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Covalent immobilization of α-amylase onto poly(2-hydroxyethyl methacrylate) and poly(styrene -2-hydroxyethyl methacrylate) microspheres and the effect of Ca^{2+} ions on the enzyme activity

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Abstract

α-Amylase (1,4-α-D-glucan-glucanohydrolase; EC 3.2.1.1, Type VI-B from porcine pancreas, extra pure 29 units mg^{-1}) was covalently immobilized on poly (2-hydroxyethyl methacrylate), p(HEMA), and poly (styrene- 2- hydroxyethyl methacrylate), p(St-HEMA) microspheres, which were activated by using epichlorohydrin (ECH). The properties of the immobilized enzyme were investigated and compared with those of the free enzyme. For the assays carried out at 25°C and pH 6.9, the relative activities were found to be 61.7 and 67.0% for ECH-activated P(HEMA) and P(St-HEMA) bound enzymes, respectively. The maximum activities were obtained at lower pH values and higher temperatures upon immobilization compared to free enzyme. Kinetic parameters were calculated as 2.51, 22.4 and 6.62 g dm^{-3} for K_m and 1.67×10^{-3}, 1.63×10^{-3} and 1.35×10^{-3} g dm^{-3} min^{-1} for V_{max} in the case of free, P(HEMA) and P(St-HEMA) bound enzymes, respectively. Enzyme activity was found to be ca. 38.9% for ECH-activated P(HEMA) bound enzyme after storage for 1 month. On the other hand, free enzyme lost its activity completely in 20 days. Immobilization, storage stability and repeated use capability experiments that were carried out in the presence of Ca^{2+} ions demonstrated higher stability. The enzymes immobilized in the presence of Ca^{2+} ions retained 90.7 and 80.0% of their original activities even after 30 days for ECH-activated P(HEMA) and P(St-HEMA) systems, respectively. In repeated batch experiments, 20 uses in 3 days, in the absence of Ca^{2+} ions, retention of 79% of the original enzyme activities was observed for ECH-activated P(HEMA) immobilized enzymes. On addition of Ca^{2+} ions to the assay medium, 90.0 and 80.0% of retention was observed for ECH-activated P(HEMA) and P(St-HEMA) systems, respectively.

Keywords: α-Amylase; Immobilization; Covalent binding; Microspheres; Poly(styrene-2-hyroxyethyl methacrylate); Poly(2-hyroxyethyl methacrylate)

1. Introduction

α-Amylase (1,4-α-D-glucan glucanohydrolase) is a widespread enzyme occurring in mammals as salivary and pancreatic α-amylase; in the seeds of plants, it is produced by large numbers of microorganisms. α-Amylase hydrolyses α-1,4 glycosidic bonds in amylose, amylopectin and glycogen in an endo-fashion. It does not hydrolyse α-1,6 linkages or any other branch points and so produces maltose and limit dextrins; the precise action pattern depends on the source of the α-amylase (Fogarty, 1983). All α-amylases are calcium metalloenzymes, binding at least one calcium ion per monomeric unit; for example *Bacillus subtilis* α-amylase strongly binds four calcium ions. The calcium ions impart resistance to pH, temperature, proteolysis and denaturation by urea and heat. The stability arises from the calcium ions contribution to the enzyme's tertiary structure, probably replacing the lack of disulphide bonds. Immobilization of enzyme on insoluble supports has been a topic of active research in enzyme technology and is essential for their application to industrial processes. Recent work on immobilized enzymes has concentrated on attachment of enzymes to water-insoluble supports such as cellulose and dextran derivatives, polyacrylamide or porous glass. α-Amylase has been commonly immobilized by entrapment within organic gels, such as acrylamide copolymers. In these studies low activities were observed and high diffusional restrictions of soluble starch were also noticed. Physical and ion-exchange adsorptions of α-amylase have also

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been carried out on a wide range of supports, sand, collagen, Sephadex, Sepharose, etc. (Roig, Slade & Kennedy, 1993).

