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Reversible immobilization of catalase on fibrous polymer grafted and metal chelated chitosan membrane

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ABSTRACT

Poly(itaconic acid) grafted and/or Fe(III) ions incorporated chitosan membranes were used for reversible immobilization of catalase (from bovine liver) via adsorption. The influences of pH and initial catalase concentration on the immobilization capacities of the CH-g-poly(IA) and CH-g-poly(IA)-Fe(III) membranes have been investigated in a batch system. Maximum catalase adsorption onto CH-g-poly(IA) and CH-g-poly(IA)-Fe(III) membrane were found to be 6.3 and 37.8 mg/g polymer at pH 5.0 and 6.5, respectively. The CH-g-poly(IA)-Fe(III) membrane with high catalase adsorption capacity was used in the rest of the study. The K_m value for immobilized catalase on CH-g-poly(IA)-Fe(III) (25.8 mM) was higher about 1.6-fold than that of free enzyme (13.5 mM). Optimum operational temperature was observed at 40 °C, a 5 °C higher than that of the free enzyme and was significantly broader. The optimum operational pH was same for both free and immobilized catalase (pH 7.0). Thermal stability was found to increase with immobilization. Free catalase lost all its activity within 20 days whereas immobilized catalase lost 23% of its activity during the same incubation period. It was observed that the same support enzyme can be repeatedly used for immobilization of catalase after regeneration without significant loss in adsorption capacity or enzyme activity. In addition, the CH-g-poly(IA)-Fe(III) membrane prepared in this work showed promising potential for various biotechnological applications.

1. Introduction

Many protocols for enzyme immobilization involve irreversible binding between an enzyme and a functionalized support. In the reversible enzyme immobilization, the supports could be regenerated using a suitable desorption agent, and they be recharged again with a fresh enzyme. On the other hand, when the covalently immobilized enzyme becomes inactivated upon use both the enzyme and the support should be eliminated as wastes. In the reversible enzyme immobilization, the expensive support can be repeatedly used and the only waste produced is a solution of inactivated enzyme [1–4].

The reversible immobilization of enzymes onto functionalized polymeric supports with high-density ion-exchange groups (such as PEI and/or dextran sulfate) has been proposed as a very suitable method for reversible but very strong protein immobilization [5,6]. In this way, a very strong adsorption of enzyme on the polymer surface is obtained with an intense multipoint ionic attachment. Thus, irreversible covalent attachment is only the best option if the enzyme stability is increased [5,6]. For reversible enzyme immobilization, ion-exchangers, hydrophobic gels and metal chelated supports have been used [7–10]. For the later case, reversible enzyme immobilization on the metal chelated support is based on multipoint interactions between chelated metal ions on the support and histidine, cysteine, glutamic acid, aspartic acid or tyrosine residues of the protein surface [11–14]. Most of the transition metal ions such as Fe^{3+}, Al^{+3}, Zn^{2+}, Ni^{2+}, Cu^{2+}, and Fe^{2+} can form stable complexes with electron-rich compounds and may coordinate molecules containing O, N and S by ion–dipole interactions. Among them, hard Lewis acids (i.e., Fe^{3+} and Al^{+3} ions) can form strong coordination complex with oxygen rich ligands such as iminodiacetic acid, nitrilotriacetic acid and ethylene diamine tetracetic acid (EDTA). Thus, the immobilization of enzyme on the metal ion chelated support is based on the interaction of a Lewis acid (electron pair donor), i.e., a chelated metal ion, with an electron acceptor group on the surface of the enzyme [2,15,16]. Metal ions chelated supports have been used extensively for separation and purification of biological macromolecules (mainly for enzyme purification) from fermentation broth or biological fluids. The low cost of metal ions and the reuse of support materials for reversible immobilization enzymes for several times without any detectable loss of metal-chelating properties can be the attractive features of metal affinity interactions with the proteins [15]. It should be noted that immobilized metal affinity chromatography

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(IMAC) is mainly advantageous when it may be associated to purification, e.g., poly His tagged proteins. Moreover, large proteins may be selectively immobilized using lowly activated IMAC supports, although this may be not a good solution to immobilize proteins [17,18].

Among the methods of modification of polymers, grafting is one of the promising method. In principle, graft copolymerization is an attractive way to impart a variety of functional groups to a polymer. For example, grafting of a functional pendant group carrying vinyl monomer such as itaconic acid onto chitosan backbone could introduce novel oxygen rich ligands for chelating of hard Lewis metal ions [19,20]. This novel comb type polymer grafted and metal ions chelated matrices, could be suitable for immobilization of enzyme due to their intrinsically high specific surfaces, providing the quantity and accessibility of the binding sites necessary for high immobilization capacity and large surface area for enzymatic reaction [4,21–23].

Catalase is a heme-containing metallo-enzyme that is regarded as one of the most common enzymes in plant and animal tissues and has a protection function related to the decomposition of hydrogen peroxide. Catalase consists of four subunits, each of which includes ferri-porphyrin as a prosthetic group [24]. Immobilized catalase has useful applications in the food industry in the removal of excess hydrogen peroxide from food products after cold pasteurization and in the analytical field as a component of hydrogen peroxide and glucose biosensor systems [25–27]. Adsorption of a protein on ionic exchangers, IMAC supports, etc. requires a multipoint enzyme–support interaction. By this way, the stability of the immobilized multi-subunit enzyme can be increased [28–31]. Some reports state that catalase is first inactivated under certain conditions via subunit dissociation [32–34]. The stabilization of the tetrameric bovine liver catalase by preventing subunit dissociation under different conditions has been discussed [35].

