different temperatures. Activity was measured in a temperature-stat at pH 5.8, 18% glucose in 0.1*M* phosphate buffer.

enzymes was determined (0.05 mg/cm^2), as well as the degree of glucose conversion by the dual enzyme system (80%). The comparison of these results with glucose conversion of bound GOD alone (40%) showed that the glucose conversion by the dual enzyme system was twice higher.

Some of the basic characteristics of the dual enzyme system were determined and compared with characteristics of separate free and bound enzymes. The pH optimum of free and immobilized GOD, free and immobilized CAT, and immobilized system GOD+CAT were determined as presented in Figure 4. The pH optimum for both free and bound GOD was found to be 5.8, whereas that of free CAT was found to be 5.8 and bound CAT was found to be 6. It should be noted, however, that the activities of the immobilized forms were less sensitive to changes in pH. As can be seen from Figure 4(b), pH optimum of the dual system was

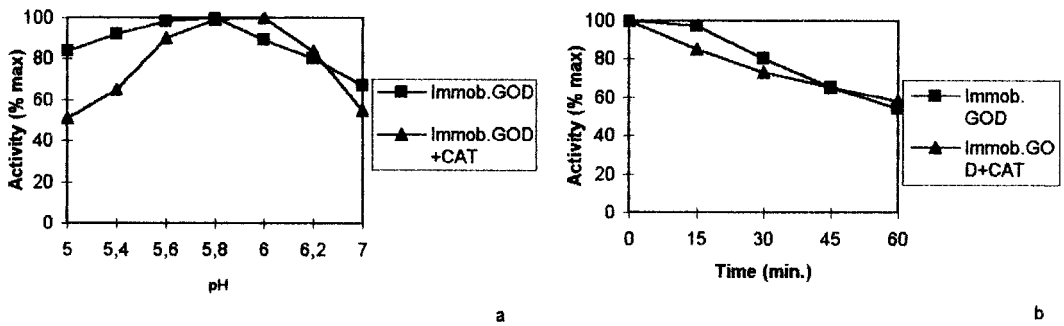

Figure 6 The pH and thermal stability: (a) pH stability of immobilized GOD and GOD+CAT, immersed in 0.1*M* phosphate buffer at different pH values for 30 min at 28°C. Activity was measured at pH 5.8, 28°C, 18% glucose in 0.1*M* phosphate buffer; (b) thermal stability of immobilized GOD and GOD+CAT, immersed in 0.1*M* phosphate buffer with pH 5.8, 50°C for 60 min. Activity was measured the same as condition pH stability.

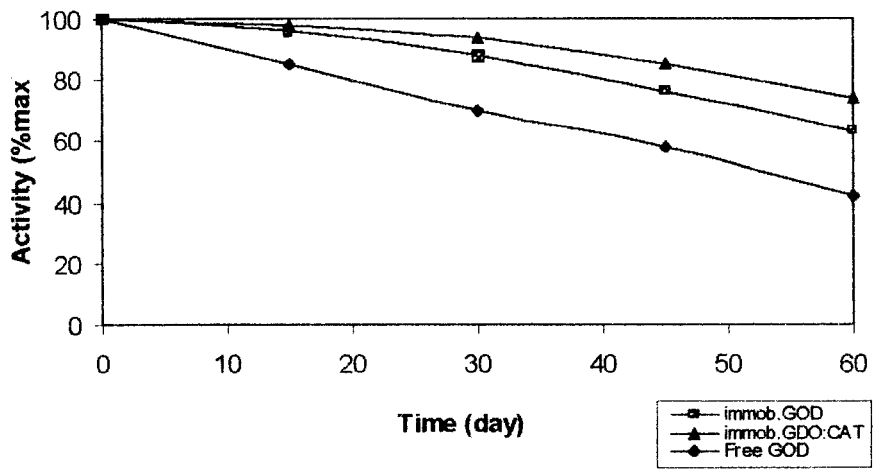

Figure 7 The storage stability of free GOD and immobilized GOD; GOD+CAT at 4°C, pH 5.8 in 0.1*M* phosphate buffer.

TABLE IV
Kinetic Constants of Immobilized GOD+CAT, Immobilized GOD, and Free GOD

Parameter	Immobilized GOD+CAT	Immobilized GOD	Free GOD
K_m (mol/l)	0.306	0.160	0.031
V_{max} 10^6, mol/(min mg)	85.11	29.65	149.1

6. Therefore, the immobilization of the enzymes did not significantly affect their pH optimum. The optimum pH of GOD+CAT was equal to this of immobilized catalase.

