ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 15 (2001) 197–206

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

Immobilization of catalase via adsorption onto L-histidine grafted functional pHEMA based membrane

Sinan Akgöl [a], Yasemin Kaçar [b,*], Serpil Özkara [a], Handan Yavuz [a], Adil Denizli [a], M. Yakup Arica [b]

[a] *Department of Chemistry, Hacettepe University, 06532 Beytepe, Ankara, Turkey*
[b] *Department of Biology, Natural and Applied Science Institute, Kirikkale University, TR-71450 Yahşihan, Kirikkale, Turkey*

Received 30 January 2001; received in revised form 10 April 2001; accepted 10 April 2001

Abstract

Poly(2-hydroxyethylmethacrylate) (pHEMA) based flat sheet membrane was prepared by UV-initiated photopolymerization technique. The membrane was then grafted with L-histidine. Catalase immobilization onto the membrane from aqueous solutions containing different amounts of catalase at different pH was investigated in a batch system. The maximum catalase immobilization capacity of the pHEMA–histidine membrane was 86 $\mu g\,cm^{-2}$. The activity yield was decreased with the increase of the enzyme loading. It was observed that there was a significant change between V_{max} value of the free catalase and V_{max} value of the adsorbed catalase on the pHEMA–histidine membrane. The K_m value of the immobilized enzyme was higher 1.5 times than that of the free enzyme. Optimum operational temperature was 5°C higher than that of the free enzyme and was significantly broader. It was observed that enzyme could be repeatedly adsorbed and desorbed without loss of adsorption capacity or enzyme activity.

Keywords: Catalase; Immobilization; Adsorption; L-Histidine; pHEMA membrane

1. Introduction

Enzymes have been utilized in a large number of practical applications, particularly in biomaterials, bioseparators and biosensors through immobilization on different shapes of supports (i.e. membranes or beads) either by adsorption, entrapment or covalent binding. Membrane-immobilized enzymes may serve as model systems for enzymes, naturally bound to membranes, or may find practical applications in membrane-based enzyme bioreactors, enzyme electrodes, and artificial organ systems [1–4].

Immobilized catalase has useful applications in the food industry in the removal of hydrogen peroxide from food products after cold pasteurization and in the analytical field as a component of hydrogen peroxide or glucose biosensor systems [4–8].

Earlier studies showed that, poly(2-hydroxyethylmethacrylate), pHEMA, is an attractive enzyme carrier [9,10]. pHEMA hydrogels are among the major synthetic polymers approved by federal agencies like the Food and Drug Administration in USA for

* Corresponding author. 90-318-357-2477; fax: +90-318-357-2329.
E-mail address: kacar@turkuaz.kku.edu.tr (Y. Kaçar).

1381-1177/01/$ – see front matter
PII: S1381-1177(01)00029-7

biomedical, pharmaceutical and industrial applications. It is a non-toxic, hydrophilic and biocompatible material which is often employed as a support in enzyme technology. In addition, the presence of hydroxyl groups offer attachment sites for enzymes via activation and derivatization by introduction of a variety of ligands [11,12]. It has been used in previous enzyme immobilization studies either by entrapment into, by covalent binding and adsorption onto membrane and microspheres [13,14].

Among immobilization techniques, adsorption may have a higher commercial potential than other methods because adsorption is simpler and less expensive and a high catalytic activity may be retained. The method also offers the reusability of expensive supports after inactivation of immobilized enzyme. However, adsorption is generally not very strong and some of the adsorbed protein will desorb during washing and other operation steps. Thus, immobilization via adsorption requires an electrostatic interaction between the enzyme and support. The natural amino acid L-histidine interacts through its carboxyl, amino and imidazole groups with several proteins at around their isoelectronic points. The immobilized L-histidine has been used as a pseudo-biospecific ligand for removal of human antibodies from plasma and for purification of monoclonal antibodies from cell culture or ascites fluids [15]. Single amino acid molecules could hold certain advantages as pseudo-affinity ligands for industrial application since they are resistance to harsh chemicals and high temperatures as well as to their low cost [15,16].

In the present study, the aim was to immobilize catalase with a significantly higher activity and stability than those described in earlier studies. In order to achieve this aim, a microporous pHEMA membrane was prepared by UV-initiated photopolymerization of HEMA. The L-histidine was grafted through the carbonyl groups of pHEMA in the presence of NaH. The L-histidine grafted functional membrane matrix was used for the immobilization of catalase via adsorption. The biocompatibility of the pHEMA based membrane matrix can be increased with incorporated L-histidine and could provide a natural microenvironment to the guest biologically active macromolecule. The resultant immobilized catalase was characterized and its activity retention, catalytic properties and reusability aspect were compared to that of free counterpart.

