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Characterization and properties of catalase immobilized onto controlled pore glass and its application in batch and plug-flow type reactors

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ABSTRACT

Bovine liver catalase was covalently immobilized onto controlled pore glass (CPG) beads modified with 3-aminopropyltriethoxysilane (3-APTES) followed by treatment with glutaraldehyde. Coupling of catalase onto CPG was optimized to improve the efficiency of the overall immobilization procedure. The optimum coupling conditions: pore diameter of CPG, pH, buffer concentration, temperature, coupling time and initial catalase amount per grams of carrier were determined as 70 nm, 6.0, 75 mM, 5 °C, 7 h and 6 mg catalase, respectively. Catalytic efficiencies (k_{cat}/K_m) and thermal inactivation rate constants (k_i) of ICPG1 were determined and compared with that of free catalase. Suitability of ICPG1 was also investigated by using it in batch and plug-flow type reactors. When the remaining activity of ICPG1 retained was about 50% of its initial activity the highest total productivity of ICPG1 was determined as 7.6×10^6 U g immobilized catalase^{-1} in plug-flow type reactor. However, the highest total productivity of ICPG1 was 6.2×10^5 U g immobilized catalase^{-1} in batch type reactor. ICPG1 may have great potentials as biocatalyst for the application in decomposition of hydrogen peroxide in plug-flow type reactor.

1. Introduction

Catalases (H_2O_2:H_2O_2 oxidoreductase; EC 1.11.1.6) are abundant enzymes in nature that decompose hydrogen peroxide to water and molecular oxygen. Catalase may present many important applications, namely:

(a) degradation of the H_2O_2 after textile bleaching [1] and after cold pasteurization of milk [2], and also to remove H_2O_2 from the reaction medium coupled with oxidases such as glucose oxidase, L-α-glycerophosphate oxidase, D-aminoacid oxidase [3–5] and
(b) in biosensor system to determine H_2O_2 or glucose amount [6,7].

Immobilization of catalase can offer several advantages for industrial and biotechnological applications, including repeated use, ease of separation of reaction products from the biocatalyst, improvement of enzyme stability, continuous operation in a packed-bed reactor and the alteration of the properties of the enzyme. Catalase has been immobilized on numerous carrier materials such as dextran [8], asymmetric cellulose membrane [9], nylon membrane [10], collagen membrane [11], kappa-carragenan gel [12], chitosan [13], alumina [14], magnesium silicate (florisil) [15], poly(ethylene terephthalate), polyamide 6.6 [16], carbon nanotubes (CNTs) [17], eggshell [18], by covalently, ultrafine silica particles [19], bioskin [20], Cibacron Blue F3GA and Fe(III)-derivatized poly(hydroxyethyl methacrylate) membranes [21], calcium hydroxyapatite [22] by adsorption, polyacrylamide gels [23] by entrapment, mesoporous silica spheres [24] and phospholipids vesicle [25] by encapsulation.

In this study, controlled pore glass (CPG) was chosen as support material for catalase and used after modification with 3-APTES and glutaraldehyde. CPG is one of the popular solid supports for covalent immobilization of enzymes. CPG contains 96% SiO_2, 3% B_2O_3, 1% Na_2O with traces of other metal oxides with Al_2O_3 as the most abundant and offers high mechanical strength, thermal stability, resistance to organic solvents or acidic conditions, easy handling and long half-life and can be prepared with a wide range of porosities and pore sizes. In addition to these CPG can withstand enormous pressures without compression or collapse. The pore size of carrier is not only related to the available surface but also to diffusion limitations and the mobility of the immobilized enzyme molecules. Pore size also can have a major impact on the activity and stability of the immobilized enzyme.

Glutaraldehyde being a bifunctional molecule has been extensively used as an enzyme immobilizing agent for many years. Although there are many discussions on the nature of the solution

Abbreviations: 3-APTES, 3-aminopropyltriethoxysilane; CPG, controlled pore glass; ICPG1, immobilized catalase onto CPG which pore diameter is 70 nm; ICPG2, immobilized catalase onto CPG which pore diameter is 100 nm; ICPG3, immobilized catalase onto CPG which pore diameter is 214.7 nm.

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species and structures responsible for its properties, it is generally accepted that reactions between the carbonyl group of glutaraldehyde and the amino functional groups of the enzyme take place yielding a Schiff base [26]. The obtention of high activities and stabilities during operational conditions usually requires an optimization of the enzyme immobilization protocol. In the literature, this is the first detailed study contained quantitative determination of free $-NH_2$ groups upon activation of CPGs with 3-APTES followed by glutaraldehyde modification, optimization of immobilization conditions of catalase and characterization of immobilized catalase. The main objectives of this study are (1) to optimize the immobilization conditions of catalase (pore size, pH, buffer concentration, temperature, duration of immobilization and initial amount of catalase) to CPG beads, (2) to determine kinetic constants (K_m, V_{max}, k_{cat}/K_m) at optimal conditions and thermal and storage stabilities of immobilized catalase and (3) to determine the operational stabilities of immobilized catalase in batch and plug-flow type reactor systems.

2. Materials and methods

2.1. Materials

Hydrogen peroxide was obtained from Merck AG (Darmstadt, Germany). Bovine liver catalase (44,500 U mg protein^{-1}), CPG beads with 200–400 mesh and an average pore diameter (specific area, $m^2\,g^{-1}$) 70 nm (39.4 $m^2\,g^{-1}$), 100 nm (21.8 $m^2\,g^{-1}$), 214.7 nm (7.4 $m^2\,g^{-1}$), glutaraldehyde solution (aqueous solution, 50% (w/w)), 3-APTES, ninhydrin reagent (2% (w/w) solution) and all the other chemicals were obtained from Sigma (St. Louis, MO).