The optimum temperature for GOD from *Aspergillus niger* was from 28 to 50°C.[31,32] Similarly, the optimal temperature of each enzyme in free and immobilized form was measured, as well as that of the dual system. Figure 5 shows that the temperature optimum of free and bound GOD was 28°C and that of free and bound CAT was 26°C. The optimal temperature of the dual system was equal to that of catalase as shown in Figure 5(b). Further, the pH and temperature stability of the GOD immobilized separately and together with CAT were compared as presented in Figure 6. The best pH [Fig. 6(a)] and temperature [Fig. 6(b)] stability was shown by GOD bound separately, followed by the dual system GOD+CAT. It can be concluded that the values of all the characteristics of the dual system were similar to these of the separate enzymes.

The stabilities of glucose oxidase and catalase immobilized on acrylonitrile copolymer membranes have been extensively studied and compared with the native enzyme. There was no loss of immobilized enzymes activity when stored for 1 month at temperatures between 4 and 5°C, whereas the native enzymes in solution were less stable and lost 30% from their initial enzyme activities, as shown in Figure 7. These results were comparable with results reported from other authors.[13,15,24] The storage activity of the immobilized enzyme system has been mainly reported as satisfactory, ranging from 1 month up to 1 year with residual activity higher than 50%.

The catalytic parameters of the enzyme reaction (K_m and V_{max}) were determined with GOD immobilized separately and with the dual system GOD+CAT. For comparison, the kinetic parameters of free GOD were also measured as shown in Table IV. The rate of the glucose oxidation reaction catalyzed with the immobilized dual system was found to be about twice as high as that catalyzed with immobilized GOD alone. The V_{max} of free GOD was the highest, because there is no impeded diffusion of the substrate to the enzyme, as at immobilized enzymes. The higher rate observed of the dual enzyme system clearly showed the advantage and efficiency of the immobilized system GOD+CAT for this reaction. It can be explained by the lower degree of inactivation of GOD by the hydrogen peroxide because of the favorable effect of the second enzyme catalase.[24,33]

To confirm this evidence, the effect of the hydrogen peroxide concentration on glucose oxidase deactivation was studied by determination of the degree of glucose conversion as present in Figure 8. It was found that GOD without catalase was deactivated most rapidly despite its immobilization. The free GOD together with free catalase was deactivated slower than immobilized GOD, but faster than the immobilized dual

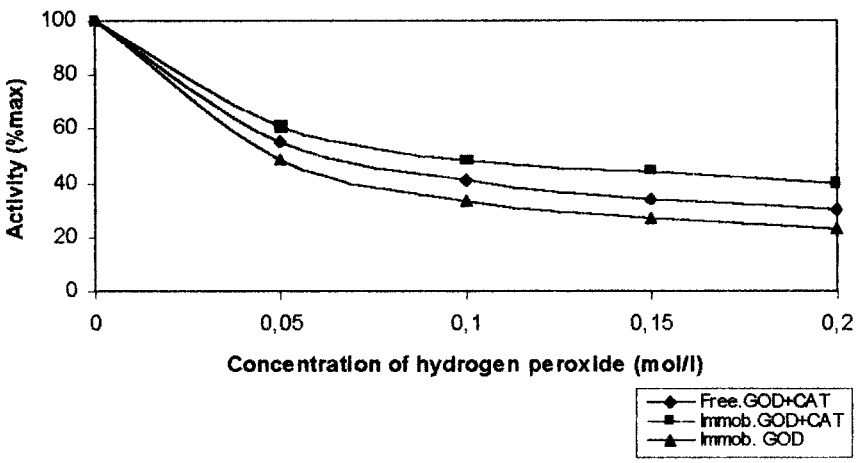

Figure 8 The effect of H_2O_2 concentration on the activity of GOD in dual enzyme system. Activity was measured at 28°C, pH 5.8, 18% glucose in 0.1*M* phosphate buffer.

system GOD+CAT. These results can be explained with the higher stability of the bound enzymes compared to the free ones and with the protection effect of the catalase on glucose oxidase inhibition by hydrogen peroxide.

CONCLUSION

Several observations result from the present study indicating that the optimal activity ratio is GOD : CAT = 1 : 5 and the optimal concentration of the modifying agent is 20% H_2O_2. The optimum pH of the dual system was 6. The optimal temperature (26°C) of the dual system was equal to that of the catalase. The pH and temperature stability of the dual system were similar to these of separate enzymes. There was no loss of the dual enzyme system activity when stored for 1 month at temperatures between 4 and 5°C. It was concluded that the rate of glucose oxidation reaction catalyzed with the immobilized dual system was about twice as high as that catalyzed with immobilized GOD alone. The higher rate observed of the dual enzyme system clearly showed the efficiency of the immobilized system GOD+CAT onto modified acrylonitrile copolymer membranes.

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