Generally, α-amylase is used together with glucoamylase to promote the hydrolysis of starch to fermentable sugar or glucose. Several methods have been developed for the preparation of immobilized α-amylase with each having its own advantages and disadvantages specific to the methods (Bagaı & Madamwar, 1997; Handa, Hirose, Akino, Watanable & Tsuchlya, 1983; Isoa, 1980; Marchal et al., 1999; Michael, 1980; Ohnishi, Iwata, Tomita, Nishikawa & Hirami, 1990; Saburo & Atsuo, 1985; Arasaratman & Balasubramin, 1993; Linko, Saarinen & Linko, 1975). Immobilization is often accompanied by some changes in the enzymatic activity, optimum pH, affinity for the substrate, and stability. The extent of these changes depend on the enzyme and carrier support and on the immobilization conditions (Kwashima & Umeda, 1975; Strumeyer, Constantinides & Freundenberger, 1974). Among these, the support is the most important (Allan, 1985; Shah, Sellappan & Madamwar, 1997; Zaborsky, 1975). Poly(2-hydroxyethyl methacrylate), due to its being a biocompatible synthetic polymer with adequate mechanical strength for most biomedical and biotechnological applications, is regarded as a suitable matrix for immobilization of enzymes (Jiri, 1987; John, 1985; Michael, 1980).

Immobilized enzymes are used in food technology, biotechnology, biomedicine and analytical chemistry because of their various advantages. Immobilization of enzymes facilitates the purification of the reaction systems (separation of the reactants and products easily from the reaction media) and recovery of enzyme and makes it possible to use the enzyme repeatedly or continuously. There are many different procedures for the immobilization of an enzyme. Covalent binding to an activated support, copolymerization of the enzyme molecules with the polymers, crosslinking between the enzyme molecules, physical adsorption of the enzyme onto a solid support and entrapment of the enzyme molecules in polymeric structures, are some of the ways of immobilization. Covalent binding to an activated support has some advantages, such as; preventing the leakage of the enzyme and diminishing the diffusion problems. When the immobilization is carried out on microspheres, because of the very high surface to volume ratio, the amount of bound enzyme, and the enzyme activity, are enhanced. In industrial applications, one important point is to prepare the carrier system in such a way that it should have the desired chemical, physical, mechanical and thermal properties.

The aim of this research is to study viability, activity and stability of α-amylase that is covalently immobilized on poly (2-hydroxyethyl methacrylate), P(HEMA), and poly (styrene-2-hydroxyethyl methacrylate), P(St-HEMA), supports. P(HEMA) and P(St-HEMA) microspheres were prepared as a new support material by applying an emulsion polymerization technique. The hydroxyl groups of the hydroxyethyl methacrylate component were activated with epichlorohydrin, then α-amylase was immobilized onto activated P(HEMA) and P(St-HEMA) microspheres by covalent binding. The effects of the immobilization process on enzyme activity and kinetic parameters of the enzyme were investigated. Experiments were carried out in the presence of Ca^{2+} ions in order to enhance the storage stability and reusability of the immobilized enzymes.

2. Materials and methods

2.1. Materials

α-Amylase (1,4-α-D-glucan-glucanohydrolase; EC 3.2.1.1, Type VI-B from porcine pancreas, extra pure 29 units mg^{-1}), starch, epichlorohydrin (ECH), styrene (St), were obtained from Merck AG (Germany). Ethylene glycol dimethacrylate (EGDMA) and 2-hydroxyethyl methacrylate (HEMA) were purchased from Aldrich Chemical Company (USA). All other chemicals were of reagent grade and used as received.

Stock iodine solution was prepared by dissolving iodine (500 mg) and potassium iodide (5.0 g) in distilled water (100 cm^3). The working iodine reagent was prepared by diluting 1.0 cm^3 of stock solution 100 times prior to each experiment. Phosphate buffer (PB) (40 mM, pH 6.9) was used for activity determination experiments.