2.2. Activation of CPG

The silanization method has been described earlier by Weetall [27]. Before silanization CPG was washed with 5% (v/v) HNO_3 solution at 80–90 °C for 60 min followed by rinsing with distilled water and dried overnight at 120 °C and then to the 1 g of the carrier, 25 mL of 4% solution of 3-APTES in acetone (v/v) was added and evaporated to dryness at 45 °C for 24 h (Fig. 1a). After that it was washed exhaustively with distilled water on a Buchnel funnel until no 3-APTES was detected in the filtrate and then the carrier was heated to 115 °C overnight. The amount of 3-APTES in the filtrate was detected by using ninhydrin-based monitoring system. Ninhydrin reacts with primary amines to form a colored complex known as Ruhemann's purple which is soluble in ethanol/water phase and highly conjugated with a strong absorption around 570 nm (blue color). This reaction also can be used to measure the amount of free $-NH_2$ groups attached to an insoluble support. Indirectly, the activation level of the matrix can be determined from the quantity of primary amine detected on the support. To 5 mg of silanized carrier 100 μL H_2O and 200 μL ninhydrin reagent were added and heated in a boiling water bath for 30 min then cooled at room temperature. Five milliliters of 50:50 (v/v) mixture of ethanol:water were added then the solution was mixed well and absorbance of the solution was measured at 570 nm with an ATI Unicam UV2 UV/vis spectrophotometer.

One gram of the silanized carrier was added into 25 mL of 2.5% (w/v) glutaraldehyde solution prepared with potassium phosphate buffer (50 mM, pH 7.0) and the reaction was allowed to continue for 2 h. After that carrier was washed exhaustively with distilled water on a Buchnel funnel until no glutaraldehyde was detected in the filtrate and then was dried at 60 °C for 2 h (Fig. 1b). Glutaraldehyde in the filtrate was detected according to Boratynski and Zal [28]. The amount of free $-NH_2$ groups onto CPG beads after glutaraldehyde modification was also determined by using ninhydrin method. The amount of functional aldehyde group introduced onto CPG after glutaraldehyde treatment was indirectly determined by subtracting the amount of $-NH_2$ groups after glutaraldehyde treatment from the amount of $-NH_2$ group after silanization.

2.3. Optimization of immobilization conditions

2.3.1. Pore size of CPG

Catalase was immobilized onto CPG beads with mean pore diameters of 70, 100 and 214.7 nm and immobilized catalases were symbolized as ICPG1, ICPG2 and ICPG3, respectively. One gram of activated CPG beads was mixed with 8 mL of catalase solution (1 mg mL^{-1}), which was prepared in 50 mM phosphate buffer pH 7.0. The mixture was kept at 25 °C for 2 h and shaken gently during this period, and then washed with the same buffer solution until no protein was detected in the filtrate (Fig. 1c). Protein values were determined by the method of Lowry et al. [29]. The amount of unbound enzyme protein was subtracted from the total amount of enzyme protein used for immobilization and the amount of bound enzyme protein was calculated as mg protein g carrier^{-1}. Immobilized catalase preparations were kept overnight at 5 °C in an incubator then stored in closed glass tubes at the same conditions. Activities of ICPG1, ICPG2 and ICPG3 were determined and optimal pore size for immobilization was revealed. The details of activity measurements are given in Section 2.4.

Optimum conditions for immobilization were determined with CPG beads by changing individually the following conditions: the range of pH 5.0–8.0 (sodium acetate pH 5.0–5.5; sodium citrate pH 6.0; potassium phosphate pH 6.0–8.0); buffer concentration 15–100 mM; temperature 5–25 °C; duration of immobilization from 1 to 24 h; initial amount of catalase 1.0–8.0 mg/g carrier. The amount of bound protein and activity of immobilized catalase was determined for all preparations.

Leo 440 Computer-controlled Digital Scanning Electron Microscope was used to obtain images of the CPG beads and immobilized catalases (ICPGs).

2.4. Enzyme activity assay

The catalase activity was determined according to the Lartillot et al. [30] which is a modification of the method described by Bergmeyer [31]. Catalase activity was measured spectrophotometrically at 240 nm using a specific absorption coefficient of 0.0392 cm^2 μmol $H_2O_2{}^{-1}$. Catalase activity was determined in reaction mixture containing 2.5 mL of substrate made up of 10 mM hydrogen peroxide in a 50-mM phosphate buffer pH 7.0 and 3.16×10^{-5} mg of free catalase or 2.5 mg of immobilized catalase. Reaction was carried out at 25 °C for 2 min and stopped by adding 0.5 mL of 1 M HCl. One unit of activity is defined as the decomposition of 1 μmol hydrogen peroxide per min at 25 °C and pH 7.0. Activity of free catalase was given as U mg protein^{-1} and immobilized catalases activities were expressed as U mg protein^{-1} or U g immobilized catalase^{-1}.

2.5. Characterization of free and immobilized catalase

Assays for activity were carried out as described in Section 2.4 in the characterization studies of free and immobilized catalases unless otherwise stated.

2.5.1. The effect of pH

Activities of free and immobilized catalase were determined by using 10 mM H_2O_2 substrate prepared in 50 mM acetate buffer (pH

