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Immobilization of a thermostable α-amylase onto reactive membranes: kinetics characterization and application to continuous starch hydrolysis

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Abstract

Epoxy groups containing porous membranes were prepared by UV-initiated photopolymerisation of hydroxyethylmethacrylate (HEMA) and glycidyl methacrylate (GMA). Epoxy supports could provide multipoint covalent attachment of enzymes, therefore, to stabilize their three-dimensional structure. α-Amylase was immobilized onto the poly(HEMA-GMA-1-3) membranes by means of the amide linkage formation between the amino groups of α-amylase and the epoxy groups of the support. The α-amylase immobilization capacity of the membranes was increased as the GMA ratio increased in the membrane structure. The retained activity of the immobilized α-amylase was 76% with poly(HEMA-GMA-2) membrane. The decrease in activity of the immobilized α-amylase could be considered to be due to reduced conformational flexibility of the immobilized α-amylase molecules for binding its large substrate, starch, as a result of the covalent immobilization. The immobilized α-amylase has more resistance to temperature inactivation than that of the free form. The optimum pH value of α-amylase was not affected by the immobilization reaction, but the pH profile was broadened for the immobilized enzyme. Kinetic parameters were determined for immobilized α-amylase as well as for the free enzyme. The values of the Michaelis constant K_m of α-amylase, were significantly larger (ca. 2.3 times) upon immobilization, indicating decreased affinity of the enzyme for its substrate, whereas V_{max} was smaller for immobilized α-amylase. In a 120 h continuous operation at 35 °C, only 4% of immobilized α-amylase activity was lost. The operational inactivation rate constant (k_{opi}) of the immobilized α-amylase with 2% starch was 8.06×10^{-6} min^{-1}.

Keywords: Polymer membrane; α-Amylase; Immobilization; Starch hydrolysis; Enzyme kinetics; Enzyme reactor

1. Introduction

Starch is hydrolysed to glucose, maltose and dextrin by α- and β-amylases and other related enzymes. Specifically, α-amylase (EC 3.2.1.1; 1,4 α-D-glucan glucanohydrolase) catalyses the hydrolysis of α-1,4 glucosidic linkages 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 dextrin (Noda & Suda, 2001; Tanyolaç, Yürüksoy, & Özdural, 1998).

Starch hydrolyzates with a high-dextrose content are extensively used in the food industry and as a source of fermentable sugars. The industrial preparation of glucose syrups involves a preliminary starch sacchanfication to maltodextrin using α-amylase, followed by a second hydrolysis to glucose using glucoamylase. Amylases, which are starch hydrolysing enzymes and widely used for different processes in food, textile and pharmaceutical industries. *Bacillus subtilis, Bacillus amyloliquefacines, Bacillus licheniformis* and *Aspergillus oiyzae* are very important α-amylase sources because their enzymes are highly thermostable (Arica, Hasirci, & Alaeddinoğlu, 1995; Arruda & Vitole, 1999; Tanyolaç et al., 1998; Tien & Chiang, 1999; Tümtürk et al., 2000).

Industrial production of maltose is generally performed in a batch reaction, which is economically disadvantageous since the enzyme can be used only once. To allow enzyme reuse, enzyme immobilization is been currently used in various industries. Covalent attachment is

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an important immobilization method (for enzymes), which has been demonstrated to induce higher resistance to temperature, denaturants, and organic solvents in several cases. The extent of these improvements may depend on other conditions of the system, i.e. the nature of the enzyme, the type of support, and the method of immobilization (Anca et al., 1995; Boy, Dominik, & Voss 1999; Kotzlski & Staude, 1996; Yavuz, Bayramoglu, Kacar, Denizli, & Arica, 2001). In recent works, α-amylase has been covalently immobilized onto poly(hydroxyethylmethacrylate) and poly(methylmethacrylate-acrylic acid) microspheres and zirconium membranes (Arica et al., 1995; Sun, Chen, & Chu, 1999; Tien & Chiang, 1999; Tümtürk, Aksoy, & Hasirci, 2000). Physical and ion-exchange adsorption of α-amylase have also been carried out on a wide range of supports, such as nitrocellulose membrane and chitosan beads (Noda et al., 2001; Tanyolaç et al., 1998).

Epoxy supports are almost-ideal for very easy covalent immobilization of enzymes on both the laboratory and industrial scale. These activated supports are very stable during storage and when suspended in neutral aqueous media. The epoxy supports are also able to form very stable covalent linkages with different protein groups (amino, thiol, phenolic types) under very mild experimental conditions (e.g. pH 7.0) (Arica, 2000; Mateo, Abian, Fernandez-Lafuente, & Guisan, 2000).