2.2. Preparation of microspheres

Microsphere preparation was carried out by the emulsion polymerization technique. For this purpose, distilled water (20 cm^3) and potassium persulfate (20 mg) were added into a three-necked flask (250 cm^3) fitted with a condenser and a thermometer. The system was heated to 70°C while flushing nitrogen through the solution. St (2.5 cm^3), HEMA (2.5 cm^3) and EGDMA (0.1 cm^3) were added and the formed emulsion was stirred vigorously for 5 h at 70°C. The mixture was kept at room temperature overnight and the precipitated P(St-HEMA), microspheres were separated by filtering, washed several times with distilled water and dried in a vacuum oven at room temperature and weighed. P(HEMA) microspheres were prepared by the same method.

2.3. Characterization of microspheres

The average particle size and size distribution curves were obtained by using a particle size analyser (Master Sizer E, Version 1.2b, Malvern Instruments Ltd., UK). The shapes of particles were examined by Scanning

Electron Microscopy (SEM) (Leitz-AMR-1000) and micrographs were obtained after coating the samples with gold under vacuum.

2.4. Activation with epichlorohydrin

P(HEMA) and P(St-HEMA) microspheres (0.1 g) were added to epichlorohydrin (5 cm^3), kept in a shaking water bath for 4 h at 25°C and stored at that temperature overnight. The activated microspheres were separated, washed three times with acetone (5 cm^3), then washed three times with phosphate buffer (5 cm^3).

2.5. Coupling of α-amylase onto activated microspheres

The activated microspheres were added to the enzyme solution (20 cm^3 containing 20 mg enzyme in PB) and the immobilization reaction was carried out for 4 h at 25°C in a shaking waterbath. Microspheres were separated and the unbound enzyme was removed by washing three times with phosphate buffer. The immobilized enzyme was stored at 4°C until use. The reactions for activation of microspheres and enzyme coupling are shown in Fig. 1.

2.6. Determination of the amount of bound enzyme

After the enzyme immobilization process and separation of the microspheres, the supernatant and the washed solutions were collected. The amount of unbound enzyme was determined from UV absorbances of the collected solutions at 280 nm (measured by using a UV-spectrophotometer, Shimadzu, Model 1201, Japan) with the help of a calibration curve.

2.7. Assay of α-amylase activity

Activities of free and immobilized α-amylases were detected according to the starch-iodine method (Yoo, Hong & Hatch, 1987). Assay solution containing free or immobilized enzyme in phosphate buffer (0.05 mg enzyme or 0.1 g microspheres in 2.5 cm^3 PB) was added to the starch solution (2% w/v, 3 cm^3) at 25°C. From this solution, 0.1 cm^3 aliquots were taken in every 5 min and added to a solution (total volume 10.9 cm^3) which consisted of stopping reagent HCl (5 cm^3, 0.1 M), iodine solution (5 cm^3) and phosphate buffer (0.9 cm^3). The absorbance due to the formed blue color was measured at 600 nm. The amount of starch was obtained from the calibration curve and used in the calculation of enzyme activity. All activity measurement experiments were carried out at least twice (generally three times) and the relative standard deviations were found to be less than 1.0%. One unit of α-amylase activity was defined as the amount of enzyme which hydrolyzes 1 mg of starch in 1 min at 25°C and pH 6.9.

2.8. Determination of K_m and V_{max} values

K_m and V_{max} values were determined by measuring the initial rates of the reaction of α-amylase with starch solution (1–5% w/v). The assay procedures were the same as described previously.

2.9. Influence of Ca^{2+} ions

In order to examine the effect of Ca^{2+} ions on the enzyme activity, the immobilization process of the enzyme was achieved in Tris–maleate buffer (TMB, pH 6.9, 0.02 M, 20 cm^3) containing 5 mM $CaCl_2$. These samples were stored in the same buffer at 4°C. The experiments on stability and reusability were carried out in the same way as described for the other immobilized samples by using 2% starch solution prepared in TMB. A sample was drawn for the determination starch content after incubating the assay solution at 25°C for 30 min. After each measurement, the remaining microspheres, containing immobilized enzyme, were washed and placed in the refrigerator.