With immobilized enzymes, improved stability, reusability, continuous operation, possibility of better control of reactions, high purity and product yields and hence more favorable economic factors can be expected [24].

In this study, chitosan membrane was prepared by phase-inversion technique and then cross-linked with epichlorohydrin under alkaline condition. Itaconic acid was grafted on the cross-linked chitosan membrane via ammonium persulfate initiation under nitrogen atmosphere. The degree of itaconic acid grafting was measured gravimetrically. The reversible immobilization of catalase (from bovine liver) was studied and the important immobilization parameters such as initial catalase concentration, and pH were varied to evaluate the nature of immobilization mechanisms of catalase on the CH-g-poly(IA)-Fe(III) membrane. The resultant adsorbed catalase was characterized and its activity retention, catalytic properties and reusability aspect were compared to that of its free counterpart.

2. Experimental

2.1. Materials

Chitosan powder, ammonium persulfate (APS) and itaconic acid monomer were obtained from Fluka AG. (Buchs, Switzerland), the monomer was distilled under reduced pressure and stored at 4 °C until use. Catalase (CAT) (hydrogen peroxide oxidoreductase; EC.1.11.1.6) from bovine liver (about 2000–5000 U/mg solid; C 9322) and epichlorohydrin were obtained from Sigma–Aldrich (St. Louis, MO, USA) and used as received. All other chemicals were of analytical grade and were purchased from Merck AG. (Darmstadt, Germany). The water used in these experiments was purified using a Barnstead water purification system (Dubuque, IA, USA).

2.2. Preparation of itaconic acid grafted chitosan membranes

The preparation of chitosan membranes was carried out via phase-inversion techniques. Chitosan solution (4.0%, 10 mL) was transferred into a flat glass dish and dried at 35 °C for 18 h. Then, the dried membrane was transferred in sodium hydroxide solution (2.5 M, 5 mL) for 2 h, and washed twice with purified water. The membrane was cross-linked with epichlorohydrin (20 mL, pH 12) at 50 °C for 2.0 h. The resulting cross-linked membrane was washed with acetone, distilled water and phosphate buffer (0.1 M, pH 7.0) in a sonicated water-bath. It was cut into disks (diameter: 0.5 cm) with a perforator.

The graft copolymerization of itaconic acid on the cross-linked chitosan membrane disks was carried out in a two-neck round-bottomed glass reactor. In a typical grafting, cross-linked chitosan membrane disks (about 2.0 g) and APS solution (6.58×10^{-3} M, 20 mL) were transferred in to the reactor and stirred magnetically at 100 rpm for 10 min. Then, the monomer was added dropwise to the reactor and, its concentration was varied between 0.1 and 0.8 mol/L). The grafting reaction was carried out at 60 °C for 3.0 h under nitrogen atmosphere. The itaconic acid grafted chitosan membrane disks, were removed and washed several times with purified water. The disks were dried under reduced pressure at 60 °C. The grafting percentage was determined from the mass of dried membrane before and after grafting by using following equation:

$$\text{The percentage of poly(itaconic acid) grafting} = \frac{(W_2 - W_1)}{W_1} \times 100 \quad (1)$$

where W_1 and W_2 are the mass of cross-linked chitosan membrane disks and poly(itaconic acid) grafted chitosan membrane disks, respectively.

2.3. Chelating of Fe(III) ions on poly(itaconic acid) grafted chitosan membrane

Chelates of Fe(III) ions with CH-g-poly(IA) membrane were prepared as follows: 5.0 g of the membrane were transferred in a flask containing of Fe(III) ions solution (50 mL) at pH 5.0. The flask was stirred magnetically at 100 rpm for 2.0 h. The Fe(III) chelating scheme is presented in Fig. 1. The amount of adsorbed Fe(III) ions was calculated by using the concentrations of the Fe(III) ions in the initial solution and in supernatant solution. The Fe(III) ion concentration was determined in the solutions using atomic absorption spectrophotometer (AAS). The metal ions chelated CH-g-poly(IA)-Fe(III) membrane disks were stored at 4 °C in phosphate buffer solution (50 mM, pH 7.0).

2.4. Characterization studies

The free amino group content of the cross-linked chitosan membrane was determined by potentiometric titration before and after grafting reaction. Briefly, the membrane (about 1.0 g) was transferred in HCl solution (0.1 M, 20.0 mL), and the medium was incubated in a shaking water-bath at 35 °C for 6.0 h. After this period, the final HCl concentration in the solution was determined by a potentiometric titration with 0.05 M NaOH solution. The amount of available surface functional carboxyl groups of the CH-g-poly(IA) membrane was determined by potentiometric titration. The CH-g-poly(IA) membrane disks (0.2 g) was allowed at room temperature for 24.0 h in purified water (10 mL). Then, sodium hydroxide solution (2 M, 10.0 mL) was added to the mixture and shaken for 1.0 h. At the end of this period, the membrane disks were removed and assayed by titration with 0.1 M HCl solution.

FTIR spectra of the CH-g-poly(IA) membrane were obtained by using a FTIR spectrophotometer (FTIR 8000 Series, Shimadzu, Japan). The dry sample (about 0.01 g) mixed with KBr (0.1 g) and pressed into a tablet form. The FTIR spectrum was then recorded.