The physical structure and chemical composition of support can also influence the microenvironment of the immobilized enzymes and consequently their biological properties (Arica, 2000; Chen, Kang, Neoh, & Tan, 2000). Enzymes are especially covalently immobilized onto polymeric supports for use in enzymatic degradation of large substrate molecules. The immobilization of enzymes onto membranes or membrane surfaces, however, offers a number of advantages over beaded supports, when operated in continuous systems, such as low pressure drop, short residence time and high operational stability with low external and internal diffusional resistances (Arica, 2000; Arica et al., 2000; Godjevargova, Konsulov, & Dimov, 1999).

Poly(hydroxyethylmethacrylate) poly(HEMA) is a hydrophilic and biocompatible synthetic polymer. It is also very stable against microbial contamination and resistant to attack by a large number of chemicals. The properties of poly(HEMA) can be modified in many ways, e.g., by copolymerisation or derivatisation. The functional epoxy groups could be introduced on the poly(HEMA) structure either by activation of functional –OH groups with epicholorohydrin or copolymerisation of HEMA with a epoxy group carrying comonomer (Arica et al., 1995).

In this study, poly(HEMA)-based supports carrying a reactive epoxy group were prepared in the membrane form by copolymerisation of 2-hydroxyethylmethacrylate (HEMA) and glycidyl methacrylate (GMA). These copolymer hydrogel membranes provide reactive epoxy groups for covalent biomolecule immobilization. In this way, the epoxy group could be readily introduced into copolymer membrane at a desired density by adjusting the concentration of epoxy group-carrying comonomer in the polymerisation mixture. Thus, the support activation procedure is eliminated for covalent biomolecule immobilization. To investigate the optimum processing conditions for preparing a high activity α-amylase-immobilized membrane, a series of poly-(HEMA-GMA-1-3) membranes with different HEMA/GMA ratios were prepared and were used for the immobilization of α-amylase. The relative activity, optimum pH and temperature, thermal and storage stabilities of the free and immobilized α-amylase were investigated. Finally, the immobilized enzyme system was applied to an enzyme reactor to study the behaviour of the enzyme in a continuous flow system.

2. Materials and methods

2.1. Materials

α-Amylase (1,4-α-D-glucan-glucanohydrolase; EC 3.2.1.1; From *B. licheniformis,* heat-stable), bovine serum albumin (BSA), starch, maltose and 3,5-dinitrosalicylic acid (DNSA) were all obtained from Sigma Chem. Co (St Louis, MO, USA). 2-Hydroxyethyl methacrylate (HEMA), glycidyl methacrylate (methacrylic acid 2,3 epoxypropyl isopropyl ether; GMA), isopropyl alcohol and α-α′-azoisobisbutyronitrile (AIBN) were obtained from Fluka AG (Switzerland). 2-HEMA was distilled under reduced pressure in the presence of hydroquinone and stored at 4 °C until use. All other chemicals were of analytical grade and were purchased from Merck AG (Germany).

2.2. Preparation of poly(2-hydroxyethylmethacrylate-co-glycidylmethacrylate) membrane

The poly(2-hydroxyethyl methacrylate-co-glycidyl methacrylate) poly(HEMA-GMA) hydrogel in the membrane form was prepared by UV-initiated photopolymerisation. The polymerisation was carried out in a round glass mould (diameter: 9.0 cm) at 25 °C under a nitrogen atmosphere for 1 h. To check the effect of monomer ratio on the α-amylase immobilization efficiency and recovered enzyme activity, in the initial polymerisation mixture three different HEMA/GMA molar ratios were used. 2-Hydroxyethyl methacrylate (1.4, 1.6 or 1.8 ml), glycidyl methacrylate (0.6, 0.4 or 0.2 ml), AIBN (10 mg), as polymerisation initiator, and, isopropyl alcohol (1.0 ml) as a monomer diluent, were mixed with phosphate buffer (2.0 ml, 0.1 M, pH 7.0). The resulting mixture was mixed and equilibrated at

25 °C for 15 min in a thermostatted waterbath. The mixture was then poured into the mould and exposed to long-wave ultraviolet radiation for 20 min. After polymerisation, the poly(HEMA-GMA) membranes were washed several times with distilled water and cut into circular pieces (diameter: 1.0 cm) with a perforator.

2.3. Characterisation of poly(HEMA-GMA) membranes

2.3.1. Swelling test

Water uptake ratios of the poly(HEMA-GMA-1-3) membranes were determined in distilled water. The experiment was carried out as follows: dry membranes sample were weighed before being placed in distilled water and allowed to soak for 48 h. The membranes were taken out from the water and were weighed after removing the excess water.

The swelling ratios of the membranes were calculated as follows:

$$\text{Swelling ratio } (\%w/w) = [(W_a - W_d)/W_d] \times 100 \qquad (1)$$

where W_s and W_d are the weights of swollen and dry membrane, respectively.