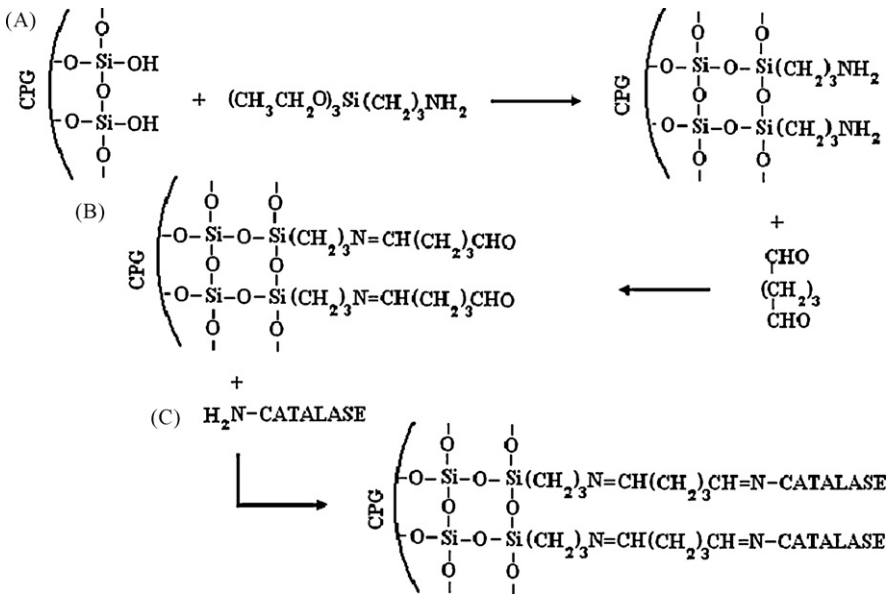

Fig. 1. Activation of carrier and immobilization of catalase by covalent binding. (a) Silanization of the surface of carrier materials. (b) Primary aminoacid groups (R-NH_2) of support are activated by glutaraldehyde ($C_5H_8O_2$) to give a carbonyl derivative by formation of a Schiff's base. (c) The formation of a Schiff's base linkage between carbonyl groups of the activated carrier and free amino groups on the protein (adapted from [27,56,57].

5.0 and 5.5), 50 mM citrate buffer (pH 6.0), 50 mM phosphate buffer (pH 6.5, 7.0, 7.5 and 8.0), and 50 mM borate buffer (pH 9.0).

2.5.2. The effect of buffer concentration

To investigate effect of buffer concentration, activities of free and immobilized catalase, depending on ionic strength, were determined by using 10 mM H_2O_2 solution prepared in 25, 50, 75 and 100 mM buffer solutions at predetermined optimal pH value.

2.5.3. The effect of temperature

The effect of temperature on the activities of free and immobilized catalase were investigated at temperatures ranging of 10–60 °C at their optimal pH and buffer concentrations.

2.5.4. Kinetic parameters

To determine maximum reaction rates (V_{max}) and Michaelis–Menten constants (K_m), the activity assay was applied for different H_2O_2 concentrations (5.0, 7.5, 10.0, 12.5 and 15.0 mM). Activities of free and immobilized catalase were determined at predetermined optimum conditions. The kinetic parameters V_{max} and K_m for free and immobilized catalases were calculated from double reciprocal plot. The catalytic efficiencies (k_{cat}/K_m) of free and immobilized catalases were also calculated.

2.5.5. Thermal stabilities of free and immobilized catalase

The thermal stability of the catalase was studied by measuring the residual activity after incubation of the free catalase (1.6×10^{-3} mg protein mL^{-1}) or immobilized catalase in 50 mM phosphate buffer pH 7.5 at various temperatures (35 and 50 °C) for different incubation times (1, 3, 7 and 15 h). The first-order inactivation constant (k_i) and half-life ($t_{1/2}$) of enzyme was calculated from the equation:

$$\ln V = \ln V_0 - k_i t$$

where V_0 and V are the initial activity and the activity after time t, respectively.

2.5.6. Storage stabilities of free and immobilized catalase

The storage stabilities of free and immobilized catalase were investigated at room temperature and 5 °C. Free enzyme preparation was stored as a solution of 1.6×10^{-3} mg protein mL^{-1} of 50 mM pH 7.5 phosphate buffer, whereas immobilized catalase was stored as solid particles in a closed glass tube and residual activities were measured during 77 days of their storages.

2.6. Operational stability of immobilized catalase

Operational stability of immobilized catalase was investigated by using batch and plug-flow type reactors. In batch type reactor, different amounts of immobilized catalase (10, 20 and 40 mg) were loaded into the reactor (7 cm length, 1 cm i.d.) and then 5 mL of 10 mM H_2O_2 solution at pH 7.5 was added and the reaction was allowed to continue for 2 min at 26 ± 1 °C. After that the reaction mixture was removed immediately from the column and the activity was determined by measuring the absorbance of H_2O_2 at 240 nm.

In plug-flow reactor experiments, immobilized catalase as 10, 20 and 40 mg was packed into small glass columns with 15 cm length, 2 mm i.d. and 0.5 mm wall thickness and reactor was connected to a peristaltic pump which fed the reactor with 10 mM H_2O_2 in 75 mM potassium phosphate buffer (pH 7.5) with varying flow rates (2.3 and 5.6 mL min^{-1}) at 26 ± 1 °C. Absorbances were measured 30 s interval in a Hellma glass quartz flow cell in spectrophotometer until immobilized catalase completely lost its original activity.

Productivities of immobilized catalase for batch type reactor system were calculated from the equation:

$$XS - K_m \ln(1 - X) = \frac{kE\pi}{v}$$

where kE is the maximum activity of the total enzyme in the reactor in mol reacted per min, v is the working volume of the reactor, π is the time period of operation, and X is the degree of conversion $S - S_t/S$, where S is the starting substrate concentration and S_t the concentration remaining after a period, t, of reaction [32].

In the same way, productivities of immobilized catalase were calculated for a plug-flow reactor from the equation:

$$XS - K_m \ln(1 - X) = \frac{kE}{q} = \frac{kE\pi}{v}$$

Table 1
Characterization of the CPG beads used to immobilize catalase, regarding pore size, surface area, the amount of $-NH_2$ groups, amount of bound catalase, activity of immobilized catalase and specific activity of immobilized catalase.

CPG ID	Pore diameter[a] (nm)	$A_{surface}$[a] ($m^2\,g^{-1}$)	$-NH_2$ amount (mol g carrier^{-1})		Bound catalase (mg protein g carrier^{-1})	Activity (U g immobilized catalase^{-1})	Specific activity (U mg protein^{-1})
			After silanization	After glutaraldehyde treatment			
ICPG1	70.0	39.4	$3.36\,(\pm0.21)\times10^{-5}$	$3.28\,(\pm0.27)1\times10^{-6}$	6.9	951.5 ± 38.9	141.25 ± 5.96
ICPG2	100.0	21.8	$1.08\,(\pm0.06)\times10^{-5}$	$1.09\,(\pm0.17)\times10^{-6}$	6.7	482.1 ± 49.6	72.95 ± 7.45
ICPG3	214.7	7.4	$2.87\,(\pm0.16)\times10^{-6}$	$2.45\,(\pm0.57)\times10^{-7}$	5.9	586.7 ± 57.7	100.12 ± 9.85

[a] Values given by the manufacturer.

where q is the volumetric rate of supply of substrate (mL min^{-1}) and π is the average residence time of substrate solution in the reactor in min.