Differential scanning calorimetry (DSC) analysis of chitosan membrane, cross-linked chitosan membrane and itaconic acid grafted membrane were obtained using a DSC instrument (Model DSC-60-DTG-60H, Shimadzu, Japan) at a heating rate of 10 °C/min under nitrogen atmosphere. Samples were embedded in nonhermetic aluminum pans.

Contact angles to water of the dry cross-linked-CH, CH-g-poly(IA) and CH-g-poly(IA)-Fe(III) membranes were measured by sessile drop method at room temperature by using a digital optical contact angle meter CAM 200 (KSV Instruments Ltd, Helsinki, Finland).

The water content of the CH-g-poly(IA) membrane was determined at room temperature in phosphate buffer (50 mM, pH 7.0 with a gravimetric method. They were weighed on a sensitive balance ($\pm 1.0 \times 10^{-4}$ g; Shimadzu, Model AX 120).

2.5. Immobilization of catalase via adsorption

Catalase immobilization on the CH-g-poly(IA) and CH-g-poly(IA)-Fe(III) membrane disks was studied at various pHs, in either acetate (5.0 mL, 50 mM, pH 3.0–5.0) or in phosphate buffer (5.0 mL, 50 mM, pH 6.0–8.0). The initial catalase concentration was 2.0 mg/mL in the corresponding buffer. The immobilization 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 washed with same buffer three times. It was then stored at 4 °C in fresh buffer until use. In order to determine the maximum immobilization capacity of CH-g-poly(IA)-Fe(III) membrane, the concentration of catalase in the medium was varied between 0.5 and 3.0 mg/mL at pH 7.0, and at 25 °C.

The amount of immobilized catalase was calculated as

$$q = \frac{(C_i - C_t)V}{w} \quad (2)$$

where q is the amount of catalase adsorbed onto g membrane (mg/g), C_i and C_t are the concentrations of the catalase in the initial solution and in the supernatant after adsorption, respectively (mg/mL), V the volume of the aqueous phase (mL), and w is the weight of the membrane (g).

2.6. Determination of immobilization efficiency

The amounts of protein in the enzyme preparations and in the wash solution were determined by the method of Bradford [36], using a Shimadzu (Model 1601) spectrophotometer. A calibration curve constructed with bovine serum albumin (BSA) solution (0.02–0.2 mg/mL) 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 immobilized enzyme. A 4.0 mL of reaction mixture was equilibrated at 25 °C, for 10 min, and the reaction was started by adding 50 μL of catalase solution (0.1 mg solid/mL). The decrease in absorbance at 240 nm was recorded for 5 min. The rate of change in the absorbance 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 enzyme activity is defined as the decomposition of 1.0 μmol hydrogen peroxide per min at 25 °C and pH 7.0. Six catalase immobilized CH-g-poly(IA) and/or CH-g-poly(IA)-Fe(III) 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 4.0–9.0 and temperature range of 15–55 °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. Thermal stability of free and immobilized catalase

The tests for the determination of the thermal stability of free and immobilized catalase were carried out by measuring the residual activity of the enzyme, exposed to two different temperatures (55 and 65 °C) in phosphate buffer (0.1 M, pH 7.0) for 120 min. A predetermined time interval, a sample was removed and assayed for enzymatic activity as described above.

2.9. Storage stabilities of free and immobilized enzymes

The activity of free and immobilized catalase after storage in phosphate buffer (50 mM, pH 7.0 at 4 °C was measured in a batch-operation mode, with the experimental conditions as given above.

2.10. Activity of catalase immobilized CH-g-poly(IA)-Fe(III) membrane in continuous system

The continuous-flow reactor system (length 8.0 cm, diameter 1.6 cm and total volume 16.0 mL), the temperature-control jacket and the upper connector were all made from Pyrex® glass. The catalase immobilized CH-g-poly(IA)-Fe(III) membrane disks (30 disks) were equilibrated overnight in phosphate buffer (50 mM, pH 7.0) at 4 °C and, were transferred into the reactor. In order to determine the effect of substrate concentration on the operational stability of the immobilized enzyme, hydrogen peroxide at three different concentrations (i.e., 10, 25 and 50 mM) in phosphate buffer was introduced into the reactor at a flow rate of 20 mL/h by means a peristaltic pump (Ismatech IPC Model, Switzerland) through the lower inlet part. The reactant solution in the reactor was stirred magnetically at 25 °C. The sample was collected and assayed for immobilized catalase activity as described above.

2.11. Reusability of the catalase immobilized CH-g-poly(IA)-Fe(III) membrane

In order to determine the reusability of the catalase immobilized CH-g-poly(IA)-Fe(III) membranes, enzyme adsorption and desorption cycle was repeated six times. Desorption experiments were carried out in phosphate buffer solution containing 0.1 M NaSCN and 50 mM EDTA at pH 8.0. Catalase immobilized CH-g-poly(IA)-Fe(III) membrane disks was placed in the desorption medium for 2 h with a stirring rate at 150 rpm, and at 25 °C. The membrane disks were washed several times with phosphate buffer (50 mM, pH 7.0), and were then reused in enzyme immobilization. Catalase concentration within the desorption medium was determined as described above.

