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ORIGINAL CONTRIBUTION

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Immobilization of α-amylase in vinyl-polymer-based interpenetrating polymer networks

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Abstract Semi-interpenetrating polymer networks (IPNs) of poly(ethylene glycol), poly(vinyl alcohol) and polyacrylamide were prepared as a support for enzyme immobilization and kinetic studies were performed for the immobilization of α-amylase. The effect of IPN composition on the extent of immobilization was investigated and the percentage of relative activity of the immobilized enzyme was evaluated as a function of the chemical architecture of the IPNs, pH and temperature, taking starch as a substrate. The kinetic constants and the maximum reaction velocity were also evaluated. The IPNs were characterized by IR spectral analysis.

Keywords Interpenetrating polymer network · α-Amylase · Immobilization · Kinetics

Introduction

A wide range of bioactive molecules, like proteins, peptides and enzymes, are commercially available as drugs. Many of these are stable only under physiological conditions and their therapeutic application is limited by their short half lives in vivo. Pulsed or self-regulated release from a polymer matrix may enhance the therapeutic effectiveness of the drug with a prolonged action: however, this is difficult to achieve owing to the complex molecular properties of macrodrugs compared to those of conventional low-molecular-weight drugs.

A significant aspect of great concern is the entrapment of enzymes into the polymeric matrices, which also provides a technique of enzyme immobilization. The main purpose of immobilizing enzymes lies in the economic application of enzymes in various industrial and technological processes. Moreover, the recovery yield and reusability of free enzymes as industrial catalysts are quite limited and, hence, attention has been paid to enzyme immobilization [1]. The choice of the support and the method of immobilization play a crucial role in governing enzymatic reactions. Suitable matrices include hydrogels that are highly biocompatible for immobilization of enzymes owing to their hydrophilic nature. Enzymes are usually immobilized by chemical and physical means to increase enzyme stability and to enable long-term operation [2, 3, 4]. Furthermore, interest in the immobilized enzymes and their application to bioprocessing [5, 6], the analytical system [7] and enzyme therapy [8] has steadily grown in the past decade. Another important aspect to use immobilized enzymes rests with the convenient handling of enzyme preparations which, in turn, are targeted owing to two benefits: easy separation of enzymes from products and reuse of the enzymes. Easy separation of enzymes from products simplifies enzyme application and permits reliable and efficient reaction technology. Enzyme reuse provides a number of cost advantages which are often essential prerequisites for estimation and economically viable enzyme catalyzed processes.

In the recent past, many approaches to the preparation of water-soluble and insoluble enzymes have been explored to study enzyme reactions and their immobilization on polymeric surfaces. Huang et al. [9] studied immobilization of lipase on partially porous poly(styrene–divinyl benzene) particles. The immobilized lipase on poly(styrene–divinyl benzene) retained less activity after the first stage of deactivation, suggesting a change in the confirmation of the enzyme molecule by immobilization. Nakane et al. [10] immobilized invertase on the composite gel fiber of cellulose acetate and zirconium alkoxide and observed that the activity for the immobilized invertase became higher with increasing fiber diameter. Arica et al. [11] studied the immobilization of catalase in poly(isopropylacrylamide– *co* –hydroxyethyl methacrylate) thermally reversible hydrogels and observed that upon entrapment, the activity retention of catalase decreased and that increasing the catalase loading of hydrogel adversely affected the activity.

The previous discussion and a thorough survey of the literature reveal that a great deal of work has been performed on the immobilization of various enzymes on polymeric supports but the study of immobilization of α-amylase has been reported less. Hence, the present article reports the results of the immobilization of α-amylase on a polymeric matrix comprising poly(vinyl alcohol) (PVA), poly(ethylene glycol) (PEG) and cross-linked polyacrylamide.

Among various synthetic polymers used in designing hydrogels of desired merits, PEGs have been the focus of interest in the biotechnical and biomedical communities [12, 13]. Primarily this is because PEG is unusually effective at excluding other polymers when in an aqueous environment. This property translates into protein rejection, the formation of two-phase systems with other polymers, nonimmunogenicity and nonantigenicity. In addition, the polymer is nontoxic and does not harm active proteins or cells although it interacts with cell membranes.

