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Simultaneous and Sequential Co-Immobilization of Glucose Oxidase and Catalase onto Florisil

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Abstract The co-immobilization of *Aspergillus niger* glucose oxidase (GOD) with bovine liver catalase (CAT) onto florisil (magnesium silicate-based porous carrier) was investigated to improve the catalytic efficiency of GOD against H_2O_2 inactivation. The effect of the amount of bound CAT on the GOD activity was also studied for 12 different initial combinations of GOD and CAT, using simultaneous and sequential coupling. The sequentially co-immobilized GOD-CAT showed a higher efficiency than the simultaneously co-immobilized GOD-CAT in terms of the GOD activity and economic costs. The highest activity was shown by the sequentially co-immobilized GOD-CAT when the initial amounts of GOD and CAT were 10 mg and 5 mg per gram of carrier. The optimum pH, buffer concentration, and temperature for GOD activity for the same co-immobilized GOD-CAT sample were then determined as pH 6.5, 50 mM, and 30°C, respectively. When compared with the individually immobilized GOD, the catalytic activity of the co-immobilized GOD-CAT was 70% higher, plus the reusability was more than two-fold. The storage stability of the co-immobilized GOD-CAT was also found to be higher than that of the free form at both 5°C and 25°C. The increased GOD activity and reusability resulting from the co-immobilization process may have been due to CAT protecting GOD from inactivation by H_2O_2 and supplying additional O_2 to the reaction system.

Keywords: Glucose oxidase, catalase, simultaneous co-immobilization, sequential co-immobilization, florisil

Glucose oxidase (GOD) (β-D-glucose: oxygen 1-oxidoreductase; E.C. 1.1.3.4) catalyzes the production of D-gluconic acid and hydrogen peroxide through the oxidation of β-D-glucose by molecular oxygen. Yet, during the catalytic turnover, GOD is inactivated by the resulting hydrogen peroxide [4, 7], along with several other oxidases, such as L-aminocyclopropane-1-carboxylic acid oxidase and L-α-glycerophosphate oxidase [12, 32]. There have already been many reports on the undesired effects of H_2O_2. For example, H_2O_2 can cause a by-reaction in the xanthine oxidase system [1], where D-amino-acid oxidase catalyzes the oxidation of D-amino acid into keto acids, yet the built-up hydrogen peroxide transforms the keto acids into carboxylic acids [16, 27]. These undesired effects of hydrogen peroxide can be reduced with catalase (CAT) (H_2O_2: H_2O_2 oxidoreductase, EC 1.11.1.6), which decomposes hydrogen peroxide into H_2O and O_2, eventually removing it from the system. When using CAT in the GOD system, some oxygen is recovered and made available for the formation of gluconic acid (Fig. 1).

Free GOD or co-immobilized GOD-CAT can be employed in food processing [9, 19, 20, 23], the production of gluconic acid [2], textile bleaching [29], analytical measurements [8, 10, 22, 30, 31], and medicine [26]. Thus, the existing literature includes many reports on the co-immobilization of GOD and CAT on various carriers and its increasing areas of application [5, 6, 8, 21, 22, 24, 25], as co-immobilized enzymes allow improved stability, reuse, continuous operation,

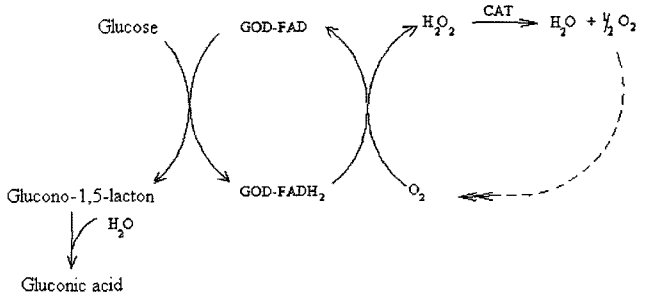

Fig. 1. Schematic representation of the GOD-CAT system.

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the possibility of better reaction control, and high purity and product yields, with valuable economic effects.

However, one of the main limiting factors of co-immobilization studies is determining the appropriate immobilization conditions for enzymes that are immobilized at the same time. GOD and CAT have already been co-immobilized onto various carriers using different methods [6, 21, 22, 25, 30], yet how the immobilization conditions were determined has not been reported. Accordingly, this study established optimum co-immobilization conditions by adjusting the results of previous studies [17, 18] by the present authors, in which GOD and CAT were immobilized individually.