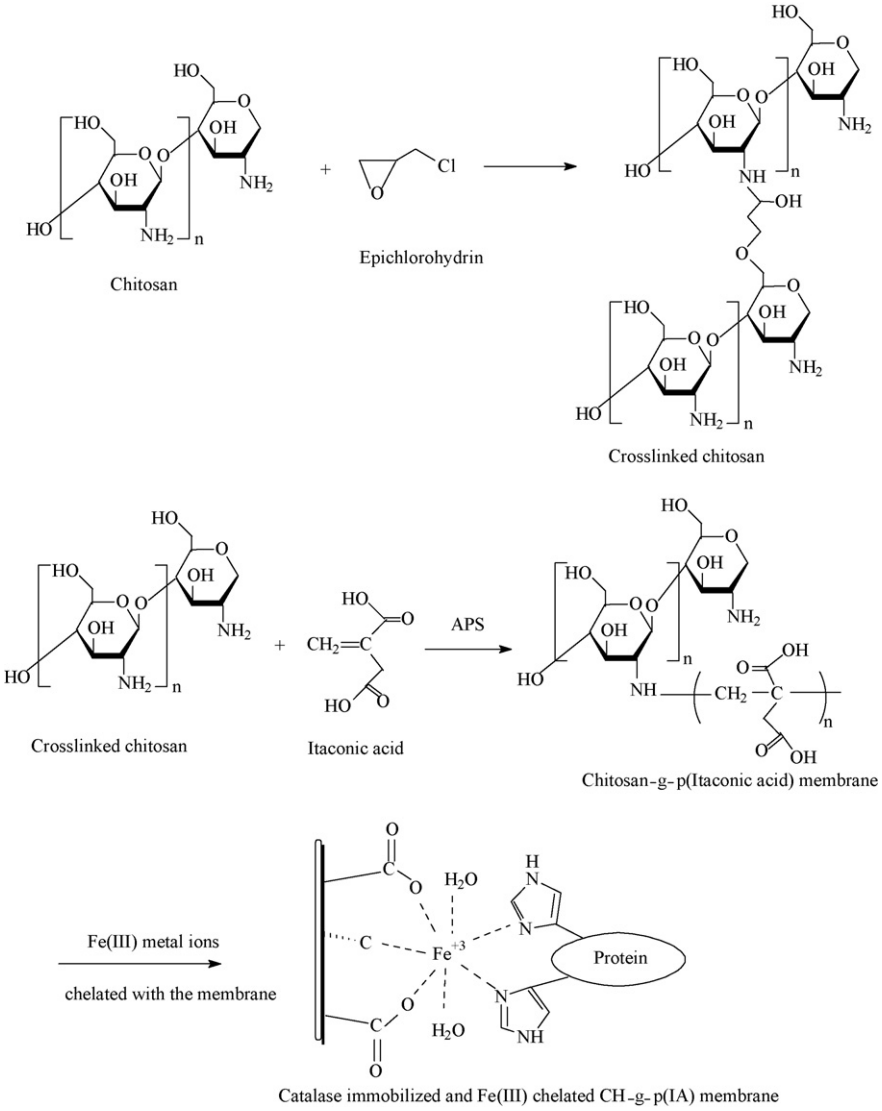

Fig. 1. Schematic representation of chemistry of the CH-g-poly(IA)-Fe(III) membrane.

3. Results and discussion

3.1. Properties CH-g-poly(IA)-Fe(III) membranes

Chitosan membrane was prepared by phase-inversion method and cross-linked with epichlorohydrin, and then itaconic acid was grafted on the membrane via free radical polymerization in the presence of ammonium persulfate. The complex formed by the reaction between $-NH_2$ and –OH groups of chitosan decomposed to generate the free radical sites at about 60 °C, facilitating the reaction for the monomer [37]. The schematic representation of grafting reaction is presented in Fig. 1. The effect of itaconic acid concentration on the grafting efficiency of the cross-linked chitosan membrane was also studied. The grafting efficiency of membrane was increased with increasing itaconic acid concentration from 0.1 to 0.6 mol/L (data not shown). On the other hand, further increase above 0.5 mol/L causes to a significant decrease in the grafting efficiency of the membrane. After certain limit, the increase in itaconic acid concentration accelerates the homopolymerization reaction rather than graft polymerization. A similar observation was reported by Wang et al. [38] grafting percentage of N-isopropylacrylamide to polyethylene membrane was proportional to the monomer concentration in the range from 0.0 to 15%. For this reason, in the remaining study, the initial concentration of itaconic acid 0.5 mol/L was used in the polymerization reaction and the percent grafting efficiency under this condition was about 8.5%.

The free amino group content of the cross-linked chitosan membrane was determined by potentiometric titration before grafting and the amount of free amino group on the cross-linked chitosan membrane was found to be as 3.63×10^{-3} mmol/g membrane. After grafting with itaconic acid, there was not a detectable free amino group on the membrane surface. The amount of carboxyl groups of the CH-g-poly(IA) membrane surface was determined by potantiometric method and was found to be 1.3×10^{-3} mmol/g.

Compared with cross-linked-CH membrane (53%), the water content of poly(IA) grafted membrane increased to 98%. This appears reasonable when it is remembered that the cross-linked-CH is not highly polar compound. After grafting poly(IA), negatively charged carboxyl groups were introduced on the composite polymer structure, and should be caused more water uptake.