The use of PVA as one of the components of the enzyme support lies in the fact that it is a hydrophilic and good-film forming polymer and that it enhances the mechanical strength of the polymer matrix. Similarly, polyacrylamide being nonionic in nature provides a neutral environment for enzyme immobilization so that the enzymes could display greater stability and reactivity.

Experimental

Materials

PEG (molecular weight 600) was obtained from Wilson Laboratories, Mumbai, India, and was used as received. PVA (hot processed, 98.7% hydrolyzed, molecular weight 30,000) was obtained from Burgoyne Burbidges and Co., India, and was used without any pretreatment. Acrylamide (Research Lab, Poona, India) was crystallized twice from methanol (general reagent) and dried under vacuum over anhydrous silica for 1 week. *N*, *N* ′-Methylene bisacrylamide (MBA) (Central Drug House, Mumbai, India) was employed as a cross-linking agent, while potassium persulfate (Loba Chemie, India) was used as a polymerization initiator. α-Amylase was obtained in powdered form (Research Laboratory, Mumbai) with an activity of 1,300 IU/g.

Method

Preparation of interpenetrating polymer networks

Highly biocompatible interpenetrating polymer networks (IPNs) of varying compositions for immobilizing α-amylase were prepared by free-radical polymerization as described in our earlier communications [14]. In brief, into a Petri dish (0.10-m diameter, Corning) were added PVA (5×10^{-4}–15×10^{-4} kg), acrylamide (10–28 mM), PEG (2.2×10^{-4}–13.2×10^{-4} kg), MBA (0.006–0.038 mM), potassium persulfate (0.003 mM) and water (1.1 M). The mixture (0.002 dm^3) was homogenized and kept at 353 K for 4 h so that the whole mass converted into thin white circular films. The films were cut into preweighed pieces of equal dimensions (0.01×0.01 m) and equilibrated with bidistilled water for 1 week. The swollen hydrogel pieces were then dried at room temperature for 72 h and weighed again. The process was continued till the constant weight of the IPNs was obtained that clearly assures the complete removal of unreacted chemicals and monomers from the IPNs. The compositions of the IPNs are presented in Table 1.

Immobilization of α-amylase

α-Amylase molecules were entrapped into the IPN by allowing the preweighed dry pieces of the IPNs to swell in 0.005 dm^3 enzyme solution (0.3% w/v) till equilibrium. The swollen pieces were dried at room temperature and stored in airtight containers.

Evaluation of activity

The activity of α-amylase was estimated by the hydrolysis of starch with the enzyme and subsequent determination of the residual starch with iodine [15]. Hydrochloric acid (1 N) was used to stop the enzyme reaction. The reducing sugar thus obtained was further diluted and an aliquot (0.005 dm^3) was taken for final estimation by recording its absorbance at 620 nm (Systronics, model 106, India). One unit of α-amylase activity was defined as the amount of enzyme catalyzing 1×10^{-6} kg maltose from starch in 3 h at pH 6.9 and 293 K. The percentage residual activity was determined as described in the following [16].

Three different reaction mixtures A, B and C were prepared for enzyme assay using starch as the substrate. Reaction mixture A consisted of the enzyme along with the substrate starch in the free state, whereas reaction mixtures B and C were made up of immobilized α-amylase but the extent of immobilization was greater in mixture B since the contact time of the solid polymeric surface with the enzyme was greater. The activity of the immobilized enzyme was expressed as residual activity in percent based on that of the free enzyme:

$$\text{Percentage residual activity} = \frac{B - C}{A - C} \times 100, \quad (1)$$

where *A* constituted the free enzyme; *B* and *C* constituted immobilized enzyme with the extent of immobilization being greater in *B*.