2.3.2. Scanning electron microscopy

Scanning electron micrographs of the dried poly (HEMA-GMA) membranes were obtained using a JEOL, JMS 5600 scanning electron microscope, after coating with gold under reduced pressure.

2.3.3. FTIR Spectra

The FTIR spectra of the poly(HEMA-GMA) membrane were obtained using an FTIR spectrophotometer (Shimadzu, FTIR 8000 Series, Japan). Poly(HEMA-GMA-2) membrane (0.1 g) and KBr (0.1 g) were thoroughly mixed and the mixture was pressed to form a tablet, and the spectrum was recorded.

2.4. Immobilization of α-amylase onto poly(HEMA-GMA) membranes

Functional epoxy group-carrying poly(HEMA-GMA) membrane disks (10 g, diameter 1.0 cm) were equilibrated in phosphate buffer (50 mM, pH 8.0) for 2 h, and transferred to the same fresh medium containing α-amylase (50 ml, 2.0 mg ml^{-1}). Immobilization of α-amylase on the poly(HEMA-GMA) membrane was carried out at 22 °C in a shaking water bath. To optimise the extent of enzyme immobilization, the coupling duration time was varied between 3 and 24 h. Physically-bound enzyme was removed first by washing the supports with saline solution (20 ml, 1.0 M) and then phosphate buffer (50 mM, pH 6.5) and this was stored at 4 °C in the same fresh buffer until used.

The amount of immobilized α-amylase on the poly (HEMA-GMA) membrane was determined by measuring the initial and final concentrations of protein within the immobilization medium using Coomassie Brilliant Blue as described by Bradford (1976). A calibration curve constructed with BSA solution of known concentration (0.05–0.50 mg $m1^{-1}$) was used in the calculation of protein in the enzyme and wash solutions.

2.5. Activity assays of free and immobilized α-amylase

The activities of both the free and the immobilized α-amylase preparations were determined by measuring the amount of reducing ends formed after enzymatic hydrolysis of starch in the medium according to a method described by Robyt and Whelan (1972).

The assay mixture (100 ml) contained sodium-potassium tartrate (25 g), NaOH (1.6 g) and DNSA (1.0 g) in distilled water. An aliquot (2.5 ml) and 0.1 ml of enzymatically-hydrolysed sample were mixed and then incubated in a boiling water for 10 mm. After cooling, distilled water (2.4 ml) was added and the intensity of the red colour developed was measured in a UV/Vis spectrophotometer (Shimadzu, Model 1601) at 530 nm. In each set of experiments, a standard curve was prepared with maltose solutions (0.1 ml) of different concentrations. Non-reducing ends of starch were determined, as described above, and used as blanks in the activity measurements. The results were converted to relative activities (percentage of the maximum activity obtained in that series). The residual activity was defined as the fraction of total hydrolytic activity recovered after covalent attachment on the poly (HEMA-GMA) membranes compared with the same quantity of free enzyme.

One unit of α-amylase activity is defined as the amount of enzyme, which produces reducing ends equal to 1.0 μmol maltose in 1 mm at 35 °C and pH 6.5.

To determine the pH and temperature profiles for the free and immobilized α-amylase activity, assays were carried out over the pH range of 4.0–8.0 and temperature range of 20–80 °C. The results of dependence of pH, temperature, storage and repeated use are presented in a normalized form with the highest value of each set being assigned the value of 100% activity. K_m and V_{max} values of the free enzyme were determined by measuring initial rates of the reaction with starch (5–30 g l^{-1}) in phosphate buffer (50 mM, pH 6.5) at 35 °C.

2.6. Immobilized α-amylase in a continuous system

The reactor (length 9.0 cm, diameter 2.0 cm, total volume 28 ml), was made from Pyrex glass. The enzyme-membrane was equilibrated in phosphate buffer (50 mM, pH 6.5) at 4 °C for 1 h. The enzyme-membrane poly(HEMA-GMA-2), wet weight 15.0 g, about 400 cm^2)

were loaded into the reactor, yielding a void volume of about 13.0 ml. To determine the effect of substrate concentration on reactor productivity, starch solution (5–30 g l^{-1}) in acetate buffer (50 mM, pH 5.5) was introduced to the reactor at a flow rate of 40 ml h^{-1} with a peristaltic pump (Cole Parmer, Model 7521-00, Miles, IL) though the lower inlet part. It was operated at 35 °C for 40 h and the solution leaving the reactor was collected in a fraction collector and assayed for α-amylase activity, as described above.