FTIR spectra of the cross-linked-CH and CH-g-poly(IA) membranes are presented in Fig. 2. The FTIR spectra of poly(IA) grafted chitosan membranes had an absorption band different from that of chitosan at 1768 cm^{-1}. It is the characteristic adsorption of carboxyl groups of the poly(IA) grafted cross-linked-CH membrane. The appearance of the new peak evidenced the successful grafting

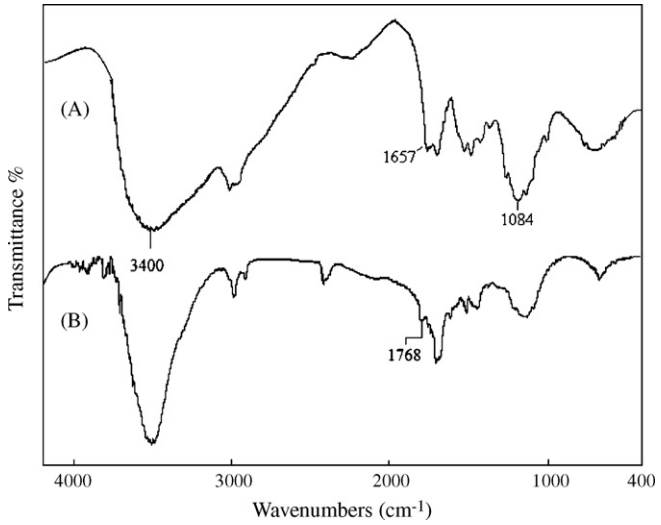

Fig. 2. FTIR spectrum: (A) the cross-linked-CH; (B) CH-g-poly(IA) membranes.

of poly(IA) on the cross-linked-CH membrane. The broad bands at ~3400 cm^{-1} corresponds to the associated –OH stretching vibration of the hydroxyl and amino groups, and the peak at 1657 cm^{-1} corresponds to the N–H deformation, due to the amino groups of chitosan in the copolymer structure. A relatively high intense peak at around 1084 cm^{-1} is due to the characteristic peak of polysaccharides.

The thermal stabilities of the CH, cross-linked-CH and cross-linked-CH-g-(IA) membranes were evaluated using thermogravimetric analysis under nitrogen atmosphere. The DSC curves of the CH, cross-linked-CH and cross-linked-CH-g-(IA) membranes are shown in Fig. 3. The CH membrane shows a distinct feature in DSC curve having one endothermic peak at about 232 °C due to the thermal degradation and one other endothermic peak is around at 269 °C. In case of cross-linked-CH membrane, two endothermic peaks are observed. The thermal degradation peak is decreased about 23 °C and the decomposition temperature is decreased to about 246 °C. In case of poly(IA) grafted membrane, thermal stability of the poly(IA) grafted membrane was further decreased to 213 °C as compared to the cross-linked-CH membrane.

All the investigated film samples yielded a different contact angle value. The values of contact angle to water were 59.3°, 49.9°, and 67.9° for the crosslink-chitosan, CH-g-poly(IA) and

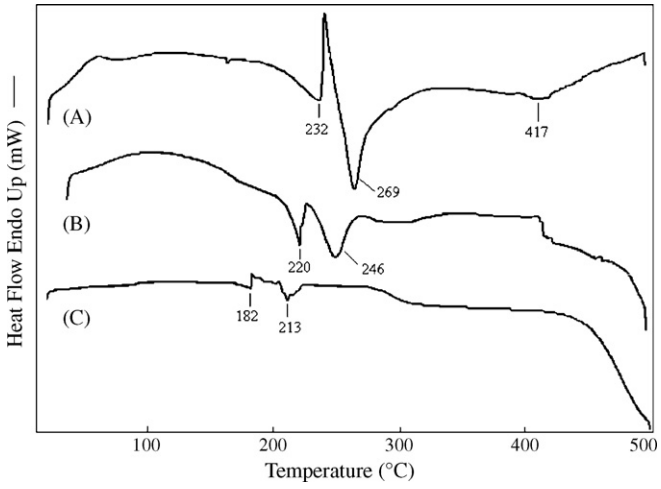

Fig. 3. The results of DSC measurements: (A) chitosan; (B) cross-linked-chitosan; (C) chitosan-g-poly(IA) membranes.

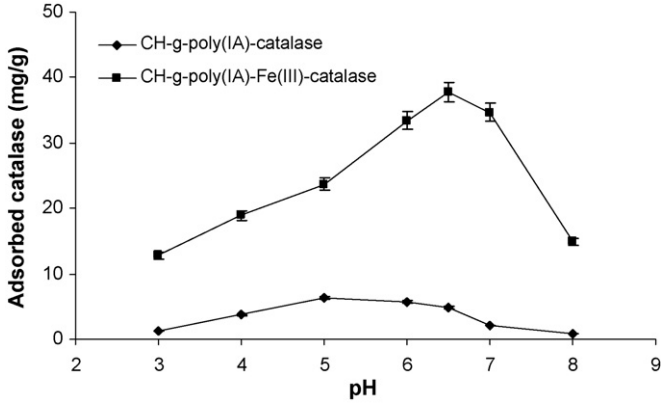

Fig. 4. Effects of pH on the immobilization efficiency of the CH-g-poly(IA)-Fe(III) membranes; Initial concentration of catalase: 2.0 mg/mL; temperature: 25 °C.

CH-g-poly(IA)-Fe(III) membranes. The poly(itaconic) acid grafted membrane surface resulted in a significant difference on the value of contact angle to water compared to cross-liked-chitosan membrane. On the other hand, after incorporation of Fe(III) ions onto the poly(itaconic acid) grafted membrane, the hydrophilicity of the membrane was decreased compared to both membrane preparations.