3. Results and discussion

3.1. Optimization of immobilization conditions

The amount of $-NH_2$ groups on the surface of the CPG beads after 3-APTES and glutaraldehyde treatment was given in Table 1. These results showed that the amount of attached $-NH_2$ group increases almost proportionally with the surface area of the CPG. As shown from Table 1 after glutaraldehyde treatment, the amounts of the remaining $-NH_2$ groups of all three types of CPGs were about 10% of the amounts of initial $-NH_2$ groups of silanized CPG beads. These results showed that CPG was successfully modified with 3-APTES and glutaraldehyde. Covalent immobilization of catalase or other enzymes onto CPG modified with 3-APTES and glutaraldehyde was commonly used immobilization technique in the literature [33–41]. However, there was not any quantitative result about the amount of $-NH_2$ groups on CPG upon silanization and also after glutaraldehyde modification. The presence of trace amount of glutaraldehyde in immobilization medium caused to decline catalase activity so unreacted glutaraldehyde should be removed completely from the CPG beads after glutaraldehyde treatment [42]. In order to be sure that the unreacted glutaraldehyde was completely removed from the CPG beads after glutaraldehyde treatment, we determined the unreacted glutaraldehyde in the filtrate and continued to wash until no glutaraldehyde was detected. However, in the studies which use the similar catalase immobilization procedures as in our study [14,38–40,42–44,46] there was not any given information concerning about the determination of unreacted glutaraldehyde in the filtrate.

The amount of bound catalase increased from 5.9 to 6.9 mg g carrier^{-1} as the surface area of the CPG increased from 7.4 to 39.4 $m^2\,g^{-1}$ (Table 1). The highest activity and specific activity as 951.5 ± 38.9 U g immobilized catalase^{-1} and 141.25 ± 5.96 U mg protein^{-1}, respectively, were obtained for the immobilized catalase onto CPG of the surface area 39.4 $m^2\,g^{-1}$ (ICPG1). Cao [47] reported that the pores need to be 3–9 times larger than the size of the enzymes for efficient immobilization. Zhang et al. [48] reported the dimensions of one bovine liver catalase molecule as 9.0 nm × 6.0 nm × 2.0 nm and therefore catalase was not excluded by all three tested CPGs. The results recorded in Table 1 indicate that a maximum loading of stable and active catalase is achieved in CPG with pore diameter of 70 nm (ICPG1).

The highest activity was determined for the preparation immobilized at pH 6.0 although the amount of bound catalase slightly increased by increasing pH from 5.0 to 8.0 (Fig. 2). These results show that the binding of the catalase onto CPG was affected from significant alterations of enzyme microenvironment, improving the retention of activity at pH 5.0–6.0 and catalase retained its active conformation after bound onto CPG at pH 6.0. Therefore, pH 6.0 was chosen as immobilization pH for subsequent studies.

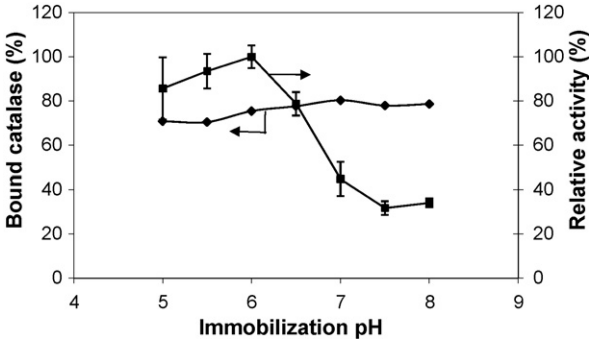

Fig. 2. The effect of immobilization pH on amount of bound catalase and activity of ICPG1 [(♦) bound catalase (%) and (■) relative activity (%)]. Catalase was immobilized in 50 mM buffer concentration at room temperature for 2 h. Initial amount of catalase was 8.0 mg/g carrier. The activity was determined using 10 mM of H_2O_2 in 50 mM potassium phosphate buffer (pH 7.0) at room temperature.

The amount of bound catalase was slightly affected from the medium buffer concentration in the range of 15–100 mM. The maximum binding occurred at 15 mM buffer concentration. However, maximum ICPG1 activity was observed at 75 mM buffer concentration. Therefore, considering the highest ICPG1 activity, we selected immobilization buffer concentration as 75 mM.

The amount of bound catalase was slightly increased when immobilization temperature was increased from 5 to 25 °C. However, the activity of ICPG1 was gradually decreased. The highest ICPG1 activity was obtained at 5 °C so we decided immobilization temperature as 5 °C.

The effect of immobilization time on catalase immobilization was investigated and the results were given in Fig. 3. When the immobilization time was 1 h, 6.9 mg enzyme protein which was about 76% of the originally added enzyme protein bound onto 1 g of carrier. The amount of bound catalase did not change signif-

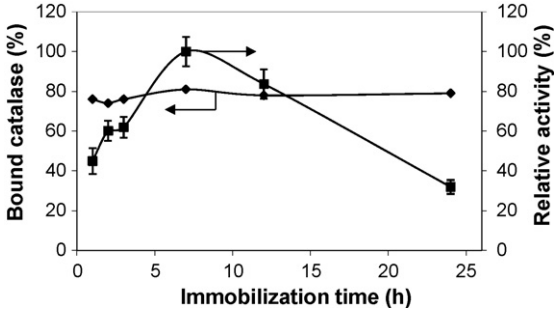

Fig. 3. The effect of immobilization time on amount of bound catalase and activity of ICPG1 [(♦) bound catalase (%) and (■) relative activity (%)]. Catalase was immobilized in 75 mM sodium citrate buffer at pH 6.0 and 5 °C. Initial amount of catalase was 8.0 mg/g carrier. The activity was determined using 10 mM of H_2O_2 in 50 mM potassium phosphate buffer (pH 7.0) at room temperature.