The performance of immobilized α-amylase in the reactor can be described under steady-state conditions by means of the integrated form of the Michaelis–Menten equation (Gacesa & Hubble, 1987).

$$-\mathrm{d}[R]/\mathrm{d}t = V_{\max}[R]/(K_{\mathrm{m}} + [R]) = V_{\max}/((K_{\mathrm{m}}/[R]) + 1) \quad (2)$$

where $[R]$ is the reactant concentration (gl^{-1}) in the reactor; $V_{\max}$ is the maximum rate of reaction (gl^{-1} s^{-1}); K_{m} is the Michaelis constant (gl^{-1}); and t is time (s).

For a bed reactor, an integrated equation can be written by replacing the reaction time with residence time, V_{tot}/Q (where V_{tot} is the reactor volume (l) and Q is the volumetric flow rate (l s^{-1}), the time that each fluid element spends in the reactor. The voidage of the reactor can be expressed as $\varepsilon = V_1/V_{\mathrm{tot}}$ V_1 is the volume of the enzyme membrane in the reactor (l), and Eq. (1) can be rearranged as:

$$V_{\max} \cdot V_{\mathrm{tot}} \cdot \varepsilon/Q = K_{\mathrm{m}} \cdot \ln([R_0]/[R]) + ([R_0] - [R]) \quad (3)$$

where $[R_0]$ is the reactant concentration in the feed (gl^{-1}). The equation for the enzyme reactor can be rearranged for the calculation of the kinetic constants from experimental data.

$$[R_0] \cdot X = K_{\mathrm{m}} \cdot \ln(1 - X) + V_{\max} \cdot V_{\mathrm{tot}} \cdot \varepsilon/Q \quad (4)$$

where X is defined as $([R_0] - [R])/[R_0]$.

Thus, plotting experimentally obtained values of $[R_0] - X$ vs $\ln(1 - X)$ will give a graph having a slope of K_{m} and an intercept of $V_{\max} \cdot V_{\mathrm{tot}} \cdot \varepsilon/Q$.

The effect of flow rate on reactor performance was studied by varying the flow rate in the range of 20–100 ml h^{-1} at 35 °C for 2 h, while keeping the concentration of starch at 20 gl^{-1} in phosphate buffer (50 mM, pH 6.5). To determine operational stability of the immobilized α-amylase, the reactor was operated at 35 °C for 120 h. The feed solution contained starch (20 gl^{-1}) in phosphate buffer (50 mM, pH 6.5), at 40 ml h^{-1}.

2.7. Storage stability

The activity of free and immobilized α-amylase, after storage in phosphate buffer (50 mM, pH 6.5) at 4 °C, was measured in a batch operation mode under the experimental conditions given above.

2.8. Thermal stability of free and immobilized enzyme

The thermal stabilities of free and immobilized α-amylase were determined by measuring the residual activity of the enzyme exposed to three different temperatures (60–80 °C) in phosphate buffer (50 mM, pH 6.5) for 2 h. After every 15 mm time interval, five membrane disks were removed and assayed for enzymatic activity, as described above. The first order inactivation rate constants, k_{i} were calculated from the equation:

$$\ln A = \ln A_0 - k_{\mathrm{i}} t \quad (5)$$

where A_0 is the initial activity and A is the activity after a time t (min).

3. Results and discussion

3.1. Properties of poly(HEMA-GMA) membrane

In the present study, a porous epoxide group-containing poly(HEMA-GMA) membrane was prepared by copolymerisation of HEMA and GMA via UV-initiated photopolymerisation in the presence of an initiator (AIBN). The most frequently used supports in enzyme technology for covalent enzyme immobilization have been obtained after activation of various natural and synthetic polymers. The present method was effective in that the reactive epoxy group was readily introduced into the matrix without any modification. The epoxide groups are very convenient for the covalent immobilization of proteins. The O–C and N–C bonds formed by the epoxide groups are extremely stable, so that the epoxy group-containing support could be used for the immobilization of enzymes and proteins (Fig. 1).

The functional poly(HEMA-GMA) membranes are hydrophilic matrices, i.e., hydro gels; therefore, they do not dissolve in aqueous media, but do swell, depending on the degree of cross-linking and on the hydrophilicity of the matrix. The water content is very important when use of matrix in enzyme immobilization is contemplated. The water content of the poly(HEMA-GMA) membranes is presented in Table 1. It is observed that the swelling ratio of the membranes decrease as the ratio of co monomer (GMA) in the membrane structure increase.

Scanning electron microscopy (SEM) micrographs, presented in Fig. 2(A and B), show the surface and cross-sectional structure of the poly(HEMA-GMA) membrane, respectively. The membrane shows a porous surface and bulk structure, which could provide a large area for immobilization of enzyme.