3. Results and discussion

3.1. Properties of microspheres

In the copolymerization process of St and HEMA, the extent of polymerization was calculated gravimetrically and the conversion was found to be more than 99%.

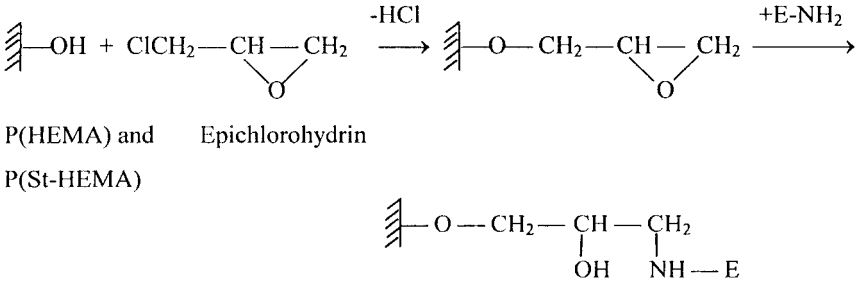

Fig. 1. The reactions for activation of microspheres and enzyme binding.

SEM micrographs of P(HEMA) and P(St-HEMA) particles demonstrated the formation of microspheres (Fig. 2a and b).

The particle size distribution curves of P(HEMA) and P(St-HEMA) microspheres are given in Fig. 3a and b. For the prepared P(HEMA) and P(St-HEMA) microspheres, the most commonly occurring particle diameters were found to be 83–182 μm, respectively.