Thus, to improve the GOD activity and prevent H_2O_2 inactivation, GOD was co-immobilized with CAT, while investigating the effect of the amount of bound CAT and immobilization method used. GOD and CAT were simultaneously and sequentially co-immobilized onto florisil using 12 different initial GOD-CAT combinations. For the simultaneous co-immobilization, the mixtures of GOD and CAT were immobilized onto the carrier at the same time, whereas for sequential co-immobilization, GOD was immobilized onto the carrier first, then CAT immobilized onto the GOD-bound carrier. The effects of the initial amounts of GOD and CAT and method of co-immobilization were investigated by comparing the resulting co-immobilized GOD-CAT samples in terms of their GOD activity and economic efficiency. The co-immobilized GOD-CAT obtained using the predetermined optimal conditions was then characterized by determining the optimum pH, buffer concentration, and temperature, along with the kinetic properties. The operational stability was also investigated.

MATERIALS AND METHODS

Materials

The florisil (magnesium silicate-based carrier containing 15% MgO and 85% SiO_2, 60–100 mesh, porous, specific surface area 170–300 $m^2 g^{-1}$) and hydrogen peroxide were obtained from Merck AG (Darmstadt, Germany). The *Aspergillus niger* origin GOD (24 mg/ml, 350 U/mg), bovine liver origin CAT (35 mg/ml, 51,199 U/mg), 3-aminopropyltrietoxysilane (APTES), Grade I aqueous glutaraldehyde solution (50%), 3,5-dinitrosalicylic acid (DNSA), and all other chemicals used were obtained from Sigma (St.Louis, MO, U.S.A.).

Methods

The preparation of the carrier, as described in our previous study [17], included three steps: cleaning with a 5% HNO_3 solution, the formation of an alkylamine derivative using APTES, and activation with a glutaraldehyde solution. The color of the carrier changed to magenta or tan after the glutaraldehyde treatment.

Table 1. Initial amounts of GOD (m_{GOD}) and CAT (m_{CAT}) used per gram of carrier for each co-immobilization study.

Enzyme sample symbol	Amount of enzyme protein (mg protein/g carrier	
	m_{GOD}	m_{CAT}
S1	5	1
S2	5	5
S3	5	10
S4	5	20
S5	10	1
S6	10	5
S7	10	10
S8	10	20
S9	20	1
S10	20	5
S11	20	10
S12	20	20

When previously immobilizing GOD [17] and CAT [18] onto florisil, the present authors established an immobilization temperature and time of 10°C and 2 h, respectively, for both enzymes. Although the optimum immobilization pH for GOD and CAT was pH 5.4 and 6.0, respectively, pH 5.7 produced about 90% activity for both enzymes. Therefore, based on these predetermined conditions, the co-immobilization pH, temperature, and time for the current study were selected as 5.7, 10°C, and 2 h, respectively. The simultaneous and sequential co-immobilization procedures also involved 12 different GOD-CAT combinations, represented by 12 different symbols (S1–S12) (Table 1), including three different GOD amounts (5, 10, or 20 mg per g of carrier) and four different CAT amounts (1, 5, 10, or 20 mg per g of carrier).

Simultaneous Co-Immobilization of GOD and CAT

One gram of florisil was incubated with 10 ml of an enzyme solution containing certain amounts of both GOD and CAT, as given in Table 1. After 2 h, the unbound enzymes were removed by extensive washing. To determine the amounts of bound GOD and CAT, independent to the immobilization experiments, GOD and CAT solutions were separately prepared at different concentrations and their absorbance values measured at both 280 and 405 nm. These wavelengths were chosen as both proteins are absorbed at 280 nm [3], whereas only the Soret band of CAT (due to its heme group) is absorbed at 405 nm [14]. The resulting values were then used to obtain standard protein curves for GOD and CAT and specific extinction coefficients (ε) at both wavelengths. The absorbance values of the combined washing solutions were measured at 280 nm and 405 nm to determine the amounts of unbound GOD and CAT using the predetermined ε values. The amounts of bound GOD and CAT per gram of carrier were then

separately calculated. The total protein in the washing solutions was also determined using the Lowry method [13]. Finally, the amount of unbound enzyme protein was subtracted from the total amount of enzyme protein initially used in the co-immobilization, and the amount of bound protein calculated as mg protein per gram of carrier for each co-immobilized sample.