2. Experimental sections

2.1. Materials

Catalase (CAT) (hydrogen peroxide oxidoreductase; EC.1.11.1.6) from bovine liver (250.000 U mg^{-1} solid) and L-histidine were obtained from Sigma (St. Louis, MO, USA) and used as received. 2-Hydroxyethylmethacrylate (HEMA) was obtained from Sigma, distilled under reduced pressure in the presence of hydroquinone and stored at 4°C until use. α-α′-Azobisisobutyronitrile (AIBN) was purchased from Fluka AG (Buchs, Switzerland) and used as received. All other chemicals were of reagent grade and were purchased from Merck AG (Darmstadt, Germany).

2.2. Membrane preparation

The pHEMA membrane was prepared by UV-initiated photopolymerization. The membrane preparation mixture (5 cm^3) contained; 2 cm^3 HEMA, 5 mg AIBN as polymerization initiator and 3 cm^3 phosphate buffer (0.1 M, pH 7.0). The mixture was then poured into a round glass mould (diameter = 9 cm) and exposed to long wave ultraviolet radiation (12 W lamp) for 30 min, while a nitrogen atmosphere was maintained in the mould. The membrane was washed several times with distilled water and cut into circular pieces (diameter = 1 cm) with a perforator.

2.3. Modification of pHEMA membrane surface by L-histidine

In order to prepare L-histidine grafted pHEMA membrane following procedure was applied. A 20 g of dry pHEMA membrane disks (diameter = 1 cm, thickness ca. 0.06 cm) was weighed and transferred into the L-histidine solution mixture (3 g L-histidine in 50 cm^3 of tetrahydrofuran) containing 1.4 g of NaH as catalyst. This grafting reaction was carried out under a constant gentle magnetic stirring at 40°C for 24 h. At the end of this reaction period, L-histidine grafted membrane disks were removed and washed extensively with methanol and water in order to remove weakly adsorbed L-histidine molecules and then dried in vacuum for 24 h. They were then stored at 4°C until use.

2.4. Immobilization of catalase via adsorption

Catalase adsorption on the pHEMA–histidine membrane disks was studied at various pHs, in either acetate (5.0 cm^3, 0.1 M, pH 4.0–5.0) or in phosphate buffer (5.0 cm^3, 0.1 M, pH 6.0–8.0). The initial catalase concentration was 2.0 mg cm^{-3} in the corresponding buffer. The adsorption experiments were conducted at 25°C while continuously stirring for 2 h. After this period, catalase immobilized membrane was removed from the enzyme solution and was washed with same buffer three times. It was then stored at 4°C in fresh buffer until use.

In order to determine the adsorption capacities of pHEMA–histidine membrane, the concentration of catalase in the medium was varied between 0.5 and 6.0 mg cm^{-3}. The absorption tests were carried out at pH 7.0 at 25°C.

The amount of immobilized catalase was calculated as

$$q = \frac{(C_i - C_t)V}{S} \quad (1)$$

where q is the amount of catalase adsorbed onto unit surface area of the adsorbent (μg cm^{-2}), C_i and C_t are the concentrations of the catalase in the initial solution and in the supernatant after adsorption, respectively (mg cm^{-3}), V the volume of the aqueous phase (cm^3), and S the external surface area of the membrane (cm^2).

2.5. Desorption of catalase from pHEMA–histidine membrane

In order to determine the reusability of the pHEMA–histidine membrane, catalase adsorption and desorption cycle was repeated five times by using the same pHEMA–histidine membrane. The catalase desorption from pHEMA–histidine membrane disks were carried out with NaSCN solution (5 cm^3, 1.0 M, pH 8.0) and stirred magnetically at 100 rpm at room temperature for 2 h. The membrane disks were removed from the desorption medium washed several times with phosphate buffer (0.1 M, pH 7.0) and were then reused in the enzyme immobilization. The catalase concentration in desorption medium was determined by the method of Bradford and the desorption ratios of catalase was calculated by using the following expression

$$\text{Desorption ratio} = \frac{(\text{amount of catalase released}) \times 100}{\text{amount of catalase adsorbed on the membrane}} \quad (2)$$

2.6. Determination of immobilization efficiency

The amount of protein in the crystalline enzyme preparation and in the wash solution were determined by the method of Bradford, using a Shimadzu (Model 1601) spectrophotometer. A calibration curve constructed with bovine serum albumin (BSA) solution (0.02–0.2 mg cm^{-3}) was used in the calculation of enzyme concentration.