3.2. Adsorption efficiency and retention of activity

The effect of pH on the adsorption of catalase onto CH-g-poly(IA) and CH-g-poly(IA)-Fe(III) membranes was studied in the pH range 3.0–8.0. With CH-g-poly(IA)-Fe(III), a maximum enzyme loading of 37.8 mg protein/g membrane was obtained at pH 6.5 while with CH-g-poly(IA) was 6.3 mg protein/g at pH 6.5 and pH 5.0, respectively (Fig. 4). Significantly lower enzyme loadings were obtained with both preparations in other tested pH regions. The maximum adsorption of catalase on the negatively charged CH-g-poly(IA) membrane was observed at around pH 5.0. On the other hand, the maximum adsorption was observed at pH 6.5 for Fe(III) ion incorporated counterpart, and a mechanism for catalase immobilization on CH-g-poly(IA)-Fe(III) membrane is proposed in Fig. 1. The chelated metal ion shows variations in affinity toward proteins, which can be predicted using hard and soft acids and bases. The ligands containing oxygen such as carboxylic groups of glutamic and aspartic acids, aliphatic nitrogen groups of asparagines, glutamine and phosphorylated groups of amino acids are classified as hard Lewis bases. The aromatic nitrogen containing amino acids on protein structure histidine and tryptophan are also considered hard Lewis bases. Ligands containing sulfur (such as in cysteine amino acid) are classified as soft bases. On the other hand, metal ions such as Ca(II), Al(III) and Fe(III) are classified as hard Lewis acids. In these concepts, hard and soft acids and bases explain that bonds between atoms with similar rating, e.g., a hard acid combined with a hard base are the strongest [39,40]. From this point of view, the interactions between incorporated Fe(III) ions and catalase at pH 6.5, may result from hard acid–base interactions caused by the carboxyl and aliphatic nitrogen groups of the amino acid side-chains of the enzyme molecules. In addition, catalase is classified as a ferric heme-containing enzyme and it has an iron protoporphyrin prosthetic group. This phenomenon could also provide additional high binding affinity for catalase to CH-g-poly(IA)-Fe(III) membrane.

An adsorption isotherm was used to characterize the interactions of the enzyme with the CH-g-poly(IA) and CH-g-poly(IA)-Fe(III) membranes. This provides a relationship between the concentration of enzyme in the solution and the amount of enzyme

adsorbed onto the solid phase when the two phases are at equilibrium. An increase in the enzyme concentration led to an increase in the adsorption efficiency, but this leveled-off at an enzyme concentration of 2.0 mg/mL (data not shown). A maximum enzyme adsorption 6.3 mg/g membrane was obtained for CH-g-poly(IA). The amount of Fe(III) ions loaded on the surface of CH-g-poly(IA)-Fe(III) membranes was 9.8×10^{-4} mmol/g. Incorporation of Fe(III) ions on to CH-g-poly(IA) membrane significantly increased catalase adsorption capacity of the membrane about 6.0 fold. The maximum enzyme loading capacity of CH-g-poly(IA)-Fe(III) membrane was 37.8 mg/g. This could be due to the specific interactions between catalase and Fe(III) molecules, because catalase has an iron protoporphyrin prosthetic group. Whereas, the recovered enzyme activity achieved with the metal chelated membrane also increased with incorporation of Fe(III) ions onto CH-g-poly(IA)-Fe(III) membrane, reaching a maximum at $\sim 6.65 \times 10^4$ U/g, on the other hand, the specific activity on the CH-g-poly(IA) preparation was $\sim 7.72 \times 103$ U/g. Thus, on the basis of metal recognition, an additional strong binding was established between ferric ions of catalase molecules and carboxyl groups of the grafted itaconic acid chains. The CH-g-poly(IA)-Fe(III) membranes yielded a high enzyme loading with a high recovered enzyme activity compared to CH-g-poly(IA) membrane. Therefore, the rest of the experimental studies, were carried out with catalase immobilized on the CH-g-poly(IA)-Fe(III) membrane.

3.3. Kinetic parameters

Kinetics parameters, the Michaelis constants K_m and V_{max} for the free and immobilized catalase were determined using H_2O_2 as substrate. The K_m values of the free and immobilized catalase were found to be 13.5 and 25.8 mM, respectively. The V_{max} values were estimated from the data as 1820 and 1103 U/mg protein for the free and adsorbed enzymes. As expected, the K_m and V_{max} values were significantly affected after immobilization onto CH-g-poly(IA)-Fe(III) membrane. The difference in K_m values between the free and 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 on the fibrous polymer layers of the membrane and the conformational changes of the catalase molecules caused by the various multipoint interactions with the carboxylic groups and Fe(III) ions on the membrane. 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 substrate [41–43]. A 1.6-fold reduction in the V_{max} value was observed upon immobilization of catalase on the CH-g-poly(IA)-Fe(III) membrane via adsorption. In the presented work, the decrease in the immobilized enzyme activity is not too large and may be compensated by a better stability. 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, Cetinus et al. [44] studied the immobilization of catalase on chitosan Cu(II) chelated beads and found that V_{max} value of the free catalase was 3200 U/mg protein whereas, upon immobilization, it was decreased about 1.7-fold (1845 U/mg protein). Arica et al. reported that the V_{max} value of catalase was decreased 2.8-fold upon immobilization in the thermally reversible hydrogels [45].