Sequential Co-Immobilization of GOD and CAT

One g of florisil was incubated with 10 ml of a GOD solution (containing 5, 10, or 20 mg of GOD) for 1 h, and then washed using an excessive buffer solution to remove any unbound GOD. The amount of unbound GOD in the washing solution was determined and subtracted from the total amount of GOD used in the immobilization. The amount of bound GOD was calculated as mg GOD per gram of carrier. Thereafter, the immobilized GOD samples were separately incubated with 10 ml of a CAT solution (containing 1, 5, 10, or 20 mg CAT) for 2 h. The unbound CAT was then removed by washing with a buffer solution and the amount of bound CAT calculated.

CAT and GOD Activities of Co-Immobilized GOD-CAT

The CAT activity was determined by measuring the decrease in the absorbance of H_2O_2 at 240 nm in a reaction mixture containing 5 mg of co-immobilized GOD-CAT and 5 ml of a 10 mM H_2O_2 solution [18]. The reaction was carried out at 25°C for 2 min and stopped by adding 1 ml of a 1 M HCl solution. The CAT activities of the co-immobilized GOD-CAT samples were expressed as μmol H_2O_2 g carrier^{-1} min^{-1}.

The GOD activities of the co-immobilized GOD-CAT samples were determined spectrophotometrically by measuring the decrease in the glucose concentration using the DNSA method [15]. Five mg of the co-immobilized GOD-CAT samples and 10 ml of a 15 mM β-D-glucose solution (saturated with air and kept at room temperature for at least 2 h for mutarotation) were used in the experiments. At the end of a 10-min reaction time, 0.5 ml of the reaction solution was added to 0.5 ml of the clear DNSA reagent. The resulting solution was kept in a boiling water bath for 10 min, and then immediately cooled in an ice bath for 1 min. The mixture was increased to a volume of 8 ml by adding distilled water, and the absorbance measured at 575 nm after 20 min.

Characterization of Co-Immobilized GOD-CAT

The co-immobilized GOD-CAT samples were characterized in terms of their GOD activities.

The GOD activity of the co-immobilized GOD-CAT samples according to the pH was investigated using a 100 mM acetate buffer at pH 5.5, 100 mM phosphate buffer at pH 6.0, 6.5, 7.0, and 8.0, and 100 mM borate buffer at pH 9.0.

The GOD activity of the co-immobilized GOD-CAT samples according to the buffer concentration was investigated using 25, 50, 75, 100, and 150 mM buffer solutions at the predetermined optimal pH value.

The effect of temperature on the GOD activity of the co-immobilized GOD-CAT samples was studied within a temperature range of 10–60°C at the predetermined optimal pH and buffer concentration.

The effect of the β-D-glucose concentration (2–80 mM) on the GOD activity of the co-immobilized GOD-CAT was investigated at the predetermined optimal conditions using the DNSA method. The maximum reaction rate (V_{max}) and Michaelis-Menten coefficient (K_M) values were determined from a Lineweaver-Burk plot.

The reusability of the co-immobilized GOD-CAT was investigated in terms of the GOD activity using a batch-type stirred column reactor. Fifty mg of the co-immobilized GOD-CAT was loaded into the reactor, followed by 5 ml of a 15 mM glucose solution, and then the reaction was allowed to continue for 5 min. Thereafter, the reaction mixture was immediately removed from the column and the retained glucose measured. This same measurement was repeated 75 times using the same enzyme reactor. Furthermore, to prevent any influence from the storage time on the enzyme activity, only 20 s was allowed between two cycles, and all the measurements were carried out on the same day.

A free enzyme solution containing 4.33 mg/ml GOD and 2.89 mg/ml CAT was stored at 25°C and 5°C, and the residual activity measured periodically for 2 months. This same measurement was also carried out on co-immobilized GOD-CAT samples incubated in a dried solid form.