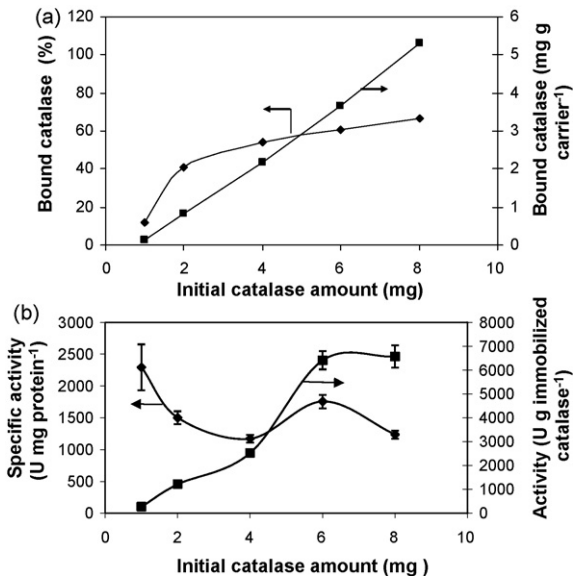

Fig. 4. (a) The effect of initial catalase amount on amount of bound catalase [(♦) bound catalase (%) and (■) bound catalase (mg protein g carrier^{-1})]. Catalase was immobilized in 75 mM sodium citrate buffer at pH 6.0 and 5 °C for 7 h. The activity was determined using 10 mM of H_2O_2 in 50 mM potassium phosphate buffer (pH 7.0) at room temperature. (b) The effect of initial catalase amount on activity and specific activity of ICPG1 [(♦) specific activity (U mg protein^{-1}) and (■) activity (U g immobilized catalase^{-1})]. Catalase was immobilized in 75 mM sodium citrate buffer at pH 6.0 and 5 °C for 7 h. The activity was determined using 10 mM of H_2O_2 in 50 mM potassium phosphate buffer (pH 7.0) at room temperature.

icantly when immobilization time increased from 1 to 24 h at a constant initial catalase amount but the highest activity was determined for the preparation for which immobilization time was 7 h. Optimum immobilization time was chosen as 7 h. In the literature, optimum immobilization time was determined as 2 and 3 h for covalent immobilization of catalase onto florisil via glutaraldehyde and poly(acrylonitrile-co-acrylic acid multi-walled carbon nanotubes (PANCAA/MWCNTs) composite nanofiber mesh, respectively [15,49].

As shown in Fig. 4a, the amount of bound catalase onto the same amount of carrier was almost directly proportional with initial catalase amount. Percentage of bound catalase sharply increased when initial catalase amount was increased from 1.0 to 2.0 mg, however, percentage of bound catalase slightly increased when initial catalase amount increased from 2.0 to 8.0 mg. The specific activity of ICPG1 was significantly decreased depending on the increase in initial catalase amount from 1.0 to 4.0 mg (Fig. 4b). Activity of ICPG1 was increased by increasing initial catalase amount from 1.0 to 6.0 mg and at higher initial catalase amount; activity of ICPG1 did not change significantly. Optimum initial catalase amount was therefore chosen as 6.0 mg by considering activities and specific activities of ICPG1.

The surface morphologies of original CPG beads (before activation) and immobilized catalase preparations were exemplified by the SEM in Fig. 5. CPGs with the different pore size are heterogeneous in shape and size. As the surface morphologies of ICPGs (Fig. 5c, e and g) compared with that of original CPGs (Fig. 5b, d and f), it is clearly seen that the surface morphologies of CPGs were not affected from activation and immobilization procedures.

3.2. Characterization of free catalase and ICPG1

Free catalase and ICPG1 showed their maximum activity at pH 7.5 (Fig. 6). ICPG1 retained 80% of its maximum activity in alkaline medium (pH 9.0), however, free catalase retained only 40% of its maximum activity at pH 9.0. The immobilized catalase was less affected with pH changes at alkaline pHs than acidic pHs as compared with that of free catalase. In our previous study, we immobilized catalase onto florisil (a basic support) and we observed the maximum activity of immobilized catalase shifted to acidic medium (pH 6.5) as compared with that of free catalase (pH 7.5) [15]. Costa et al. [14] reported that the treatment of the immobilized preparations with glutaraldehyde further increased their ability to retain enzymatic activity at alkaline pHs (8–11). Immobilized catalase on gellan, PANCAA/MWCNTs and crosslinked polystyrene ethylene glycol acrylate resin (CLPSER) showed their maximum activity at pH 6.5, 7.2 and 7.5, respectively [50,51,49]. All these results showed that optimum pH of ICPG1 took place between in range in which the literature was given.

Free catalase and ICPG1 showed maximum activity at 50 and 75 mM phosphate buffer pH 7.5, respectively. When the buffer concentration was increased from 25 to 100 mM, the activity of ICPG1 more affected than the activity of free catalase, and at 100 mM buffer concentration free catalase retained 96.9% of its maximum activity, ICPG1 retained 65.6% of its maximum activity. These results showed that when the buffer concentration was increased, activity of ICPG1 was more affected than the activity of free catalase.

Fig. 7 shows the activities of free catalase and ICPG1 as a function of temperature. Free catalase and ICPG1 had their maximum activities at 25 and 35 °C, respectively. They both showed high relative activity in the temperature range of 25–35 °C. ICPG1 activity was more affected than free catalase activity with the rise of temperature from 10 to 25 °C. The activation energies of free catalase and ICPG1 were determined as 14.4 ± 3.5 and 22.6 ± 3.0 kJ mol^{-1} by using the Arrhenius equation, respectively. The slope of the Arrhenius plot for the immobilized enzyme was not equal to that of the soluble enzyme, this result may be explained with internal diffusion restrictions or reduced conformational flexibility of the catalase molecule to reorganize to the appropriate conformation for catalysis to occur at 10–25 °C. In our previous study, the maximum activities of catalase immobilized via glutaraldehyde and via glutaraldehyde + 3-aminopropionic acid (spacer) onto florisil were 35 and 25 °C, respectively [15]. Our results showed that immobilization via glutaraldehyde onto CPG or florisil did not change the optimum temperature of immobilized catalase.