Table 1 Network parameters of the interpenetrating polymer networks of different compositions

Poly (ethylene glycol) ($kg \times 10^3$)	Poly (vinyl alcohol) ($kg \times 10^3$)	Acrylamide (mM)	*N*, *N* ′-Methylene bisacrylamide (mM)	Potassium persulfate (mM)	M_c	$q \times 10^3$	$V_e \times 10^{-20}$	Amount of α-amylase immobilized ($kg \times 10^3$)
0.22	0.75	14	0.006	0.003	7,664	9.2	0.75	14.0
0.88	0.75	14	0.006	0.003	3,602	19.7	1.60	20.0
1.32	0.75	14	0.006	0.003	3,001	21.8	1.82	30.0
0.55	0.50	14	0.006	0.003	5,517	12.8	1.04	20.0
0.55	1.0	14	0.006	0.003	5,400	16.8	1.82	24.0
0.55	1.5	14	0.006	0.003	2,124	33.4	2.72	28.0
0.55	0.75	10	0.006	0.003	7,525	9.4	0.76	19.0
0.55	0.75	21	0.006	0.003	7,369	9.6	0.79	25.2
0.55	0.75	28	0.006	0.003	3,602	19.7	1.60	27.0
0.55	0.75	14	0.012	0.003	4,075	17.4	1.42	22.0
0.55	0.75	14	0.025	0.003	3,602	19.7	1.60	19.5
0.55	0.75	14	0.038	0.003	2,780	25.5	2.08	17.0

The residual activities obtained for various compositions of the IPNs are summarized in Table 1.

IR spectra

IR spectra of the α-amylase immobilized polymeric support were recorded using a Fourier transform IR spectrophotometer (PerkinElmer).

Results and discussion

Characterization of IPN

Prior to discussing the results obtained, it is worth characterizing the structure of the IPN.

IR spectral analysis

The IR spectra of nonimmobilized and immobilized IPNs are shown in Fig. 1. Spectrum a clearly marks the presence of hydroxyls of alcohol at 3,650 cm^{-1} (O–H stretching), an amide group at 3,424 cm^{-1} (N–H stretching), 1,596 cm^{-1} (N–H bending), and at 1,656 cm^{-1} C = O stretching. In addition to these peaks, the IR spectrum also confirms the presence of PEG in the semi-IPN as evident from the observed absorption bands at 1,351 cm^{-1} (interaction between O–H bending and C–O stretching), and 1,026 cm^{-1} (asymmetric C–O–C stretching). The spectrum also contains characteristic vibrational modes at 699 and 761 cm^{-1}, which are due to out of plane C–H bending vibrations.

α-Amylase contains a major percentage of aspartic and glutamic acids [17] along with other amino acids. Thus, the IR spectra of α-amylase immobilized IPN, as shown in Fig. 1, spectrum b, clearly indicates the presence of carboxylate ion groups at 1,648 cm^{-1} [asymmetrical $(C–O)_2$ stretch] and 1,397 cm^{-1} [symmetrical $(C–O)_2$ stretch], respectively. In addition to these bends, the other observed peaks are the asymmetrical $(–NH_3)^+$ N–H band at 1,617 cm^{-1}, and the symmetrical $(–NH_3{}^+)$N–H band at 1,507 cm^{-1}. Thus, spectrum b also presents evidence of the immobilization of α-amylase on the IPN.

Network studies

The cross-linked polymers are best characterized by an important structural parameter M_c, the average molar mass between cross-links, which is directly related to cross-link density. The magnitude of M_c affects the physical and mechanical properties of cross-linked polymers and its determination has a wide range of practical significance. Equilibrium swelling is widely used to determine M_c. Early research by Flory and Rehner laid the foundation for the analysis of equilibrium swelling. According to the theory of Flory and Rehner, for a perfect network

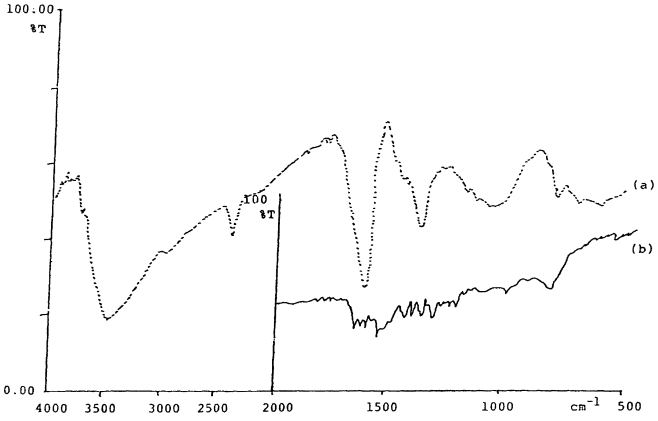

Fig. 1 IR spectra of (*a*) nonimmobilized and (*b*) immobilized interpenetrating polymer networks (*IPNs*)

$$M_c = -V_1 d_p \frac{\left(V_s^{1/2} - V_s/2\right)}{\ln(1 - V_s) + V_s + \chi V_s^2}, \quad (2)$$

where M_c is the number-average molar mass of the chain between cross-links, V_1 is the molar volume, d_p is the polymer density (grams per milliliter), V_s is the volume fraction of polymer in the swollen gel, and χ is the Flory–Huggins interaction parameter between solvent and polymer [18].