2.7. Activity assays of free and immobilized catalase

Catalase activity was determined spectrophotometrically, by direct measurement of the decrease in the absorbance of hydrogen peroxide at 240 nm due to its decomposition by the enzyme. Hydrogen peroxide solutions (5–30 mM) were used to determine the activity of both the free and the immobilized enzyme. A 4 cm^3 of reaction mixture was preincubated at 25°C, for 10 min and the reaction was started by adding 50 μl of catalase solution (100 μg solid cm^{-3}). The decrease in absorbance at 240 nm was recorded for 5 min. The rate of change in the absorbance (ΔA_{240} min^{-1}) was calculated from the initial linear portion with the help of the calibration curve (the absorbance of hydrogen peroxide solutions of various concentrations (5–30 mM) at 240 nm). One unit of activity is defined as the decomposition of 1 μmol hydrogen peroxide per min at 25°C and pH 7.0.

Four catalase immobilized pHEMA–histidine membrane disks were introduced to the assay mixture to initiate the reaction as above. After 10 min, the reaction was terminated by removal of the membrane disks from the reaction mixture. The absorbance of the reaction mixture was determined and the immobilized catalase activity was calculated.

These activity assays were carried out over the pH range of 4.0–8.0 and temperature range of 4–65°C to determine the pH and temperature profiles for the free and immobilized enzyme. The effect of substrate concentration was tested in the 5–30 mM H_2O_2 concentrations range. The results of pH and temperature are

presented in a normalized form with the highest value of each set being assigned the value of 100% activity.

2.8. Stability of immobilized catalase during repeated reuses

The retention of immobilized catalase activity was tested in a batch system as described in Section 2.7. After each reaction period, the enzyme–membrane disks were removed from reaction medium and washed with phosphate buffer (0.1 M, pH 7.0) at 25°C for 15 min to remove any residual substrate on the membrane disks. They were then reintroduced into fresh reaction medium containing 10 mM H_2O_2.

2.9. Elemental analysis

The amount of L-histidine grafting to the plain pHEMA membrane was determined by measuring the C, H, N, and O contents with a Leco (CHNOS-932, USA) elemental analyzer. The L-histidine content of the dry membrane was calculated from nitrogen stoichiometry and expressed as mg L-histidine per cm^2 of dry membrane.

2.10. Scanning electron microscopy

Scanning electron micrographs of the pHEMA–histidine membrane were obtained using a JEOL-JMS 5600 (Japan) after coating with gold under vacuum.

3. Results and discussion

3.1. Properties of pHEMA–histidine membrane

The pHEMA membrane prepared in this study are rather hydrophilic, i.e. hydrogels. There have been

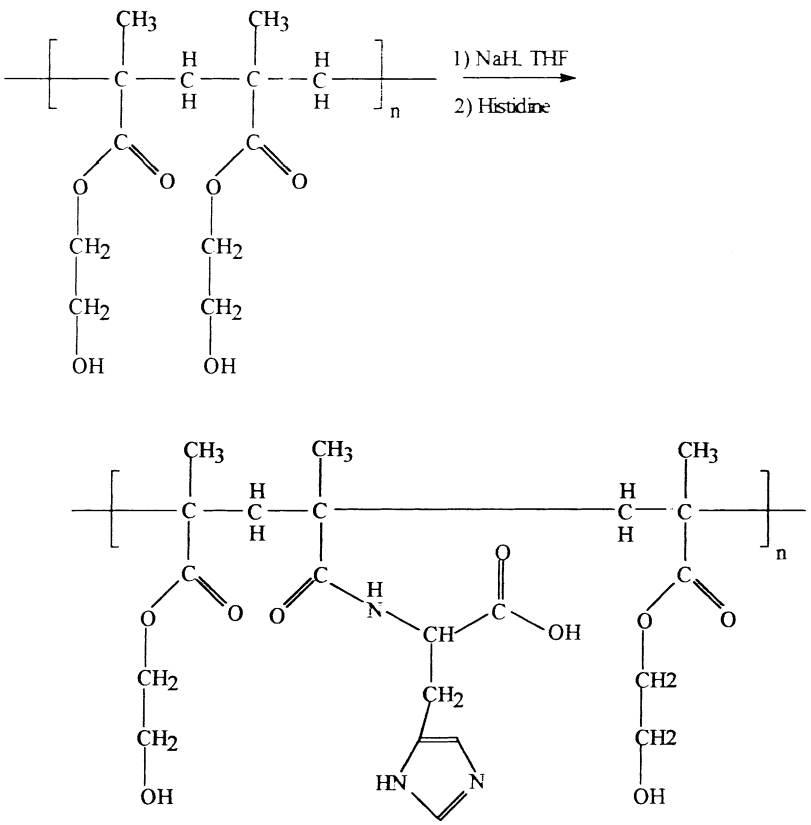
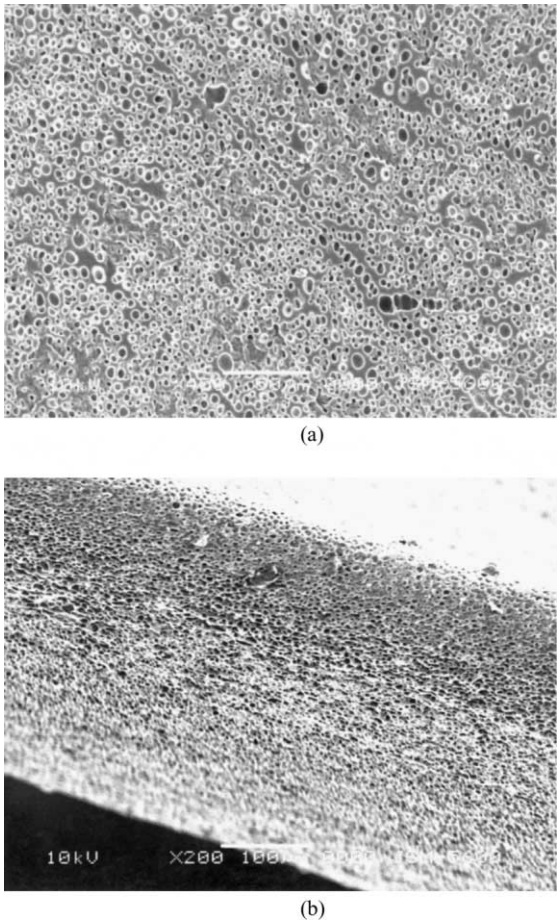