The apparent Michaelis–Menten constant, K_m, was higher for ICPG1 (48.3 ± 4.6 mM) than for free catalase (28.6 ± 3.6 mM). The higher K_m values for the solid-phase enzymes may be a result of a number of effects. The migration of substrate from the solution to the microenvironment of an immobilized enzyme can be a major factor increase in K_m values. A diffusion film which covers the surface of an insoluble particle was proposed and within which the substrate concentration is lower than in the solution. The rate at which substrate passes over the diffusion film, which in turn determines the concentration of substrate in the vicinity of the enzyme and hence the K_m values. V_{max} value of ICPG1 ($1.9 (\pm 0.2) \times 10^4$ U mg protein^{-1}) was 14% of V_{max} value of free catalase ($1.4 (\pm 0.2) \times 10^5$ U mg protein^{-1}). Catalytic efficiency (k_{cat}/K_m) of ICPG1 (1.6×10^6 M^{-1} s^{-1}) was 8% of catalytic efficiency of free catalase (2.1×10^7 M^{-1} s^{-1}). There is not given any data about the kinetic parameters of immobilized catalase onto CPG in the literature [37–39] so we could not compare our experimental results. In our previous study, k_{cat}/K_m value of immobilized catalase onto florisil was found as 2.0×10^4 M^{-1} s^{-1} and this was about 0.1% of catalytic efficiency of free catalase [15]. Catalytic efficiency of catalase immobilized onto CPG increased about 80 times with respect to catalytic efficiency of catalase immobilized onto florisil. In the literature, V_{max} values of immobilized catalases onto cellulose, chitosan, silica gel, copolymer hydrogel and silica glass were reported as 4%, 5%, 10%, 28% and 36% of V_{max} value of free catalase [13,52,45,53,54]. Wan et al. [17] and Wang et al. [49] immobilized catalase onto CNTs and they reported that V_{max} values of immobilized catalase

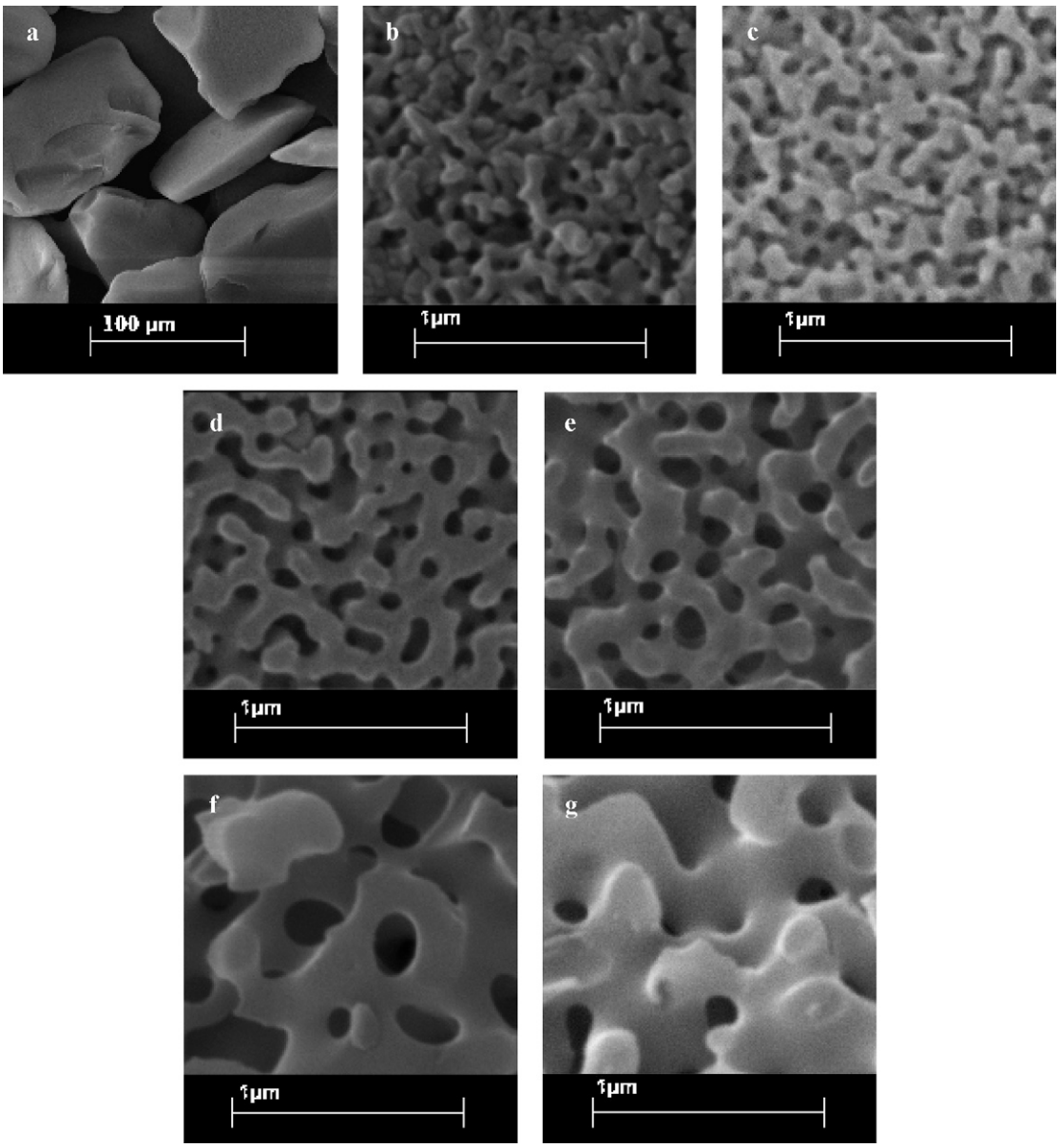

Fig. 5. Scanning electron micrographs of the surfaces of CPGs, ICPG1, ICPG2 and ICPG3. (a) CPG (70 nm) 200×, (b) CPG (70 nm) 30,000×, (c) ICPG1 30,000×, (d) CPG (100 nm) 30,000×, (e) ICPG2 30,000×, (f) CPG (214.7 nm) 30,000× and (g) ICPG3 30,000×.

preparations were 84% and 92% of V_{max} value of free catalase, respectively. The high activities obtained with these preparations were explained by the fact that CNTs behave as electrons transferors during enzyme catalysis.