The swelling ratio is equal to $1/V_s$. Here, the cross-link density, q, is defined as the mole fraction of cross-linked units:

$$q = M_o/M_c, \quad (3)$$

where M_o is the molar mass of the repeating unit.

Some other authors defined a cross-link density, V_e, as the number of elastically effective chains, totally included in a perfect network, per unit volume. V_e is simply related to q since

$$V_e = d_A N_A / M_c. \quad (4)$$

The values of V_1, d_p and χ were taken from related literature [19, 20]. The values of M_c, q and V_e of the networks were calculated and are summarized in Table 1 for various compositions of the IPNs.

Effect of composition of the IPN on immobilization

The amount of the enzyme immobilized onto the three-dimensional IPN matrix depends mainly on the potential of the IPN to imbibe and retain the enzyme molecules when placed in its reservoir. In other words, the loading of enzyme is regulated by the extent of the swelling, which, in turn, depends on the chemical architecture of the network.

IPNs of different compositions were prepared by varying the amounts of PEG, PVA, acrylamide and MBA in the feed mixture and the enzyme was loaded by equilibrating the IPNs in the enzyme reservoir. The following discussion clearly reveals that the chemical architecture of the IPN has a pronounced effect on the percentage loading of the α-amylase. The results obtained are summarized in Table 2 and may be interpreted as follows.

PEG and PVA are nonionic and hydrophilic polymers. When their concentrations in the feed composition are increased in the ranges 2.2×10^{-4}–13.2×10^{-4} and 5×10^{-4}–15×10^{-4} kg, respectively, the amount of immobilized α-amylase is found to increase. The observed increase in immobilization is due to the increased amounts of PEG and PVA in the IPNs causing them to swell more and more in the enzyme solution and consequently the amount immobilized α-amylase increases.

Table 2 K_m and V_{max} of free and immobilized α-amylase

Sample	K_m	V_{max}
Free enzyme	2.77	2.63
Immobilized enzyme	5	2.38

A similar trend is noticed when the hydrophilic monomer acrylamide increases in the feed mixture in the range 10–28 mM. The results may be explained by the fact that increasing concentration of acrylamide increases the mesh sizes of the gel to such an extent that it favors the movement of water and enzyme molecules into the IPN and hence the percentage loading increases.

On varying the concentration of cross-linker (MBA) in the range 0.006–0.38 mM in the feed mixture of the IPNs, there was a significant fall in the swelling of the IPNs and, therefore, the amount immobilized decreases. The observed fall may be explained by the fact that on increasing the cross-linker content there is a prominent decrease in the mesh sizes of the free volumes available between the chains of the macromolecular network and thus the immobilization decreases.

Effect of the nature of the polymeric carrier on enzyme activity

The nature of the macromolecular matrix entrapping the enzyme within its network also affects enzyme activity. The individual effect of the carrier components on the percentage relative activity of α-amylase may be explained as follows.

Effect of PVA

PVA, being a hydrophilic polymer, has been found to have a profound effect on the relative activity of α-amylase when varied in the range 5×10^{-4}–15×10^{-4} kg in the feed mixture of the hydrogel. The results are shown in Fig. 2, which clearly indicates that initially the relative activity increases up to 10×10^{-4} kg PVA, while beyond it, a fall in the activity of α-amylase is noticed. The results obtained could be explained by the fact that with increasing PVA in the matrix the hydrophilicity also increases, which results in an increased swelling of the carrier. This obviously gives rise to widening of pores of the network and so the substrate (starch) molecules could easily penetrate and approach the active sites of the entrapped enzyme, thus resulting in an increase in the relative activity of the α-amylase. Another consequence of greater swelling is that the available surface area for the substrate