Fig. 1. Schematic representation of L-histidine grafting on the pHEMA membrane.

several reports for the use of hydrogels as support for enzyme immobilization. Hydrogels are polymeric materials that do not dissolve in water at physiological temperature and pH but swell considerably in aqueous medium. The simple incorporation of water weakens the secondary bonds within the hydrogels [17]. This enlarges the distance between the polymer chains and causes uptake of water. The equilibrium water uptake ratio of pHEMA membrane was about 58% in weight base. It should be noted that the percent water content of the pHEMA–histidine did not change after histidine grafting.

The natural amino acid molecule L-histidine was grafted on the pHEMA membrane via covalent bonding and it is accepted that amide bonds are formed between the amine groups of the L-histidine and carbonyl groups of the pHEMA (Fig. 1). Elemental analyses of the plain pHEMA and the pHEMA–histidine membranes were performed, and the amount of attached L-histidine was found to be $1.52\,mg\,cm^{-2}$ membrane from nitrogen stoichiometry.

The SEM micrographs of the surface and cross-section of pHEMA–histidine membrane are presented in Fig. 2a and b, respectively. These figure show that, both surface and bulk structure of pHEMA membrane are very porous. Note that an affinity system using microporus membranes as the support matrix with sites on the pore wall for attachment has even better potential to provide higher efficiency since it offers high surface area and reduce the diffusion distance.



(a)

(b)

Fig. 2. SEM micrographs of pHEMA membrane. (a) Surface; (b) cross-section.

3.2. Absorption efficiency and retention of activity

The optimal pH values for adsorption of catalase ($2\,mg\,cm^{-3}$) onto pHEMA–histidine membrane were investigated in the pH range 4.0–8.0 at 25°C. As observed in Fig. 3, with pHEMA–histidine the maximum enzyme loading of $69\,\mu g\,cm^{-2}$ was obtained at around pH 7.0. Significantly lower enzyme loading was obtained for pHEMA–histidine in alkaline and acidic pH regions. It has been shown that enzymes have no net charge at their isoelectric points (IPs), and therefore, the maximum adsorption from aqueous solution is usually observed at their IPs. The IP of catalase is 6.4. In the present study, the maximum adsorption was not observed at this pH, but slightly shifted to rather neutral pH values. This could be resulted from preferential interaction between catalase and immobilized histidine molecules at neutral pH. These interactions may result from ion-exchange effects, caused by the free carboxyl group and imidazole ring on the grafted L-histidine and the amino acid side-chains of the enzyme molecules. In addition, catalase is classified as a ferric haem-containing enzyme and it has an iron protoporphyrin prosthetic group [18]. This phenomenon could also provide additional high binding affinity for catalase to the pHEMA–histidine membrane. The trivalent metal ions such as Fe(III) are considered hard Lewis acids and interact with hard Lewis bases such as oxygen. Molecules that have hard bases such as phosphates, carboxylates, sulfates, and phenolic groups have high binding constants. From this point of view, grafted L-histidine molecules on the pHEMA mem-