The copolymer is composed of HEMA and GMA units as proven by FTIR-spectroscopy. The FTIR spectra of poly(HEMA-GMA) have the characteristic stretching vibration band of the hydrogen-bonded alco-

hol of HEMA at ~3500 cm^{-1}. Among the characteristic vibrations of both HEMA and GMA are the methylene vibration at ~2930 cm^{-1} and the methyl vibration at 2960 cm^{-1}. The vibration at 1740 cm^{-1} represents the ester configurations of both HEMA and GMA. The epoxide group gives the band at 910 cm^{-1}.

3.2. Effect of epoxy group density and coupling time on the immobilization efficiency

The effects of HEMA-GMA mole ratio on the immobilization capacity and on α-amylase activity are presented in Table 1. An increase in the GMA ratio led to an increase in the immobilization efficiency from 51 to 109 μg protein cm^{-2} membrane and 70–80% of activity retained upon immobilization. The poly(-HEMA-GMA-3) membrane has a maximum epoxy group density (containing 1.75 mmol GMA g^{-1} membrane) and resulting in a higher enzyme immobilization capacity with a decrease in the recovered enzyme activity. It should be noted that, α-amylase is immobilized either at the external surface or within the pore space of the support, and thus immobilized α-amylase on the surface could be more readily available to the large substrate molecule, starch, than when present in the pore space. As the loading increases, this renders the enzyme immobilized within the pore of the membrane useless because the large substrate cannot diffuse easily through the blocked pore mount to the internal portion of the porous membrane. As previously reported, β-amylase acts on large substrates of low diffusivity, so when immobilized in high concentration it may be

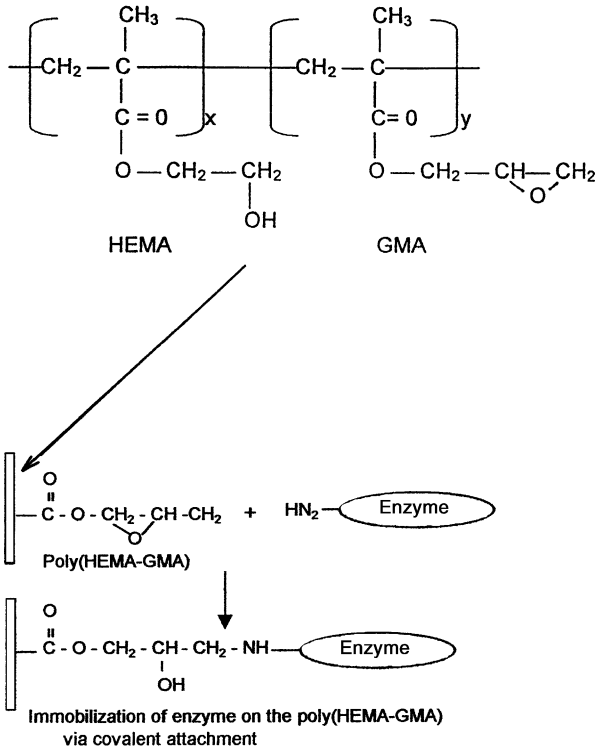

Fig. 1. Covalent immobilization of biomolecules on the poly(HEMA-GMA) membrane.

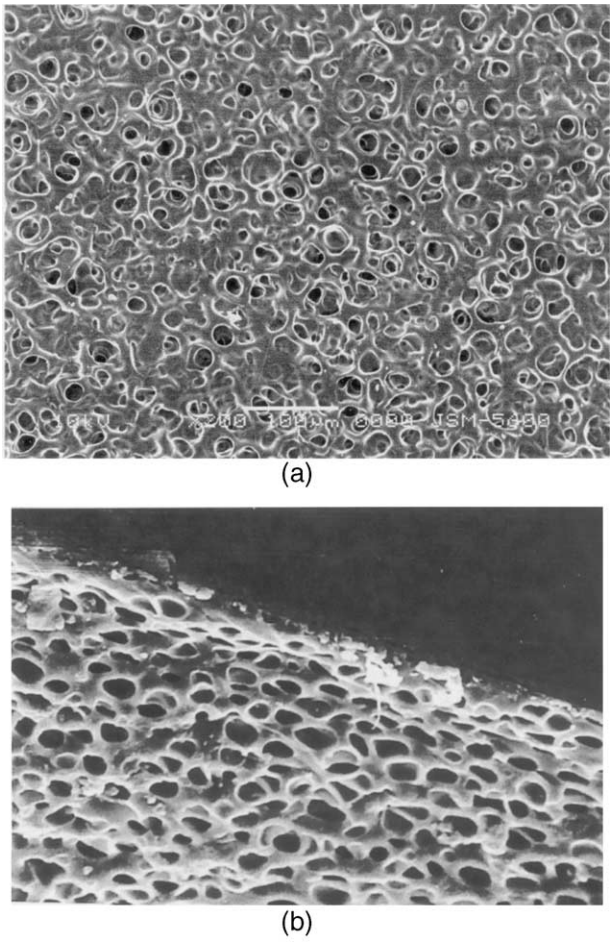

Fig. 2. SEM micrographs of the poly(HEMA-GMA) membrane: (a) surface; (b) cross-section.