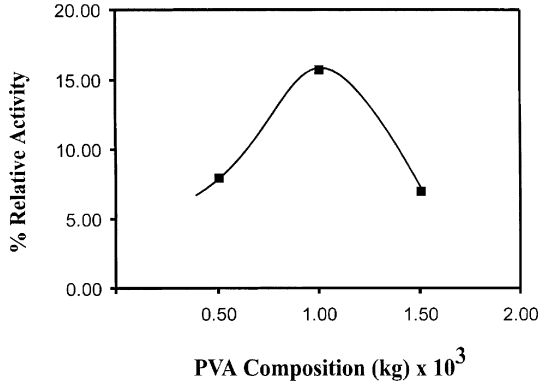

Fig. 2 Variation in percentage relative activity of α-amylase with poly(vinyl alcohol) (*PVA*) content of the IPNs at fixed concentrations of poly(ethylene glycol) (*PEG*) (0.55×10^{-3} kg), acrylamide (*AM*) (14 mM), *N*, *N*′-methylene bisacrylamide (*MBA*) (0.006 mM) and potassium persulfate (*KPS*) (0.003 mM)

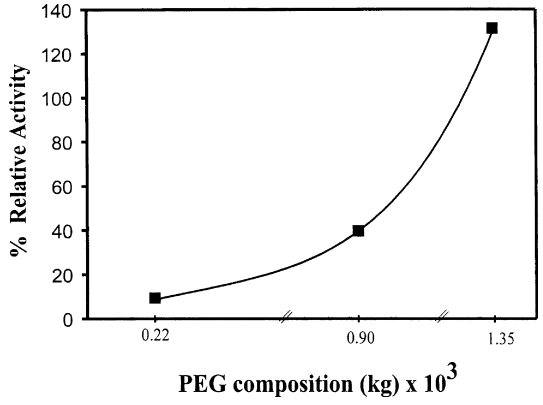

Fig. 3 Variation in percentage relative activity of α-amylase with PEG content of the IPNs at fixed [PVA] = 0.75×10^{-3} kg, [AM] = 14 mM, [MBA] = 0.006 mM and [KPS] = 0.003 mM

diffusion was also higher owing to the swollen gel volume. These two factors provide a high rate of substrate diffusion through the hydrogel carrier according to Fick's law and, therefore, resulted in an increased relative activity. Moreover, it is also likely that the entrapped enzyme molecules are released and react with starch molecules. However, beyond 10×10^{-4} kg PVA, the relative activity decreased because the hydrogel acquired a high density of macromolecular chains at high PVA content and, therefore, this resulted in a hindered diffusion of substrate molecules into the network. This clearly brings about a fall in the relative activity of α-amylase.

Effect of PEG

When the amount of PEG is varied in the feed mixture of the IPN in the range 2.2×10^{-4}–13.2×10^{-4} kg, the percentage relative activity of α-amylase is found to increase as shown in Fig. 3. The observed increase in relative activity is attributed to the fact that increasing PEG in the IPN imparts greater hydrophilicity to the gel, which because of large swelling opens the pores of the network for diffusion of substrate molecules into the gel and that of α-amylase molecules into the bulk. Therefore, the effective diffusion coefficient of substrate becomes higher owing to lower internal mass transfer resistance. This obviously enhances the percentage relative activity of the enzyme.

Effect of acrylamide

Variation of acrylamide in the feed mixture of the IPN in the range 10–28 mM brings about a constant fall in the relative activity of the immobilized enzyme as shown in Fig. 4. The observed decrease in relative activity may be due to the fact that with increasing acrylamide in the IPN, the mesh sizes of the gel increase and so the movement of water and enzyme molecules into the IPN is facilitated and subsequently a greater loading results (Table 1). Now, because of the greater loaded network the pores of the IPN shrink owing to which diffusion of substrate molecules into the gel becomes restricted. This obviously results in a fall in the percentage relative activity of the immobilized enzyme.