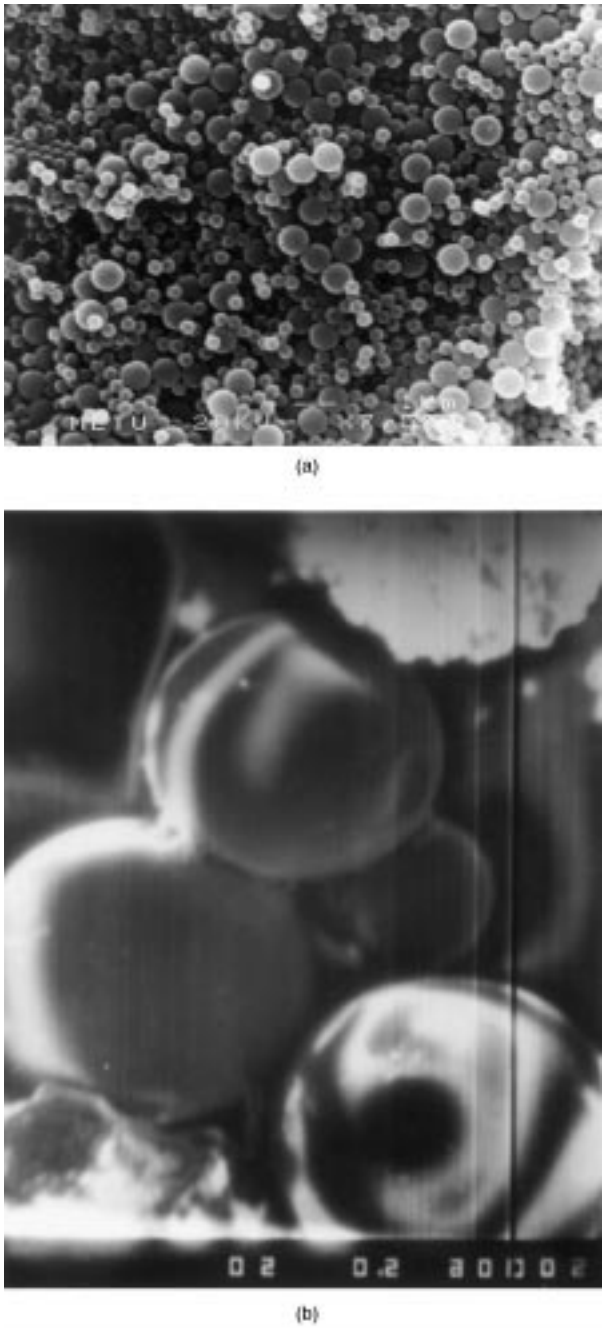

Fig. 2. Micrographs of (a) P(HEMA) and (b) P(St-HEMA) microspheres.

3.2. Enzyme loading

The amounts of bound enzymes were found to be 5.0 and 6.8 mg per g of P(HEMA) and P(St-HEMA) microspheres, respectively, and the preserved activities were 67.0 and 61.7% for ECH-activated P(HEMA) and P(St-HEMA) bound enzymes, respectively. In the literature, for immobilized α-amylase, various values for binding capacities and preserved activities are given. For example, when immobilization was achieved on polystyrene- and silica-based supports, coupling capacities and preserved activities were reported as 3–29 mg per g support and 7–40%, respectively (Ulbrich, Schellenberger & Damerav, 1986; Kvétsitadze & Dvali, 1982); on dextran and cellulosic supports these values are given as 25–90 mg per g support and 25–67%, respectively (Kennedy & Paterson, 1993; Wykes, Dunny & Lilly, 1971). Similar coupling capacities (e.g. 11–34 mg/g support) are given for different polymeric structures with 20–54% preserved activities (Arıca, Hasırcı & Alaeddinoglu, 1995; Ohtsuka, Kawaguchi & Yamamoto, 1984).

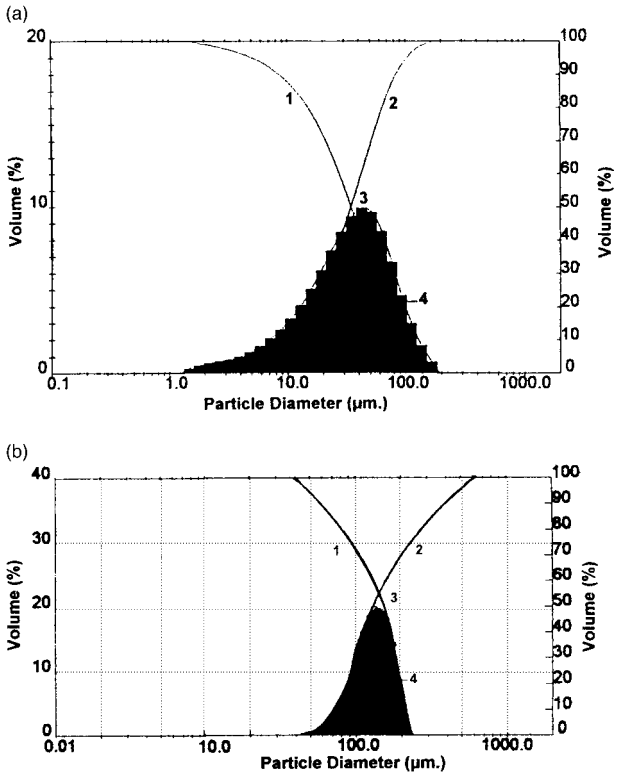

Fig. 3. Particle size distribution curves of (a) P(HEMA) and (b) P(St-HEMA) microspheres. In this figure, undersize curve (1) and oversize curve (2) show the percentages below and above a certain size, respectively. Both curves use the right hand scale to take their percentages scale. The frequency curve (3) is obtained by differentiating the cumulative undersize curve. The peak of this curve gives the most commonly occurring particle diameter. Histogram plot (4) shows the percentages of the volume of the sample that is within a particular size band and uses the left hand scale.