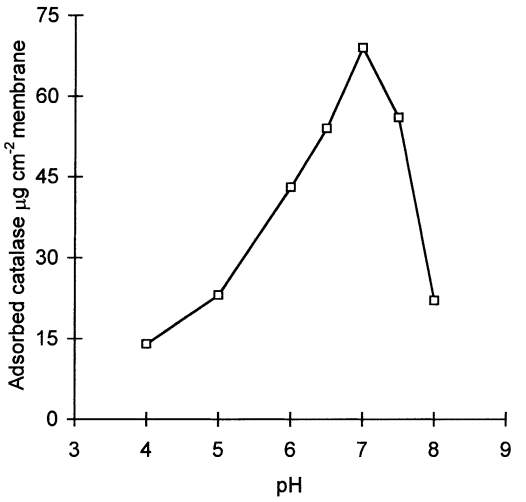


Fig. 3. Effect of pH on catalase adsorption onto pHEMA–histidine membrane.

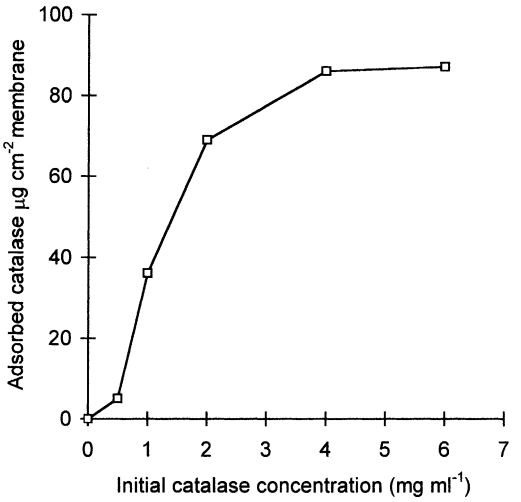


Fig. 4. Effect of catalase concentration onto adsorption efficiency of pHEMA–histidine membrane.

brane have free carboxyl and imidazole groups, the free carboxyl group of histidine could easily chelate with the ferric haem-containing catalase. In this way, on the basis of metal recognition, an additional strong binding could be established via ferric ion of catalase and histidine molecule on the membrane surface.

The adsorption tests were carried out at pH 7.0 at 25°C and the concentration of catalase in the adsorption medium was varied between 0.5 and 6.0 mg cm^{-3}. The results of the adsorption tests for pHEMA–histidine membrane are presented in Fig. 4. An increase in enzyme concentration led to an increase in adsorption efficiency but this leveled off at an enzyme concentration of 4.0 mg cm^{-3}. The enzyme adsorption reached a saturation level 86 μg cm^{-2} on the pHEMA–histidine membrane.

The effect of loading on the activity of catalase adsorbed on the pHEMA–histidine membrane was determined by varying the enzyme content on the pHEMA–histidine membrane. As observed in Fig. 5, the highest retention of enzyme activity (78%) was obtained with the lowest enzyme loading (5 mg cm^{-2} pHEMA–histidine membrane). As the enzyme content increased (from 5 to 86 μg cm^{-2} pHEMA–histidine membrane), retention of activity decreased, reaching a value of 48% (Table 1). A high enzyme loading on the support generally leads to a low retained activity. This is brought either by over-saturation of the pore space of the membrane with the enzyme, as a result substrate diffusion limitations occur, or the presence of protein–protein interactions becomes more important and these hinder the substrate conversion. With an increase in catalase content on the pHEMA–histidine membrane, the enzyme activity of the unit membrane was also increased (915–9718 U cm^{-2} pHEMA–histidine membrane), but not at the same

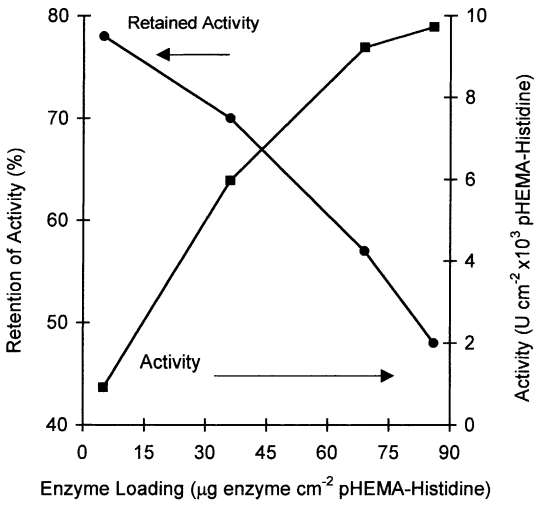


Fig. 5. Effect of catalase loading on the activity retention and enzyme membrane activity.