3.4. Effect of temperature and pH on the catalytic activity

The temperature dependence activities of the free and immobilized catalase were studied in phosphate buffer (0.1 M, pH 7.0) in the temperature range 15–55 °C (Fig. 5). In general, the effects of changes in temperature on the rates of enzyme-catalyzed reactions

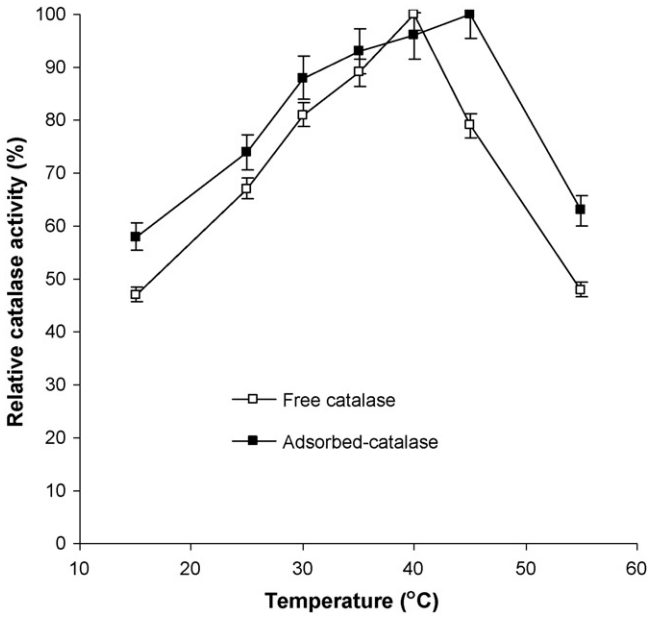

Fig. 5. Temperature profiles of the free and immobilized catalase preparations.

do not provide much information on the mechanism of catalysis. However, these effects could be important in indicating structural changes in enzymes [46]. As seen in Fig. 5, the optimum temperature for the immobilized catalase at 45 °C, 5 °C higher than that of the free enzyme at 40 °C, and the support has a slightly protecting effect at the high temperatures at which enzyme deactivation takes place. The non-covalent multipoint interactions between the enzyme and the CH-g-poly(IA)-Fe(III) support may reduce the degrees of freedom of the molecular structure of the enzyme, thus, protecting it in some extent from denaturation by high temperature [47–49].

The effect of pH on the free and immobilized enzymes was investigated in the pH range between 4.0 and 9.0 in acetate or/and phosphate buffers and the results are presented in Fig. 6. The immobilized catalase has the same optimum pH 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 CH-g-poly(IA)-Fe(III) metal chelated support [50,51].

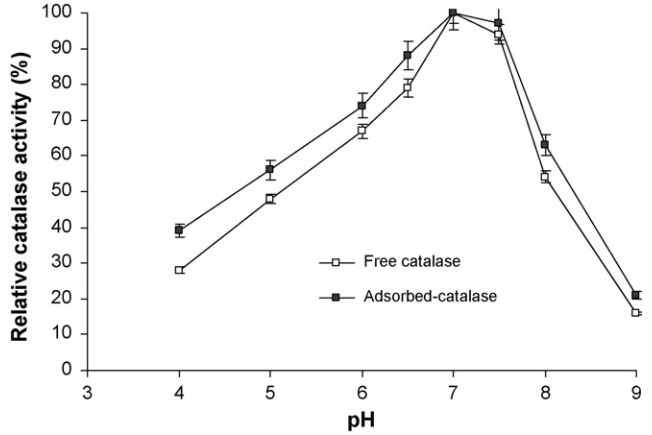

Fig. 6. The effect of pH on the activity of the free and immobilized catalase preparations.

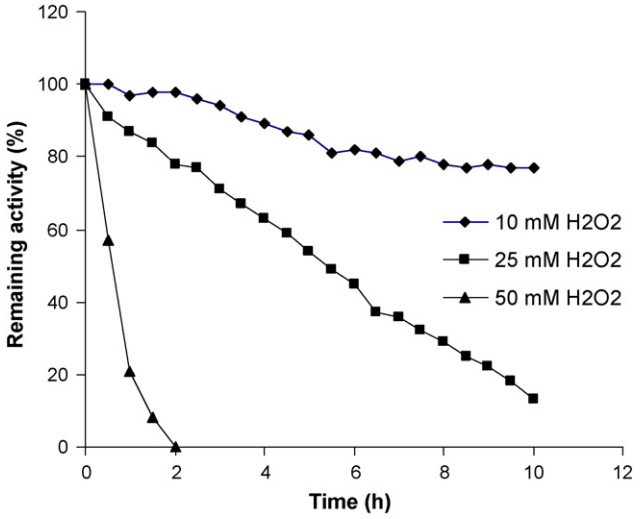

Fig. 7. Operational stability of the immobilized catalase at three different substrate concentrations.

3.5. Activity of the immobilized catalase

Activity of the catalase immobilized on the CH-g-poly(IA)-Fe(III) membrane was determined in a continuous-flow system for 10 h. Hydrogen peroxide concentration in the feed solution was varied (10, 25 and 50 mM) at 30 °C. The results are presented in Fig. 7, at low H_2O_2 concentration, the immobilized catalase lost about 13% of its initial activity during 10 h continuous operation. At 25 mM H_2O_2 concentration, a steady decrease in the activity of immobilized catalase was observed and this loss reached 83% after 10 h of continuous operation. At high-hydrogen peroxide concentrations (50 mM), catalase seems to be more labile, and activity of immobilized catalase decreased much more rapidly with a higher slope (Fig. 7). The immobilized catalase loss all its initial activity within 2 h continuous operation. This should be a result of a poisoning effect brought about by the substrate "hydrogen peroxide".