In this study, the coupling capacities of the immobilized enzymes were found to be less, while the preserved activities were found to be much higher than the literature values.

3.3. Parameters affecting enzyme activity

The activities of free and immobilized α-amylase were calculated by measuring the absorbances of the starch solutions at 600 nm. The reactions were carried out at various pH values, temperature and substrate concentrations and the effects of these parameters, as well as storage stabilities and repeated use capabilities, were examined. The slopes of the linear part of the absorbance–time curves were used in the calculation of enzyme activities with the help of a starch calibration curve.

3.3.1. Effect of pH

The pH dependence of the immobilized α-amylase activity was compared with that of the free enzyme in the pH range of 5.0–8.0 at 25°C and the results are presented in Fig. 4. Maximum substrate conversion was found at pH 7.5 for free α-amylase, and at pH 6.5 for ECH-activated P(HEMA) and P(St-HEMA) bound enzymes. The trends of the curves are similar for both cases. The shift in the optimum pH towards a lower pH value upon immobilization might be because of the difference in the hydronium ion concentrations in the vicinity and in the bulk of the solution. It is known that polyionic matrices cause partitioning of protons between the bulk phase and the enzyme microenvironment (Strumeyer et al., 1974). For amylase immobilization, shifts in the optimum pH towards both the acidic and alkaline directions have been observed (Arıca et al., 1995; Bayramoğlu, Akbulut & Sungur, 1992).

3.3.2. Effect of temperature

The temperature effect on the activity of free and immobilized enzymes is shown in Fig. 5. The free enzyme exhibited a temperature optimum of 40°C and this value shifted to 55°C for both immobilized systems. The increase in the optimum temperature may arise from the change of the conformational integrity of the enzyme structure upon covalent binding to the support material (Arıca et al., 1995; Bayramoğlu et al., 1992; Demircioğlu, Beyenal, Tanyolaç & Hasırcı, 1994; Lee, Lee & Siaw, 1993).

3.3.3. Kinetic parameters

Kinetic parameters, K_m and V_{max} for free and immobilized enzymes were determined by using soluble starch as substrate. The activities of free and immobilized enzymes for various substrate concentrations were plotted in the form of Lineweaver–Burk plots (Fig. 6). V_{max} values were calculated to be 1.67×10^{-3}, 1.63×10^{-3} and 1.35×10^{-3} g dm^{-3} min^{-1} for free, ECH-activated P(HEMA) and P(St-HEMA) immobilized enzymes, respectively, K_m values were found to be 2.51, 22.40 and 6.62 g dm^{-3} for free, ECH-activated P(HEMA) and P(St-HEMA) immobilized enzymes, respectively. The increase in K_m values for immobilized enzymes has been reported by various authors (Arıca & Hasırcı, 1993; Arıca et al., 1995; Demircioğlu et al., 1994; Demirel, Akovali, Tanyolaç & Hasırcı, 1992; Ohtsuka et al., 1984; Pozniak, Krajewska & Trochimczuk, 1995; Tarhan, 1989; Demirel, Akovali, Tanyolaç & Hasırcı, 1992). These changes in kinetic parameters may be a consequence of either structural changes in the enzyme occurring upon immobilization or lower accessibility of substrate to the active sites of the immobilized enzyme.

3.3.4. Storage stability

Enzymes in solution are not stable and during storage their activities decrease gradually over time. The storage

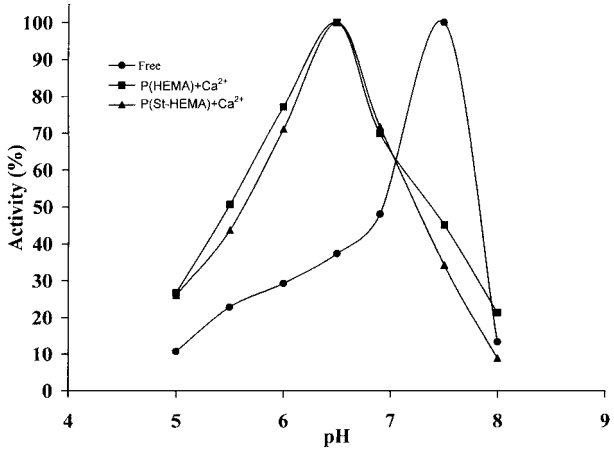

Fig. 4. Effect of pH on the activity of free and immobilized α-amylases.