Table 1
Composition of the poly(HEMA-GMA) membranes and activities of the immobilized α-amylase obtained with these membrane compositions

Membrane type	HEMA/GMA (mole ratio)	GMA density (mmol g^{-1} membrane)	Water content (%)	Immobilised enzyme (μg cm^{-2})	Enzyme activity (U cm^{-1})	Activity retention (%)
Poly(HEMA-GMA-1)	7.65	0.58	54	51	61.5	76
Poty(HEMA-GMA-2)	3.41	1.16	49	89	77.9	73
Poty(HEMA-GMA-3)	1.98	1.75	45	96	77.2	67

limited by slow intraparticle substrate diffusion. For the immobilized amylases, the porosity of the support is not critical to efficiency of hydrolysis rate since the starch or dextrin molecules are too large to penetrate the pore space of the matrix (Lee, Lee & Reilly, 1980).

As observed in Fig. 3, an increase in the coupling time led to increase in extent of immobilization, but this relation levelled of after 18 h. Thus, a maximum enzyme loading of 83 μg cm^{-2} was observed. No enzyme leakage was observed during washing of the freshly prepared poly(HEMA-GMA-2) membrane or during continuous or batch operation mode. This indicates that the immobilization process was irreversible.

Several publications have appeared describing the investigation of new supports for α-amylase immobilization, for example, α-amylase covalently immobilized onto poly(methylmethacrylate-acrylic acid) microspheres. The amount of enzyme immobilized on 1.0 g of microspheres was about 4 mg and immobilized enzyme retained about 65% of its initial activity (Aksoy, Tumturk & Hasirci, 1998). Tien and Chiang (1999) used a zirconium dynamic membrane for covalent immobilization of α-amylase. The immobilized α-amylase activity was about 59.8 U g^{-1} wet gel. A thermostable α-amylase was immobilized onto a nitrocellulose membrane and the maximum enzyme immobilization yield was around 21% (Tanyolac et al., 1998). α-Amylase was immobilized in thermal-responsive composite hydrogel membranes, and the immobilized α-amylase was retained about 33% of its initial activity (Sun et al., 1999). Sweet potato β-amylase was immobilized on chitosan beads and the beads exhibited an activity of 142 U g^{-1} (Noda et al., 2001). The α-amylase was covalently immobilized onto poly(HEMA) microspheres. The immobilized α-amylase activity was 390 U g^{-1} of support. The reactive poly(HEMA-GMA) membranes gave a good loading of α-amylase that retains high specific activity after immobilization when compared to other immobilized α-amylase systems. The highest enzyme activity observed (77.9 U cm^{-2}) with the present novel poty(HEMA-GMA-2) membrane support appears to be quite promising.

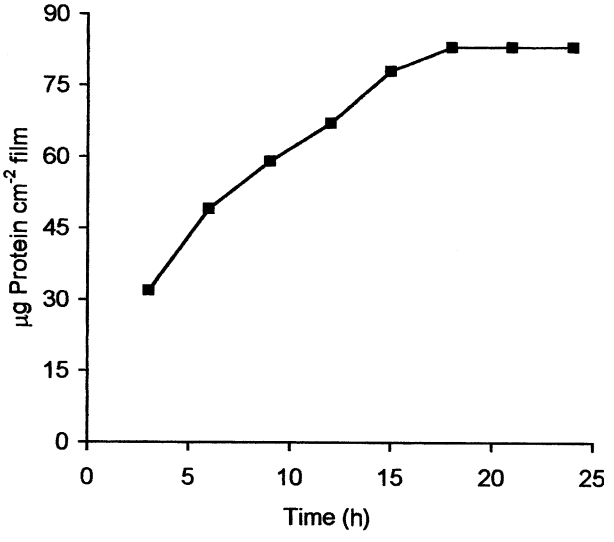

Fig. 3. Effect of coupling time on the immobilization efficiency of α-amylase on the poly(HEMA-GMA) membrane.