Thermal stabilities of free catalase and ICPG1 were investigated at 35 and 50 °C. The half-lives of free catalase at 35 and 50 °C were 9.0 and 6.7 h, respectively and; these correspondingly were 70.0 and 9.7 h for ICPG1. The thermal inactivation rate constants (k_i) at 35 and 50 °C were calculated as 7.70×10^{-2} and 10.4×10^{-2} h^{-1}, respectively, for free catalase, and as 1.0×10^{-2} and 7.2×10^{-2} h^{-1}, respectively, for ICPG1. These results show that the thermal stability of ICPG1 was comparably higher than that of the free catalase at 35 and 50 °C. Wan et al. [55] reported that the residual activity of free catalase, immobilized catalases onto poly(acrylonitrile-co-N-vinyl-2-pyrolidone (PANCNVP) and PANCNVP/CNTs nanofibers were 60%, 45% and 33% of their original activities, respectively, after 1 h preincubation at 50 °C.

The storage stability of ICPG1 was greater than that of free catalase both at room temperature and at 5 °C. As shown in Fig. 8

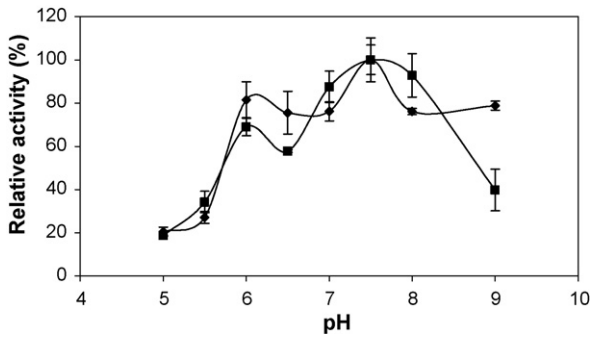

Fig. 6. The effect of the pH on the activity of ICPG1 and free catalase [(♦) ICPG1 and (■) free catalase]. Assays were carried out at 25 °C using 50 mM buffer, pH 5–9. The reaction time was 2 min, and 10 mM H_2O_2 was used as substrate.

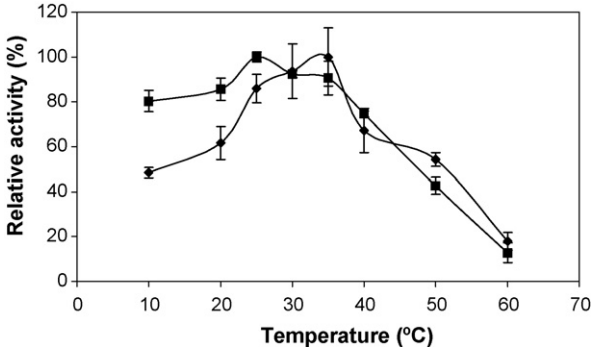

Fig. 7. Effect of the temperature on the activity of ICPG1 and free catalase [(♦) ICPG1 and (■) free catalase]. Assays were carried out in 50 mM phosphate buffer pH 7.5 for free catalase and 75 mM phosphate buffer for ICPG1. The reaction time was 2 min, and 10 mM H_2O_2 was used as substrate.

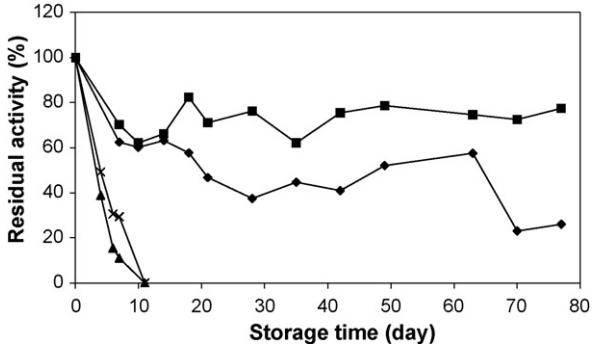

Fig. 8. Storage stability of ICPG1 and free catalase at 5 °C and room temperature (RT). [(♦) ICPG1 (RT), (■) ICPG1 (5 °C), (×) free catalase (RT), (▲) free catalase (5 °C)]. Free catalase was stored in 50 mM potassium phosphate buffer, pH 7.5. Immobilized catalase was stored as solid particles. The remaining activity was determined using 10 mM of H_2O_2 in 50 mM potassium phosphate buffer (pH 7.5) at room temperature. It is expressed as a percentage of the original activity.

free catalase completely lost its initial activity at the end of 11 days both at room temperature and also at 5 °C. However, ICPG1 retained 26% of its initial activity at room temperature and 77% of its initial activity at 5 °C at the end of 77 days. It is obvious that there is a remarkable difference in the activity retentions with storage time. In other words, catalase immobilized onto CPG showed high stability, whereas free catalase lost most of its initial activity very soon. Çetinus et al. [13] immobilized catalase on glutaraldehyde-pretreated chitosan films. They reported that the free catalase retained about 50% of its activity for 18 days, immobilized catalase stored wet retained about 50% of its activity for 25 days and stored dry retained about 50% of its activity for 5 days at 5 °C. Wan et al. [17] reported that immobilized catalase onto acrylonitrile-based copolymers bearing porphyrine pendants were blended with carbon nanotubes (PANAACoPP/CNTs PAN) retained 70% of its initial activity at 4 °C at the end of 26 days. Wang et al. [49] reported that immobilized catalase onto PANCAA and PANCAA/MWCNTs nanofiber mesh retained about 30% and 80% of their initial activities, respectively, at 4 °C at the end of 20 days. As we compared our storage stability results with that of the above given literatures it is obvious ICPG1 has the highest storage stability at 5 °C.