Table 1
Activity of catalase adsorbed onto pHEMA–histidine membrane[a]

Catalase concentration in the adsorption medium (mg cm^{-3})	Enzyme loading (μg enzyme cm^{-2} membrane)	Activity (U cm^{-2} membrane)	Activity (10^{-3} U mg of immobilized enzyme)	Recovered activity (%)
0.5	5	915	183	78
1.0	36	5976	166	70
2.0	69	9246	134	57
4.0	86	9718	113	48

[a] Effect of initial catalase concentration in the adsorption medium on the enzyme loading and activity retention.

rate because of the loss in retained activity with increased loading (Fig. 5). As presented in Table 1, pHEMA–histidine membrane with a loading of 69 μg enzyme per cm^2 was found to be optimum and it was used in the rest of study. In our previous study, catalase was immobilized on the Cibacron Blue F3GA and Fe(III) derivatized pHEMA membrane via adsorption, the maximum enzyme immobilization capacity of the membrane was 23.6 mg cm^{-2} [3]. In this case, we achieved a higher immobilization capacity with the pHEMA–histidine membrane (up to 86 mg cm^{-2}) than that of the previously reported one.

In the earlier studies, catalase was immobilized on the various types of supports and a wide range of retention activities were reported for the immobilized catalase in the literature. Tarhan [5] reported that the activity retention of catalase immobilized on the protein coated support was 24.5 %. Çetinus and Öztop [19] studied the immobilization of catalase on the chitosan film and the retained activity of immobilized catalase was about 4.3%. Tarhan and Uslan report that with a non-porous acrolein/styrene copolymer support the retained activity of immobilized catalase was 22% [20]. Solas et al. [21] studied the adsorption of catalase on bioskin and reported that the retained activity of adsorbed catalase was 70%. The maximum activity retention of the immobilized catalase we achieved with the pHEMA–histidine membrane developed in this study was 78%; this was quite comparable with relevant literature values.

Kinetics parameters, the Michaelis constants K_m and V_{max} for free and immobilized catalase were determined using H_2O_2 as substrate. For the free enzyme the K_m of was found to be 16.5 mM, whereas V_{max} value was calculated as 236×10^3 U mg^{-1} protein. Kinetic constants of the immobilized catalase were also determined in the batch system. The K_m values were found to be for 25.8 mM. The V_{max} values of immobilized enzyme was estimated from the data as 118×10^3 U mg^{-1} adsorbed protein onto pHEMA–histidine membrane. As expected, the K_m and V_{max} values were significantly effected after immobilization onto pHEMA–histidine membrane. The difference in K_m values between the free and the immobilized catalase can be attributed to the limited accessibility of substrate molecules to the active sites of the immobilized catalase, as a result of the spatial distribution of catalase molecules in the porous structure of the membrane matrix and the conformational changes of the catalase molecules caused by the various interactions with the L-histidine immobilized support. The decrease in V_{max} value as a result of immobilization is considered to be associated with the K_m value since the lower the value of K_m, the greater the affinity between the enzyme and the substrate [22].

A two-fold reduction in the V_{max} value was observed upon immobilization of catalase on the pHEMA–histidine membrane via adsorption. When compared with the other catalase immobilization studies reported in the literature with respect to reduction in the V_{max} values upon immobilization, for example, Çetinus and Öztop [19] studied the immobilization of catalase on chitosan film and found that V_{max} value of the free catalase was 24,042 μmol min^{-1} mg protein whereas, upon immobilization, it was decreased about 24-fold (1022 μmol min^{-1} mg protein). Arica et al. [13] reported that the V_{max} value of catalase was decreased 2.8-fold upon immobilization in the thermally reversible hydrogels. Thus, the results obtained in the present study appear to be quite promising.

3.3. Effect of temperature and pH on the catalytic activity

The temperature dependence of the activities of the soluble and immobilized catalase were studied in

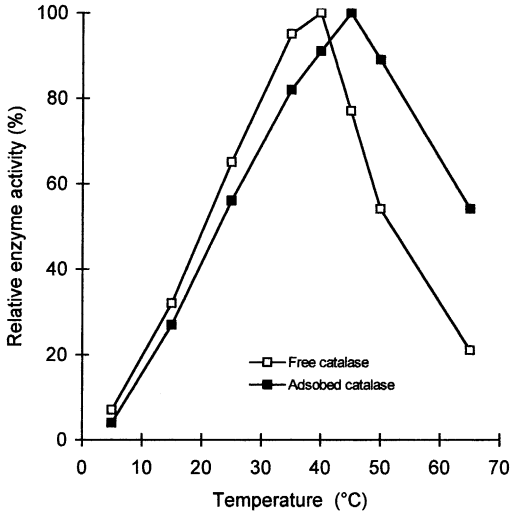


Fig. 6. Temperature profiles of free and adsorbed catalase.