Results and Discussion

Simultaneous Co-Immobilization of GOD-CAT

In previous studies, GOD and CAT have been co-immobilized using either just one GOD-CAT combination [6, 11, 21, 22, 30] or different combinations [24–26]. Yet, the individual amounts of bound GOD and CAT have not been reported. Only Podual *et al.* [21] measured the absorbance of the washing solution at 280 and 450 nm to determine the total protein and GOD content in the washing solution after immobilization, and identified the bound ratios of GOD and CAT as 0.83 and 0.70, respectively.

In the present study, the amounts of bound GOD and CAT were determined separately, and the ε values for GOD and CAT were 1.8517 cm^2 mg^{-1} and 2.0068 cm^2 mg^{-1} at 280 nm, respectively, and 0.1965 cm^2 mg^{-1} and 1.7408 cm^2 mg^{-1} at 405 nm, respectively.

Table 2. Measured (A_M) and calculated (A_C) absorbances of GOD-CAT mixtures initially used in co-immobilization studies (enzyme solutions were used after eight-fold dilution).

Sample symbol	280 nm			405 nm		
	A_M	A_C	%*	A_M	A_C	%*
S1	0.137	0.141	103	0.029	0.034	117
S2	0.237	0.241	102	0.110	0.121	110
S3	0.361	0.367	102	0.217	0.230	106
S4	0.595	0.617	104	0.423	0.448	106
S5	0.254	0.257	101	0.042	0.046	110
S6	0.350	0.357	102	0.124	0.133	107
S7	0.468	0.463	99	0.228	0.242	106
S8	0.716	0.733	102	0.439	0.460	105
S9	0.488	0.488	100	0.073	0.071	97
S10	0.590	0.588	99	0.157	0.158	101
S11	0.728	0.714	98	0.26	0.267	103
S12	0.954	0.960	100	0.485	0.484	99

*=$(A_C/A_M)\times 100$.

The total absorbance at 280 nm or 405 nm of an enzyme mixture with known GOD and CAT concentrations (C_{GOD} and C_{CAT}) can be calculated using equations 3.1 or 3.2.

$$A_{280}=2.0068\ C_{CAT}+1.8517\ C_{GOD} \quad (3.1)$$

$$A_{405}=1.7408\ C_{CAT}+0.1965\ C_{GOD} \quad (3.2)$$

Furthermore, the measured absorbances of a GOD and CAT mixture at 280 and 405 nm can also be used to calculate the concentrations of both GOD and CAT using the same equations. Thus, to confirm the reliability of equations 3.1 and 3.2, the absorbance value of an enzyme solution containing known amounts of GOD and CAT (A_M) was measured, and then compared with the calculated value (A_C). The results are given in Table 2.

As seen from Table 2, the A_M and A_C values were very similar. Therefore, the absorbances of the washing solution at 280 and 405 nm were used to determine the amounts of unbound GOD and CAT, and then the amounts of bound GOD and CATs per gram of carrier were calculated, as presented in Table 3.

Table 3. Amounts of bound GOD and CAT per gram of carrier for simultaneously and sequentially co-immobilized GOD-CAT samples.

Sample symbol	Simultaneous co-immobilization (mg enzyme protein/ g carrier)		Sequential co-immobilization (mg enzyme protein/ g carrier)	
	M_{GOD}	M_{CAT}	M_{GOD}	M_{CAT}
S1	2.41	0.28	2.68	0.25
S2	2.18	1.78	2.68	2.52
S3	1.68	4.81	2.68	5.02
S4	1.22	8.66	2.68	8.51
S5	4.68	0.41	4.33	0.34
S6	4.14	3.27	4.33	2.89
S7	3.77	6.98	4.33	5.99
S8	2.49	12.62	4.33	9.22
S9	10.44	0.01	11.20	0.31
S10	10.40	1.20	11.20	3.18
S11	9.58	4.20	11.20	5.53
S12	8.93	1.60	11.20	10.62

Table 4. Theoretical (M_T) and experimental (M_E) amounts of bound GOD and CAT in simultaneously co-immobilized samples.

Sample symbol	Total amount of bound protein (mg protein. g carrier^{-1})	
	Theoretical (M_T)	Experimental (M_E)
S1	2.69	2.78
S2	3.96	4.04
S3	6.49	6.78
S4	9.88	9.66
S5	5.09	5.34
S6	7.41	7.78
S7	10.80	10.90
S8	15.11	14.43
S9	10.60	9.41
S10	9.89	9.35
S11	13.78	14.50
S12	20.53	21.27

The amounts of bound CAT increased with an increased initial amount of CAT. A similar tendency was also observed for GOD. However, for each initial amount of GOD amount, the amounts of bound GOD decreased slightly when increasing the initial amount of CAT. Furthermore, the amounts of bound GOD and CAT differed in all 12 co-immobilized GOD-CAT samples.