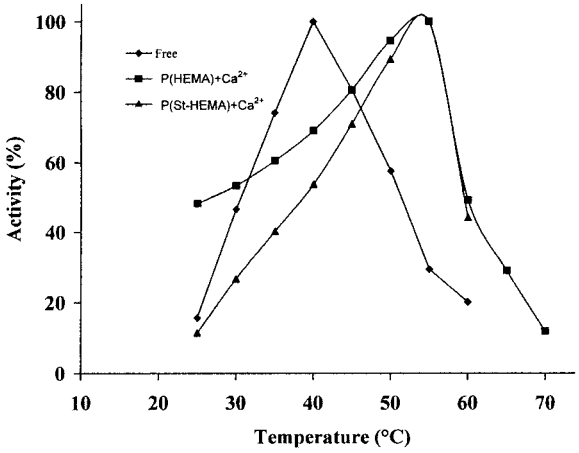

Fig. 5. Effect of temperature on the activity of free and immobilized α-amylases.

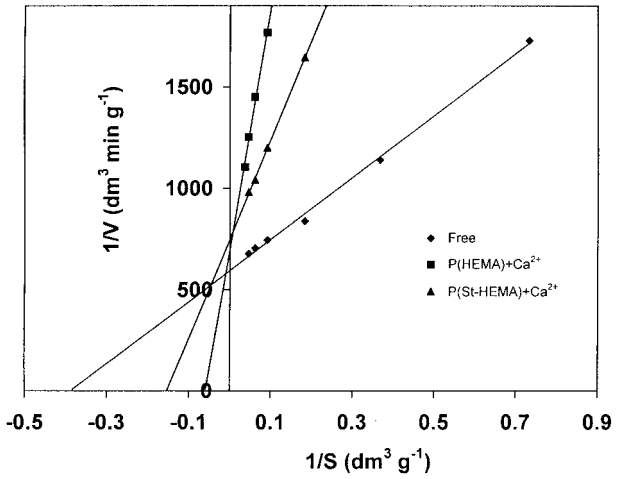

Fig. 6. Lineweaver–Burk plots for free and immobilized α-amylases.

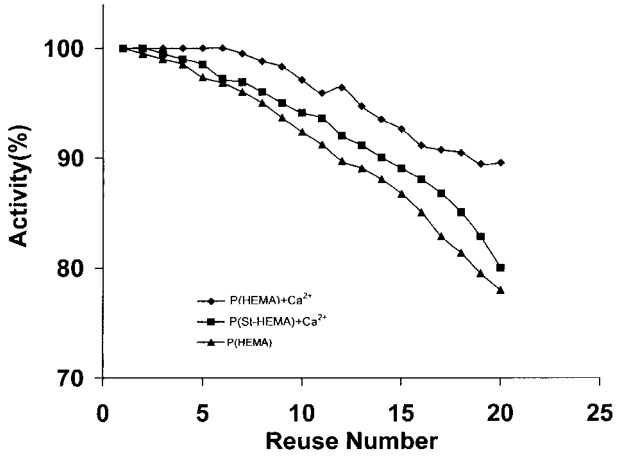

Fig. 8. Reuse of ECH-activated P(HEMA) and P(St-HEMA) bound enzymes.