3.3. Kinetic constants

Kinetic parameters, the Michaelis constant K_m and the maximum activity V_{max} for free and the immobilized α-amylase were determined using soluble starch as substrate. The K_m value for free enzyme, estimated from the Lineweaver–Burk plot, was 6.7 gl^{-1}, whereas V_{max} value was 1285 U per mg protein for starch. Kinetic parameters of the covalently bound α-amylase were determined in the continuous system (plotted according to Eq. 4). The K_m for immobilized enzyme was 15.7 g l^{-1}, approximately 2.3-fold higher than that of the free enzyme and V_{max} value was calculated as 1015 U mg^{-1} bound protein. In general, the K_m of an immobilized enzyme is different from that of the free enzyme due to diffusional limitations, steric effects and ionic strength (Bayramoglu, Denizli, & Arica, 2002). The change in the affinity of the enzyme for its substrate is also caused by structural changes in the enzyme introduced by the immobilization procedure and by lower accessibility of the substrate to the active site of the immobilized enzyme (Arica et al., 1995). However, K_m and V_{max} values of the free and immobilized α-amylase for starch are of the same order of magnitude. This indicates that the catalytic function of α-amylase was not very much impaired by this immobilization method.

3.4. Effect of flow rate on the reactor performance

Eqs. (3) and (4) reveal that an increase in flow rate would lead to decrease in the starch hydrolysis rate, where the residence time is inversely proportional to flow rate. As can be seen in Fig. 4, when the residence time is increased, as expected, the efficiency of starch degradation is also increased. This effect is not linear and further increase in residence time does not yield a higher degree of hydrolysis. As previously reported, this could be a result of either substrate depletion in the reaction medium or possible interference with the enzyme activity at lowest substrate concentration by the product membrane diffusion limitation (Arica et al., 1995).

3.5. Effects of pH and temperature on the catalytic activity

The effect of pH on the activity of the free and immobilized α-amylase for starch hydrolysis was examined in the pH range 4.0–8.0 at 35 °C. The reactions were carried out in acetate and phosphate buffers and

the results are presented in Fig. 5. After immobilization, the optimal pH for starch hydrolysis did not change (pH 6.5). On the other hand, the pH profile of the immobilized enzyme was broader than that of the free enzyme, which means that this method preserves the enzyme activity in a wider pH range. Similar observations, for immobilization of α-amylase and other enzymes, have been reported by other researchers (Chen et al., 1997; He, Cai, Wei, Nie, & Yao, 2000; Ida, Matsuyama, & Yamamoto, 2000; Tanyolac et al., 1998).

The activities obtained in a temperature range of 20–80 °C were expressed as a percentage of the maximum activity. In the resultant bell-shaped curve, the free and the immobilized enzymes activities are retained above 50 °C. The optimum reaction temperature for the free and immobilized α-amylases were 50 °C and 60 °C, respectively, and the temperature profile of the immobilized enzyme was slightly broader than that of the free one. The increase in optimum temperature was caused by the changing physical and chemical properties of the enzyme. The covalent bond formation, via amino groups of the immobilized α-amylase, might also reduce the conformational flexibility and may result in higher activation energy for the molecule to reorganize the proper conformation for binding to the substrate. One of the main reasons for enzyme immobilization is the anticipated increase in its stability to various deactivating forces, due to restricted conformational mobility of the molecules following immobilization (Arica et al., 1995; Bayramoglu et al., 2003; Tien & Chiang, 1999). These results indicated that the covalent immobilization of α-amylase on the poly(HEMA-GMA2) membrane strengthens the enzyme structure via multi-point attachments (Yavuz et al., 2001).

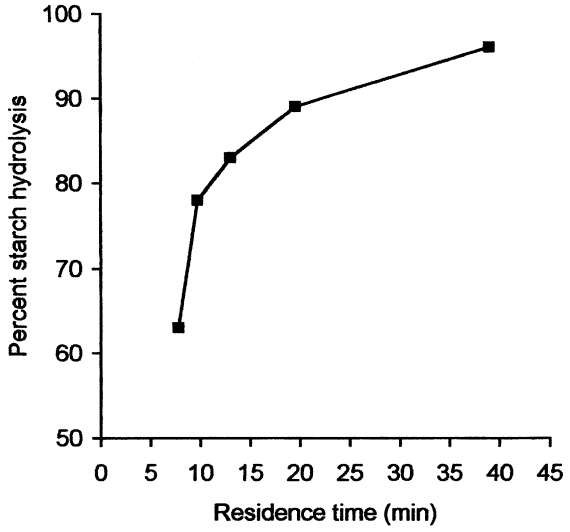

Fig. 4. Effect of residence time on the starch degradation rate in the continuous system.