The total amount of GOD and CAT in each co-immobilized sample was considered as the theoretically bound protein amount (M_T). Furthermore, the total protein in the washing solution was determined using the Lowry method, and then the experimental amount of bound protein was calculated for each co-immobilized sample (M_E). The M_T and M_E values for the co-immobilized samples were very similar, with a less than 5% difference (Table 4), confirming the reliability and accuracy of the amounts of GOD and CAT determined using equations 3.1 and 3.2.

Sequential Co-Immobilization of GOD-CAT

Although simultaneous co-immobilization of GOD and CAT has already been carried out in various studies, this is apparently the first report on sequential co-immobilization.

The amounts of bound GOD and CAT as a result of the sequential co-immobilization are given in Table 3. The amount of bound GOD was 2.7, 4.3, and 11.2 mg/g carrier when the initial amount of GOD was 5, 10, and 20 mg/g carrier, respectively. The amount of bound CAT increased

with an increased initial amount of CAT when the carrier contained a constant amount of bound GOD. Yet, the amount of bound CAT remained almost the same when solutions with the same concentration of CAT were used in the co-immobilization onto carriers with different contents of bound GOD. This result may be explained by the large surface area of the porous florisil carrier, as mentioned in previous literature [28].

CAT Activities of Co-Immobilized GOD-CAT Samples

The CAT activities of the simultaneously and sequentially co-immobilized GOD-CAT samples are represented in Fig. 2A. All the sequentially co-immobilized GOD-CAT showed a higher CAT activity than the simultaneously co-immobilized GOD-CAT samples. It was also found that the CAT activity increased when increasing the initial amount of CAT with the same initial GOD amount. However, the CAT activities of the sequentially co-immobilized GOD-CAT decreased when increasing the initial amount of GOD with any initial amount of CAT.

GOD Activities of Co-Immobilized GOD-CAT Samples

The amounts of bound GOD and CAT differed in all the simultaneously co-immobilized GOD-CAT samples, making a comparison impossible. Therefore, the sequential co-immobilization was used to obtain co-immobilized samples with a constant GOD content while increasing the initial content of CAT to reveal the effect of the amount of CAT on the GOD activity.

The resulting sequential co-immobilization samples showed higher GOD activities than the simultaneously co-immobilized samples (Fig. 2B). In addition, the increased GOD activity of the sequentially co-immobilized samples was more pronounced than that of the simultaneously co-immobilized samples when the initial amount of GOD was increased from 5 to 20 mg per gram of carrier.

As seen from Fig. 2C, the GOD activity of the sequentially co-immobilized samples was higher than that of the simultaneously co-immobilized samples when the initial amount of GOD was 10 and 20 mg per gram of carrier. However, the GOD activity of the simultaneously co-immobilized samples prepared with an initial GOD amount of 5 mg/g carrier was generally higher than that of the corresponding sequentially co-immobilized GOD-CAT.

Fig. 2D shows the GOD activity per mg of total protein used in the co-immobilization experiments, where the activity was higher for the sequentially co-immobilized GOD-CAT samples and the maximum value was obtained for sample S6.

The GOD activity of the sequentially co-immobilized GOD-CAT samples was consistently higher than that of