Effect of cross-linker

The cross-linking agent employed in the IPN preparation was MBA. When the MBA content is varied in the feed mixture of the network in the range 0.006–0.038 mM, a steep increase in the percentage relative activity is observed (Fig. 5), which is quite unexpected. The observed increase in relative activity with increasing cross-linker content may be explained by the

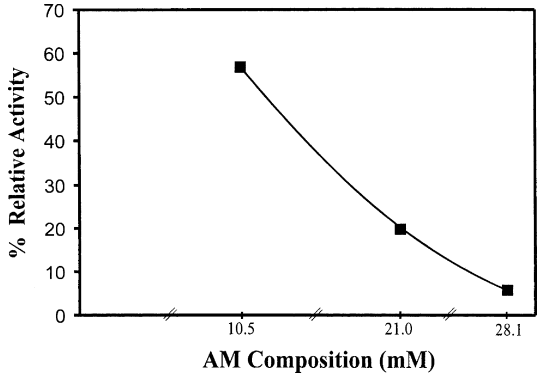

Fig. 4 Variation in percentage relative activity of α-amylase with AM content of the IPNs at fixed [PVA] = 0.75×10^{-3} kg, [PEG] = 0.55 g, [MBA] = 0.006 mM and [KPS] = 0.003 mM

fact that at higher cross-link density the pores of the IPN are narrow and, therefore, will allow less amylase for immobilization. Thus, because of the lower loading of the enzyme, the pores of the IPN will not be saturated and as a consequence the penetration of substrate molecules will be facilitated, which then will result in an enhanced percentage relative activity of the enzyme. Similar types of results have also been reported elsewhere [21].

Determination of Michaelis constant

It seems interesting to analyze the enzymic hydrolysis with immobilized α-amylase in the framework of the Michaelis–Menten mechanism. The kinetic parameters, the Michaelis constant K_m and V_{max} for free and immobilized α-amylase, were determined by varying the concentration of starch in the reaction medium. Lineweaver–Burk plots for free and immobilized α-amylase are shown in Fig. 6. The kinetic parameters of free and immobilized enzymes are summarized in Table 2. The apparent K_m values of the immobilized α-amylase were higher than those of free α-amylase. This may be caused by the limitation of diffusion resistance. On the other hand, the V_{max} values follow the opposite trend, suggesting the residual activity of the immobilized α-amylase decreased in the course of entrapment.

Effect of pH on activity

The effect of pH on the activity of free and immobilized α-amylase for the starch hydrolysis was examined from pH 4 to 11. The activities obtained are presented in Fig. 7. The activities of the free enzyme were more

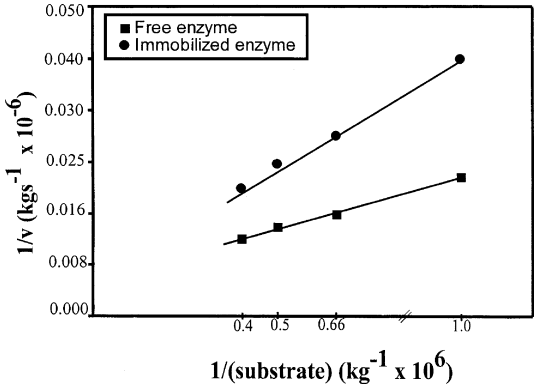

Fig. 6 Lineweaver–Burk plots for evaluation of K_m and V_{max} for free and immobilized α-amylase

dependent on pH than those of the immobilized enzymes.

The maximum activity was observed at pH 7.0 for both free and immobilized α-amylase. The pH profile of the immobilized enzyme gave a lower amplitude of enzyme activity. Activity loss of the immobilized enzyme may be attributed to partial destruction of enzyme activity during entrapment into the IPN. This change was depicted by an apparent distinction in the kinetic parameters that were obtained before and after immobilization.

The pH profiles also imply that whereas the immobilized enzyme exhibits a broader profile in the acidic region, the two profiles were further widened in the alkaline range. The former observation could be attributed to the diffusional limitation [22], whereas the later could be explained possibly owing to secondary interactions (e.g. ionic and polar interactions, hydrogen bonding) between the enzyme and the polymeric carrier. Similar type of observations have been frequently reported in the literature [23, 24].