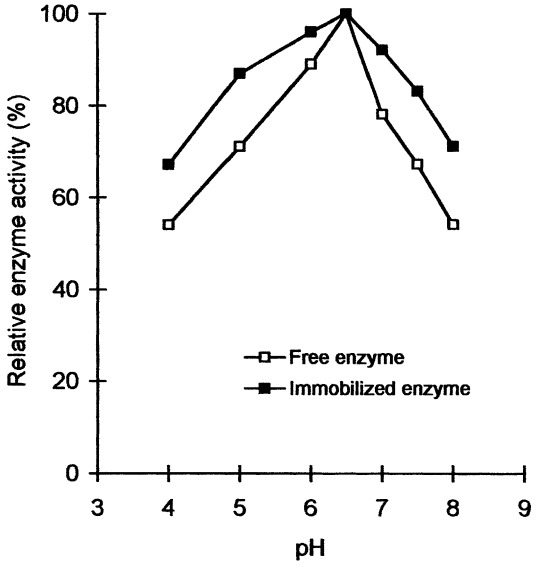

Fig. 5. pH profiles of the free and immobilized α-amylase.

3.6. Thermal stability

Thermal stability experiments were carried out with free and immobilized enzymes, which were incubated in the absence of substrate at various temperatures. The immobilized α-amylase preserved its all-initial activity at 60 °C and free enzyme retains about 71% of its initial activity during a 120 min incubation period. At 70 °C the immobilized and free enzymes retained their activity about to a level of 85 and 41%, respectively. The immobilized heat-stable α-amylase was inactivated at a much slower rate than that of the native form. Both the free and immobilized enzymes lost their initial activity at 80 °C after 45 min and 75 min treatments, respectively. The half-life values at 70 and 80 °C were determined for the free enzyme as 102 and 16 min; these were 400 and 34 min for the immobilized enzyme, respectively. The thermal inactivation rate constants (k_i) were calculated as 7.42×10^{-2} and 8.03×10^{-1} for free, and 1.35×10^{-1} and 3.53×10^{-2} for the immobilized α-amylase at 70 and 80 °C, respectively. These results suggest that the thermostability of immobilized α-amylase increased considerably as a result of covalent immobilization onto poly(HEMA-GMA-2). The activity of the immobilized enzyme, especially in a covalentty bound system, is more resistant than that of the soluble form against heat and denaturing agents. If the thermal stability of an enzyme were enhanced by immobilization, the potential utilization of such enzymes would be extensive. In principle, the thermal stability of an immobilized enzyme can be enhanced, diminished, or unchanged relative to free counterparts, and several examples of each kind have previously been reported (Arica, 2000; Chen & Liao, 2002; Tien & Chiang, 1999).

3.7. Operational stability of α-amylase in the continuous system

It is important for economical use of an enzyme, as a means for the mass production of the desired product, that the enzyme reaction is continuous. One of the problems in continuous enzyme reactions is the operational stability of the enzyme immobilized on the support.

The operational stability of covalently linked α-amylase in the packed bed reactor was monitored at 35 °C for 120 h. During the initial 95 h of continuous operation, the immobilized enzyme preserved all of its initial activity. After this time, a small decrease in enzyme activity is observed with time. After 120 h, the immobilized enzyme lost about 4% of its initial activity, possibly resulting from the inactivation of α-amylase upon use. The operational inactivation rate constant of the immobilized α-amylase at 35 °C with 20 g l^{-1} starch was calculated as $k_{opi} = 8.06 \times 10^{-1}$ min^{-1}.

3.8. Storage stability

In general, if an enzyme is in solution, it is not stable during storage, and the activity is gradually reduced. The free and the immobilized enzymes preserved all their initial activity during the first week of the storage period, followed by a gradual decrease over time. The free enzyme lost all its activity within five weeks. For the immobilized enzyme, there was a slight decrease in the activity during the same storage period (about 16%) and the immobilized enzyme preserved about 71% of its initial activity during a 2 month storage period. This decrease in activity is explained as a time-dependent natural loss in enzyme activity.

4. Conclusion

In this study, reactive poly(HEMA-GMA) membranes were prepared and used as supports for the immobilization of α-amylase. Among the advantages of its application are: (1) easy immobilization protocol, which obviates the need for a pre-activation step; (2) the epoxy group density could be easily adjusted to a required level by changing the monomer/co monomer ratio; (3) it also has a high immobilization capacity, comparable with previously reported membranes or beads supports; (iv) it has good mechanical stability for various biotechnological applications such as being part of an enzyme electrode or enzyme reactor. The optimum pH and temperature profile of the immobilized enzyme were not drastically modified upon immobilization. More importantly, the immobilized α-amylase showed higher thermostability than the free counterpart. The enzyme-membrane was used continuously for hydrolysis of starch, with a 4% initial activity loss during continuous operation in the enzyme reactor for 120 h. The poly(HEMA-GMA) membrane has desirable properties, and could be used in bioactive macromolecule immobilization. A high operational stability obtained with the immobilized enzyme indicates that the immobilized α-amylase could successfully be used in a continuous system for the production of maltose and dextrin from starch.

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