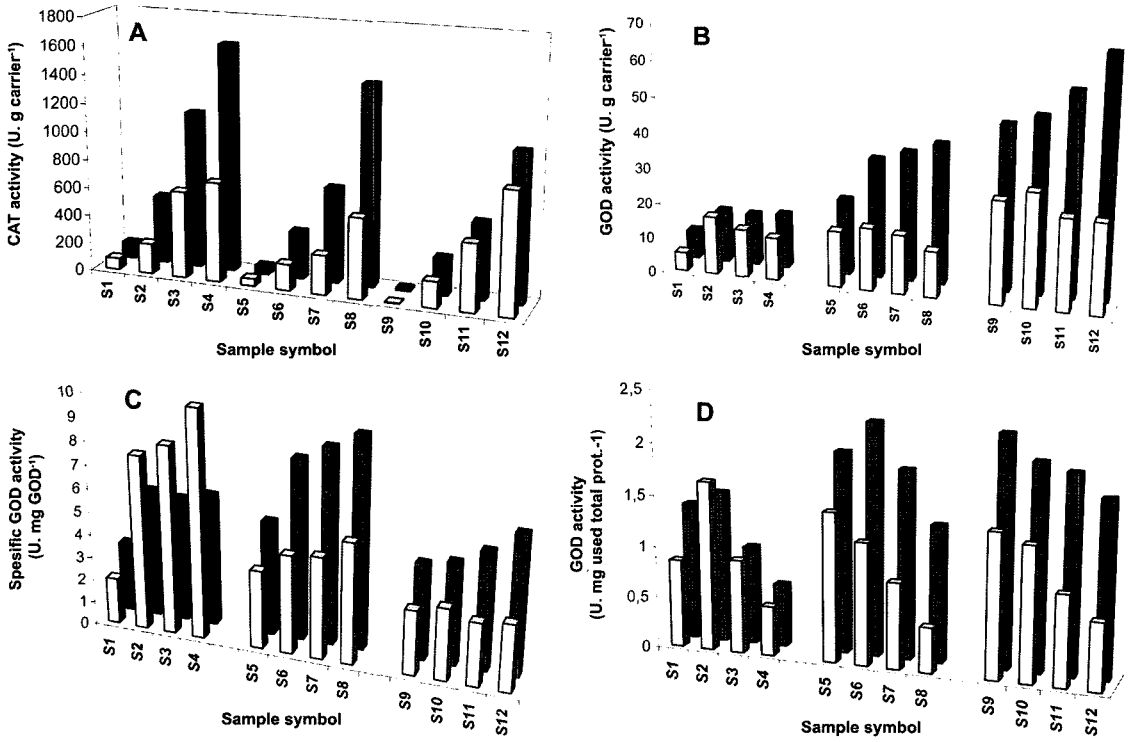

Fig. 2. **A**. CAT activities of simultaneously (□) and sequentially (■) co-immobilized GOD-CAT samples. **B**. GOD activities of simultaneously (□) and sequentially (■) co-immobilized GOD-CAT samples. **C**. Specific GOD activities of simultaneously (□) and sequentially (■) co-immobilized GOD-CAT samples. **D**. GOD activities per mg total protein used in simultaneous (□) and sequential (■) co-immobilization experiments.

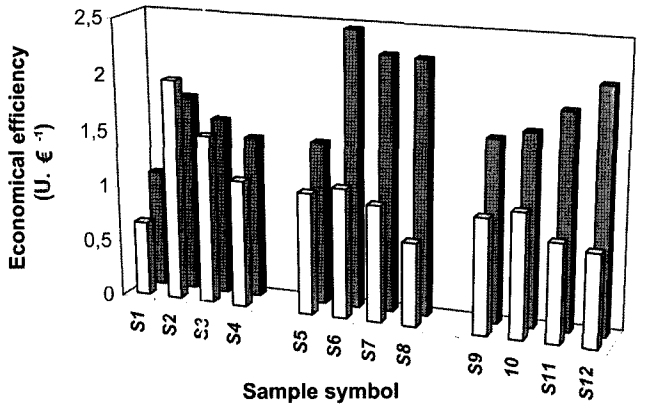

Fig. 3. Economic efficiencies of simultaneously (□) and sequentially (■) co-immobilized GOD-CAT samples.

the simultaneously co-immobilized GOD-CAT samples, except for sample S2, which showed a slightly higher GOD activity than its sequentially co-immobilized counterpart.

Since the aim of the co-immobilization of GOD with CAT is to protect GOD from H_2O_2 inactivation, the GOD activities of the co-immobilized samples were compared, and the GOD activities of the sequentially co-immobilized GOD-CAT samples found to be higher than those of the simultaneously co-immobilized samples.