3.3. Operational stability of ICPG1

Operational stabilities of ICPG1 were determined in batch and plug-flow type reactors. The results were compared in terms of reuse numbers, operation times and total productivities of ICPG1

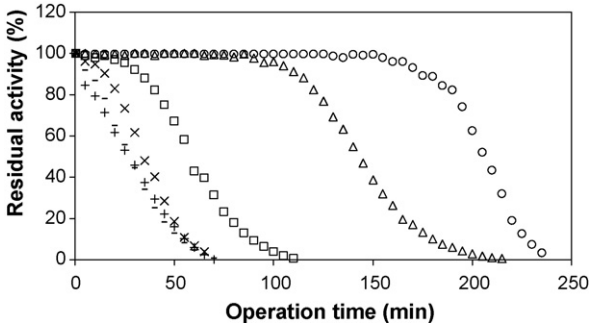

Fig. 9. Operational stabilities of ICPG1 in plug-flow type reactor. Flow rate 2.3 mL min^{-1} [(+) 10 mg, (□) 20 mg and (△) 40 mg], flow rate 5.6 mL min^{-1}[(−) 10 mg, (×) 20 mg and (○) 40 mg]. The substrate solution contained 10 mM H_2O_2 in 75 mM potassium phosphate buffer, pH 7.5. The reaction was carried out at 26 ± 1 °C.

when the remaining activity of ICPG1 was about 50% of its original activity. In batch type reactor, in order to prevent the influence of storage time on ICPG1 activity all measurements were carried out at the same day. The reuse numbers were 34, 72 and 180 cycles of batch operation in the reactors loaded with 10, 20 and 40 mg of ICPG1, respectively. The total productivities of ICPG1 in batch type reactor loaded with 10, 20 and 40 mg of ICPG1 were calculated as 1.5×10^5, 1.8×10^5 and 6.2×10^5 U g immobilized catalase^{-1}, respectively. In the cases of plug-flow reactor loaded with 10, 20 and 40 mg of ICPG1 the operation times were determined as 27, 58 and 143 min, respectively, for 2.3 mL min^{-1} flow rate of substrate, however, when the substrate flow rate was increased to 5.6 mL min^{-1} corresponding operation times were determined as 26.5, 34 and 205 min (Fig. 9). In plug-flow reactor loaded with 10, 20 and 40 mg of ICPG1 the total productivities were calculated as 4.0×10^5, 8.0×10^5 and 15.7×10^5 U g immobilized catalase^{-1}, respectively, for 2.3 mL min^{-1} flow rate of substrate, however, when the substrate flow rate was increased to 5.6 mL min^{-1} corresponding total productivities were calculated as 5.8×10^5, 8.0×10^5 and 69.7×10^5 U g immobilized catalase^{-1}. In plug-flow type reactor when ICPG1 load was high, total productivity was more affected from the flow rate of substrate. Increased productivity in a plug-flow reactor with increased flow rate may be related to the decrease the external diffusion limitation [32]. The highest total productivity (69.7×10^5 U g immobilized catalase^{-1}) obtained in plug-flow reactor was about 11-fold higher than the highest productivity (6.2×10^5 U g immobilized catalase^{-1}) obtained in batch type reactor. The use of ICPG1 in plug-flow type reactor may be suggested due to its high performance as compared with that of ICPG1 in batch type reactor. In our previous study, immobilized catalase onto florisil via glutaraldehyde and via glutaraldehyde + spacer retained 50% of their initial activities after 80 and 3 cycles of batch operation, respectively [15]. Arıca et al. [53] reported that immobilized catalase in poly(isopropylacrylamide-co-hydroxyethyl-methacrylate) retained 50% of its initial activity after about 17 cycles of batch operation. Wan et al. [17] reported that immobilized catalase onto PANAACoPP/CNTs retained 70% of its initial activity after 10 cycles of batch operation. Wang et al. [49] reported that immobilized catalase onto PANCAA and PANCAA/MWCNTs nanofiber mesh retained 40% and 33% of their initial activities, respectively, after 10 cycles of batch operation. As we compared operational stabilities of ICPG1 with that of the above given literatures it is obvious ICPG1 had the highest operational stability.

4. Conclusion

The surface modification of CPG was successfully carried out with 3-APTES and glutaraldehyde for covalent immobilization of

catalase onto CPG. The activation levels of CPGs were determined from the quantity of primary amine detected on the CPG after 3-APTES and also glutaraldehyde treatment. The tested pore sizes (70, 100 and 214.7 nm) were not the limiting factor for immobilization of catalase onto CPG and a maximum loading of stable and active catalase is achieved in CPG with pore diameter of 70 nm (ICPG1). Optimization studies on catalase immobilization onto CPG clearly showed that a high catalase binding yield did not correspond necessarily with a high activity. Therefore, a successful immobilization should combine a maximal load and also a maximal activity. Catalytic efficiency (k_{cat}/K_m) of ICPG1 was 8% of catalytic efficiency of free catalase. However, the thermal stability of immobilized catalase was higher than that of free catalase at 35 and 50 °C. The thermal stabilities of ICPG1 were increased 8- and 1.5-fold at 35 and 50 °C, respectively. The storage stabilities of the ICPG1 were obviously improved compared with the free catalase at room temperature and also 5 °C. The highest productivity of ICPG1 was obtained in plug-flow type reactor so ICPG1 may have great potentials as biocatalyst for the application in decomposition of hydrogen peroxide in plug-flow type reactor.

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