stabilities of amylase at 4°C, either in the presence or absence of Ca^{2+} ions at 4°C, were investigated by measuring the enzyme activities at certain time intervals and the results are given in Fig. 7. The presence of Ca^{2+} ions enhanced the enzyme stability. For free enzyme, the presence of Ca^{2+} ions caused a retention of 37.4% of the original activity in 20 days of storage at 4°C although, in the absence of Ca^{2+}, the free enzyme lost its activity completely in the same time period. The retained activities were found to be 38.9 and 90.7% for ECH-activated P(HEMA) immobilized enzyme in the absence and presence of Ca^{2+} ions, respectively, upon 30 days of storage at 4°C. The retained activity was found to be 74.0% for ECH-activated P(St-HEMA) immobilized enzyme in the presence of Ca^{2+} ions, upon 20 days of storage at 4°C. From these results, it was seen that immobilization definitely put the enzyme into a more stable position in comparison to free enzyme and, also, stabilities of free and immobilized enzymes were substantially increased in the presence of Ca^{2+} ions. Addition of Ca^{2+} ions causes saturation of the enzyme by formation of an enzyme–Ca^{2+} complex. It is known that each molecule of α-amylase contains at least one Ca^{2+} ion in its structure. The presence of Ca^{2+} ions and the saturation promote the stability of the three-dimensional structure of the enzyme (Aksoy, Tümtürk & Hasırcı, 1998; Bayramoğlu et al., 1992; Cord, Vanhoof, Hu, Maesmans, Hendrickx & Tabback, 1992; Flor & Hayashida, 1980; Violet & Meunier, 1989).

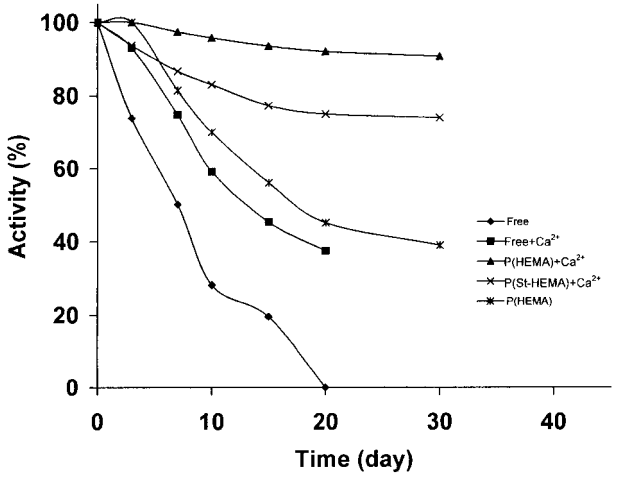

Fig. 7. Effect of storage on the activity of free and immobilized α-amylases.

3.3.5. Repeated use capability

The immobilized samples that were prepared in the presence and absence of Ca^{2+} ions were used repeatedly 20 times within 3 days and the measured activities are presented in Fig. 8. It was observed that, after the 20th use, ECH-activated P(HEMA) bound enzymes retained 79.0 and 90.0% of their original activities in the absence and in the presence of Ca^{2+} ions, respectively. The retained activities for ECH-activated P(St-HEMA) bound enzyme was found as 80.0% in presence of Ca^{2+} ions. These results achieve the positive effect of Ca^{2+} ions on the enzyme stability on the reuse experiments.

4. Conclusion

The present study shows that P(St-HEMA) and P(HEMA) microspheres are promising carriers for α-amylase enzyme. These are new matrix materials, which have both hydrophilic and hydrophobic domains coming from HEMA and St, respectively. It is possible to

change the properties of copolymeric matrix by altering the molar ratios of the added monomers. The immobilization of α-amylase on P(St-HEMA) microspheres by using ECH, promoted enzyme stability and as a result, the enzyme became more stable against temperature, storage and reuse compared to the free enzyme. The immobilization process, which is carried out in the presence of Ca^{2+} ions, demonstrated a substantial increase in enzyme stability. The obtained results achieved the usefulness of the examined matrix materials and immobilization techniques in enzyme immobilization. The system can be a good candidate for practical use in continuous reactors in biotechnological applications.

Acknowledgements

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