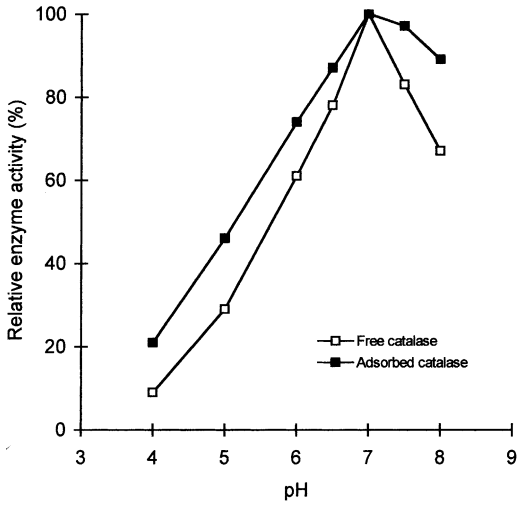


Fig. 7. The pH profiles of free and adsorbed catalase.

phosphate buffer (0.1 M, pH 7.0) in the temperature range 5–65°C (Fig. 6). The behavior of the activity with temperature was as expected: increased temperature caused an increased activity, up to optimum reaction temperature and then overlapped with the deactivation of the enzyme in a reversible or irreversible ways or both. As a result, the activity increased to a peak and then fell down. In this case, the peak was obtained at 40 and 45°C for the free and immobilized catalase, respectively. As seen in Fig. 6, the support has a slightly protecting effect at the high temperatures at which enzyme deactivation takes place. The non-covalent bonds between the enzyme and the membrane matrix, may reduce the degrees of freedom of the protein molecular structure of the enzyme, thus protecting it in some extent from denaturation by high temperature [11–13,22].

The pH effect on the activity of the free and immobilized catalase for hydrogen peroxide degradation was studied at various pHs at 35°C. The reactions were carried out in acetate and phosphate buffers and the results are presented in Fig. 7. The immobilized catalase has the same optimum as the free enzyme (around pH 7.0). The pH profile of the immobilized catalase was much broader with respect to the free enzyme, probably due to secondary interactions (e.g. ionic and polar interactions, hydrogen bonding) between the enzyme and the pHEMA–histidine membrane.

3.4. Repeated loading of catalase onto pHEMA–histidine membrane

Desorption of adsorbed catalase from pHEMA–histidine membrane was carried out in a batch system. The catalase immobilized pHEMA–histidine membrane disks was placed within the desorption medium containing NaSCN (1.0 M, pH 8.0) at room temperature for 2 h as described above and was then

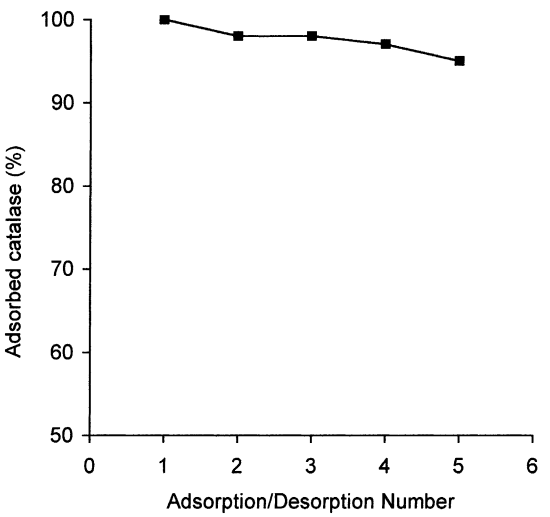


Fig. 8. Absolute membrane activity as a function of the number of repeated adsorption–desorption cycles.

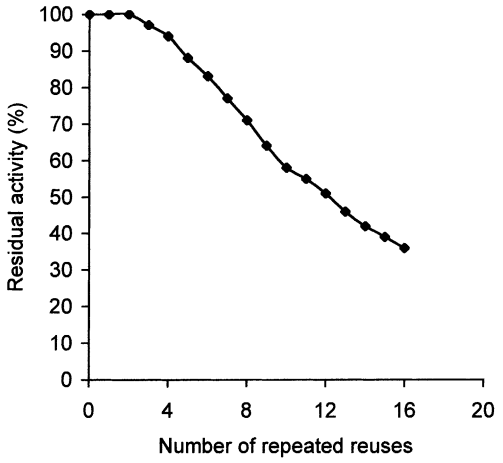


Fig. 9. The stability of immobilized catalase during repeated reuse in a batch system.

repeatedly used in adsorption of catalase. The catalase adsorption capacities and the absolute retained activity were not changed significantly during five successive adsorption–desorption cycles of the pHEMA–histidine membrane disks (Fig. 8). Enzyme activity of the pHEMA–histidine membrane did not significantly change during these adsorption–desorption cycles. These results showed that the novel pHEMA–histidine membrane can be repeatedly used in enzyme immobilization without detectable losses in their initial adsorption capacities.