Economic Efficiencies of Co-Immobilized GOD-CAT Samples

The cost of the carrier was calculated as 0.61 € g carrier^{-1} using the unit prices of the chemicals used. The unit prices for the GOD and CAT used in this work were 1.4 € mg GOD^{-1} and 0.15 € mg CAT^{-1}, respectively (Sigma Catalog). The amounts of GOD and CAT used for each sample are given in Table 1. Therefore, these values were used to calculate the cost of each co-immobilized GOD-CAT sample as € g carrier^{-1}. The economic efficiencies were then determined as U €$^{-1}$ based on dividing the GOD activities in Fig. 2.3 by their costs, as represented in Fig. 3. Generally, the GOD activity per € for the sequentially co-immobilized samples was higher than that for the simultaneously co-immobilized samples, except for sample S2.

The maximum economic efficiency was obtained for sequentially co-immobilized sample S6, which also had the highest GOD activity per mg of total protein used. Therefore, this sample was chosen to characterize the co-immobilized GOD-CAT system.

Thus, the sequentially co-immobilized GOD-CAT samples showed a higher GOD activity and economic efficiency than the simultaneously co-immobilized GOD-CAT samples.

Characterization of Co-Immobilized GOD-CAT

Sequentially co-immobilized sample S6, which showed the highest GOD activity, was carried out using 10 mg GOD per gram of florisil and 5 mg CAT per gram of florisil. Therefore, this co-immobilized GOD-CAT sample was used for the characterization studies. The results, summarized in Table 5, were compared with those for free and immobilized GOD given in our previous study [17].

As shown in Table 5, the optimum pH for free GOD was 5.5, the immobilization of GOD caused the optimum pH to shift to 6.0, and the co-immobilization of GOD with CAT caused the optimum pH to shift to 6.5. This considerable shift of the optimal pH for the immobilized GOD to a higher pH value was expected because of the production of gluconic acid, causing a decrease in the pH of the microenvironment of the immobilized enzyme. However, the deviation in the optimal pH was less than expected, possibly due to the basic property of the florisil [17]. In the case of the co-immobilized GOD-CAT, the one-unit shift to the less acidic side may be explained by increased production of gluconic acid in the microenvironment of the bound GOD because of the increased catalytic efficiency.

When investigating the effect of the pH, ionic strength depending on the buffer concentration, and temperature on the GOD activity of the co-immobilized GOD-CAT, the maximum activity was observed at pH 6.5, 50 mM buffer concentration, and 30°C, respectively.

The GOD activity of the co-immobilized GOD-CAT was also determined in various concentrations of glucose

Table 5. Comparison of free GOD, immobilized GOD, and co-immobilized GOD-CAT samples in terms of their optimal conditions and kinetic properties.

Determined parameters	Free GOD*	Immobilized GOD*	Co-immobilized GOD-CAT
Optimum pH	5.5	6.0	6.5
Optimum buffer concentration (mM)	100	50	50
Optimum temperature (°C)	35	35	30
Activation energy (kJ mol^{-1}K^{-1})	32.8	44.8	39.9
K_M (mM)	68.2	258.9	359.7
V_{max} (U mg GOD^{-1})	434.8	217.4	370.4
K_{cat} (mol glucose mol GOD^{-1}s^{-1})	1.2×10^3	0.6×10^3	1.0×10^3
K_{cat}/K_M (s^{-1})	1.7×10^4	2.2×10^3	2.8×10^3

*Ozyilmaz *et al.* [16].

solutions. The results were used to obtain a Lineweaver-Burk plot and the K_M and V_{max} values determined as 370.4 mM and 359.7 U mg GOD^{-1}, respectively.

As seen from Table 4, the GOD activity of the immobilized GOD and co-immobilized GOD-CAT was approximately 50% and 85% of that of the free GOD, respectively. The highest K_M value was related to the dual enzyme system (359.7 mM), whereas the K_M value for the free GOD (68.2 mM) was the smallest. A similar tendency was also previously reported by Godjevargova *et al.*, who immobilized GOD alone and with CAT onto a chemically modified acrylonitrile copolymer. The V_{max} values reported for the free GOD, immobilized GOD, and co-immobilized GOD-CAT were 149.1, 19.65, and 85.11 U mg^{-1}, respectively, and the K_M values were reported as 31, 160, and 306 mM, respectively.