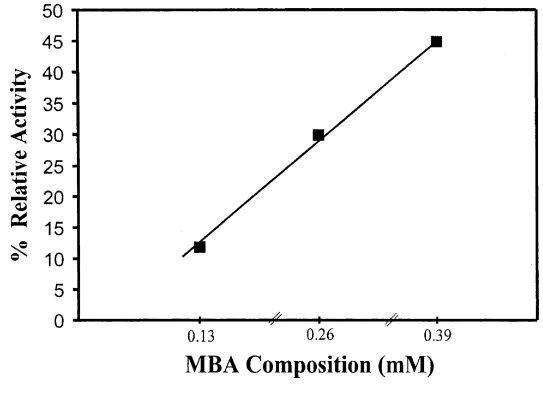

Fig. 5 Variation in percentage relative activity of α-amylase with MBA content of the IPNs at fixed [PVA] = 0.55×10^{-3} kg, [AM] = 14 mM, [PEG] = 10^{-3} kg and [KPS] = 0.003 mM

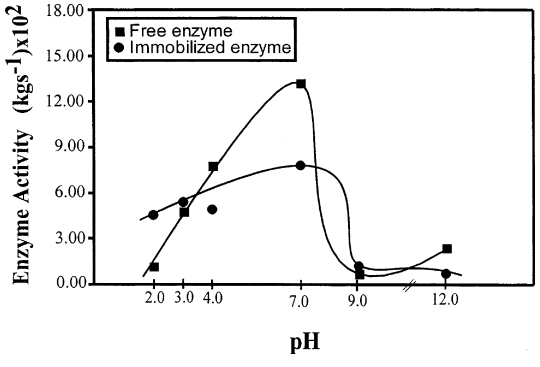

Fig. 7 Effect of pH on percentage relative activity of free and immobilized α-amylase

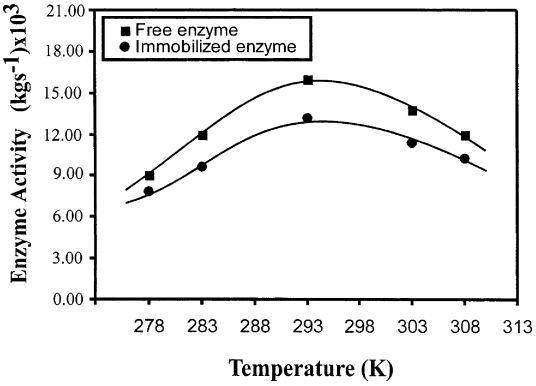

Fig. 8 Influence of temperature on percentage relative activity of free and immobilized α-amylase

Effect of temperature on activity

The temperature dependence of the free and immobilized α-amylase activity was studied in the temperature range 278–308 K. The results obtained are depicted in Fig. 8. Optimum catalytic activity was observed at 293 K, for both free and immobilized α-amylase; however, the immobilized enzyme had a lower activity than the free one. This may be due to decreased affinity of the enzyme for the substrate caused by internal diffusion restriction of the immobilized α-amylase.

The immobilized α-amylase exhibited a broader profile at the optimum temperature, thereby showing better thermostability, whereas the free enzyme was less stable towards heat. Increased thermal stability has been reported for a number of immobilized enzymes, and the polymer network is supposed to preserve the tertiary structure of the enzyme. In addition, it has also been reported that polymeric carriers such as Sephadex, Sepharose, poly(2-hydroxyethyl methacrylate) and polyacrylamide protect enzymes from thermal inactivation and yield higher thermal stabilities [25, 26].

Conclusions

The IPNs prepared by cross-link polymerization of acrylamide in the presence of PEG and PVA yield a potentially compatible support for the immobilization of α-amylase. Because of the hydrophilic nature of the IPNs they are swollen in enzyme solution, thus entrapping the enzyme within their network. The extent of immobilization increases with increasing PEG, PVA and polyacrylamide content in the IPN, while increasing cross-linker tends to reduce the amount of immobilized enzyme.

The chemical architecture of the IPN also affects the relative activity of the enzyme. When the amounts of PEG, PVA and cross-linker (MBA) are increased in the feed mixture of the IPNs, an increase in relative activity is found (except for PVA, where a decrease in noticed at higher content). In the case of polyacrylamide variation, a constant decrease in relative activity is observed.

The pH and temperature of the substrate medium (starch) also influence the relative activity. It is found that upon immobilization, the optimum pH and temperature remain unaltered, while a fall in the relative activity is noticed.

The IR spectra of α-amylase immobilized IPN provide evidence of immobilization.

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