3.6. Thermal stability measurement for free and immobilized catalase

The thermal stabilities of free and immobilized enzymes were studied at two different temperatures at 55 and 65 °C (Fig. 8). Residual activities for the free and immobilized catalase were found to be 71, and 87% of their original activities, respectively at 55 °C for a 120 min incubation period. Free catalase remained about 17% of its original activity at 65 °C for 120 min incubation period. Immobilized catalase protected about 43% of its original activity for the same period at 65 °C. As incubation time increased, the activity of immobilized enzyme decreased at a constant temperature value. This situation was more pronounced especially at higher temperature (at 65 °C). However, the results showed that the thermal stability of the immobilized catalase on the Fe(III) incorporated membrane was greater than that of the free catalase.

3.7. Storage stability

Storage stability is an important advantage of immobilized enzymes over the free enzymes, because free enzymes can lose their activities fairly quickly. In general, if an enzyme is in aqueous solution, it is not stable during storage, and the activity is gradually decreased. Free and immobilized catalase (i.e., CH-g-poly(IA)-Fe(III)) were stored in a phosphate buffer (50 mM, pH 7.0) at 4 °C and the activity measurements were carried out for a

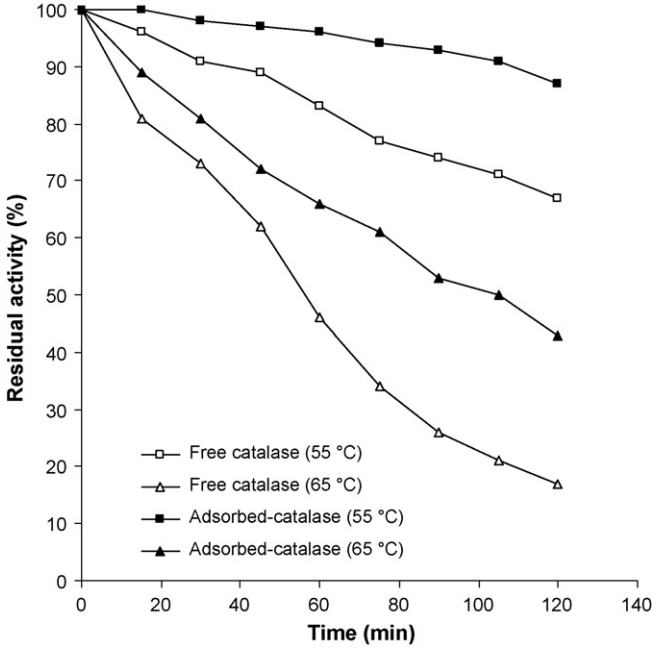

Fig. 8. Thermal stability of the free and immobilized catalase at two different temperatures.

period of 30 days (data not shown). During this incubation period, no enzyme leakage from the support was detected in the storage solution. The free enzyme lost all of its activity within 20 days. On the other hand, immobilized catalase lost 23% of its activity during the incubation period. The result readily indicates that the immobilized catalase exhibits an improved stability over that of the free enzyme. Of the immobilization methods, fixation of enzyme molecule on a surface often gives rise to the highest stabilization effect on enzyme activity, because the active conformation of the immobilized enzyme is stabilized by multipoint interactions with support [52–54]. The decrease in the activity of catalase preparations can be explained as a time-dependent natural loss in enzyme activity and this was prevented to a significant degree upon immobilization.

3.8. Reusability of CH-g-poly(IA) membranes

Desorption of catalase from CH-g-poly(IA)-Fe(III) membranes was carried out in a batch system as described above. Then, the same CH-g-poly(IA) membrane disks were used for reload of Fe(III) ions. After reloaded with Fe(III) ions, the same membranes were used for immobilization of catalase. The reusability of the CH-g-poly(IA) membrane disks, the adsorption–regeneration cycle was repeated six times using the same membrane disks. At the end of the sixth cycles, catalase immobilization capacity of the CH-g-poly(IA) membrane was decreased about 7%.

4. Conclusion

In this study, the poly(IA) grafted and/or metal ions incorporated metal chelate-affinity membranes were used for reversible immobilization of catalase. The presented new method has many advantages over conventional techniques. An expensive and critical step in the preparation process of metal-chelating adsorbent is coupling of a chelating ligand on the adsorption matrix. In this method, grafted poly(itaconic acid) acted as a metal-chelating ligand, and there is no need any reaction step to activate the matrix for the chelating-ligand immobilization. In addition, grafted poly(itaconic acid) brush provides a hydrophilic microenviron-

ment for the guest enzyme. The immobilization conditions of the catalase have been investigated with detailed characterization of poly(itaconic acid) fibrous layers on the membrane surfaces. Catalase adsorption capacity of the Fe(III) ion incorporated membrane is higher than that of CH-g-poly(IA) membrane. The optimum pH was not affected by immobilization, but optimum temperature was shifted from 40 to 45 °C upon immobilization. The improved stability of the immobilized catalase as observed in temperature and pH profiles (showed by the Figs. 5 and 6) may be also just associated to a reduction of the dissociation of the enzyme subunits, this should greatly improve enzyme stability. Immobilized catalase on the CH-g-poly(IA)-Fe(III) membrane also showed a better thermal and storage stabilities. The properties of the metal incorporated affinity membrane seem to provide an adequate approach to catalase immobilization. The CH-g-poly(IA)-Fe(III) membrane revealed good properties as an adsorptive material and will find useful applications in biotechnology.

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