3.5. Operational stability of adsorbed catalase in a batch system

The stability of immobilized enzyme systems is very important for various biotechnological applications; an increased stability could make the immobilized enzyme more advantageous than its free counterparts. Operational stability of the adsorbed catalase was determined for 16 successive batch run at 25°C for 60 min. The results presented in Fig. 9 shows that the immobilized catalase activity remained almost the same as the original activity after four cycles. After that, a steady decrease in degradation capability of the immobilized catalase was observed, and this loss reached about 58% after 10 cycles of batch operation. At the end of the last use the activity was 36% of the initial value. This activity loss could result of a poisoning effect brought about by the substrate.

4. Conclusion

Surface modification of pHEMA membrane was achieved through covalent attachment of L-histidine and was used in the immobilization of catalase. The natural amino acid carrying membrane support may provide an artificial natural microenvironment for the enzyme. The adsorbed catalase retained 48–78% of its activity when compared to that of the free form. The pH value for optimum activity of catalase was not affected by the immobilization. Optimum temperature was 5°C higher than that of the free enzyme and was significantly expanded at high temperature. The L-histidine grafted pHEMA membrane revealed good properties as an adsorptive membrane and will find useful applications in biotechnology. In addition, the reusability of the membrane matrix may provide economic advantages for large-scale biotechnological applications.

References

[1] J.F. Kenedy, M. Paterson, Polym. Int. 32 (1993) 177.
[2] R. Ulbrich-Hofmann, U. Arnold, J. Mansfeld, J. Mol. Catal. B: Enzymatic 7 (1999) 125.
[3] M.Y. Arica, A. Denizli, B. Salih, E. Piskin, V. Hasirci, J. Membr. Sci. 129 (1997) 65.
[4] M.S. Alvesda, M.H. Gill, A.P. Piedace, J.S. Redinha, A.M.O. Brett, J.M. Costa, J. Polym. Chem. 29 (1991) 269.
[5] L. Tarhan, Biomed. Biochim. Acta 49 (1990) 307.
[6] A.P. Piedada, M.H. Gill, M.C. Cavaco, M.E. Andreda, Polym. Int. 38 (1995) 269.
[7] P.T. Vasudevan, D.S. Thakir, Appl. Biochem. Biotechnol. 49 (1995) 173.
[8] T.H.S. Puis, J.K. Leypoldt, D.A. Gough, Biotechnol. Bioeng. 29 (1987) 696.
[9] M.Y. Arica, Y. Kaçar, A. Ergene, A. Denizli, Process Biochem. 36 (8/9) (2001) 847.
[10] M.Y. Arica, G. Akin-Öktem, A. Denizli, Colloids Surf. B: Biointerface, in press.
[11] M.Y. Arica, J. Appl. Polym. Sci. 77 (2000) 2000.
[12] M. Yoshida, M. Kumakura, I. Kaetsu, Polymer 20 (1979) 3.
[13] M.Y. Arica, H. Avni Öktem, Z. Öktem, A. Tuncel, Polym. Int. 48 (1999) 48.
[14] B. Schulz, A. Riedel, P.U. Abel, J. Mol. Catal. B: Enzymatic 7 (1999) 85.
[15] J.J. Gooding, E.A.H. Hall, Biosens. Bioelectron. 11 (1996) 1031.

[16] M.Y. Arica, V. Hasirci, Biomaterials 8 (1987) 489.
[17] M.V. Risbud, A.A. Hardikar, S.V. Bhat, R.R. Bhonde, J. Control. Rel. 68 (2000) 23.
[18] C.E. Cooper, Biochim. Biophys. Acta 1141 (1999) 290.
[19] S.A. Çetinus, H.N. Öztop, Enzyme Microbiol. Technol. 26 (2000) 497.
[20] L. Tarhan, H. Uslan, Process Biochem. 25 (1990) 1.
[21] M.T. Solas, C. Vicente, L. Xavier, M.E. Legaz, J. Biotechnol. 33 (1994) 63.
[22] J.M. Gozalez-Saiz, C. Pizarro, Eur. Polym. J. 37 (2001) 435.