Therefore, despite the favorable effect of CAT, it may also prevent the interaction between GOD and glucose molecules, owing to steric hindrances and diffusion limitations. The higher reaction rate of the co-immobilized GOD-CAT clearly showed the advantage and efficiency of CAT. It has been reported that the V_{max} for free GOD is the highest, as there is no impeded diffusion of the substrate to the enzyme, as in immobilized enzymes [4]. Also, immobilized GOD can be readily inactivated by H_2O_2 because of the limited diffusion of H_2O_2 from the carrier pores to the bulk solution.

One of the main benefits to immobilized enzymes is their high economic efficiency due to the possibility of repeated successive use. Thus, operational stability is an important parameter that needs to be further investigated to establish the importance of immobilization studies.

Fig. 4 shows the relative GOD activities of the co-immobilized GOD-CAT after multiple use. After reusing 50 times, the residual activity was about 85% of the initial activity, whereas 72.5% remained after 75 times. However, in our previous study, the immobilized GOD only retained 40% of its initial activity after being used 50 times [17]. Thus, the GOD activity was clearly improved by co-immobilization with CAT, possibly due to its protection of

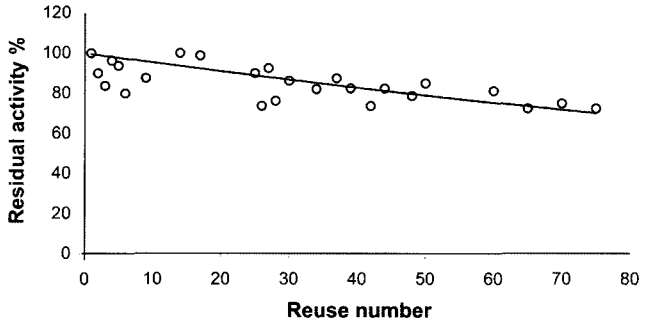

Fig. 4. Operational stability of a sequentially co-immobilized GOD-CAT sample.

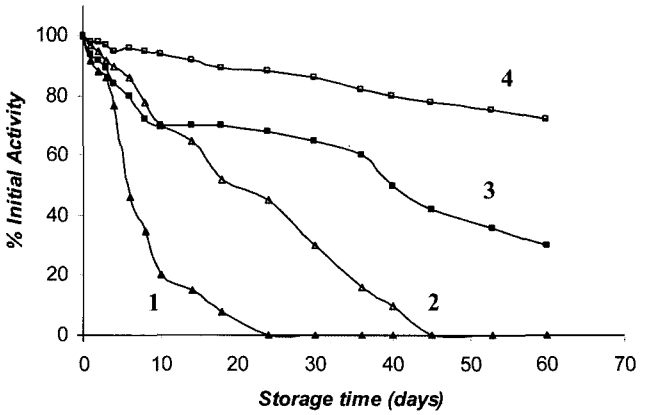

Fig. 5. Storage stabilities of free and co-immobilized GOD-CAT. **1**, Free at 25°C; **2**, Free at 5°C; **3**, Bound at 25°C; and **4**, Bound at 5°C.

GOD against H_2O_2 inhibition. The co-immobilization of GOD with CAT, which is about 10 times cheaper than GOD, provided an effective increase in GOD activity, possibly due to CAT protecting GOD from inactivation by H_2O_2 and supplying additional O_2 to the reaction system.

The storage stabilities of the free and co-immobilized GOD-CAT were measured and the results are represented in Fig. 5, where the co-immobilized GOD-CAT clearly showed a higher storage stability than the free forms. In particular, the co-immobilized enzyme retained 72% of its initial activity when stored at 5°C. In contrast, the free forms did not show any catalytic activity after 24 and 45 days of storage when stored at 25°C and 5°C, respectively.

The co-immobilization of GOD and CAT was investigated in detail, including the method of co-immobilization and different initial enzyme amounts. The co-immobilized GOD-CAT showed a 70% higher catalytic activity and more than 2-fold better operational stability than the individually immobilized GOD. Therefore, these results clearly showed that the GOD activity was improved by co-immobilization with CAT, possibly due to CAT protecting of GOD from inactivation by H_2O_2 and supplying additional O_2 to the reaction system. This would also appear to be the first comparison of the simultaneous co-immobilization and sequential co-immobilization of GOD and CAT, and the first time the amount of bound GOD and CAT was determined separately for simultaneous co-immobilized samples. The sequential co-immobilization procedure was found to be more effective.

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