In Situ Entrapment of α-Chymotrypsin in the Network of Acrylamide and 2-Hydroxyethyl Methacrylate Copolymers

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ABSTRACT: A mild and reproducible method has been developed for the entrapment of α-chymotrypsin into a crosslinked copolymer. A porous copolymer was synthesized at 293 K by solution copolymerization of acrylamide and 2-hydroxyethyl methacrylate. α-Chymotrypsin was entrapped during copolymerization at different polymerization stages. The effect of crosslinking on enzyme loading and retention of its activity was examined. Copolymer with 2% crosslinking could entrap >90% of the enzyme. The activity of free and immobilized α-chymotrypsin was determined by using N-benzoyl-L-tyrosine ethyl ester and casein as low and high molecular weight substrates respectively. Storage as well as thermal stability of the immobilized enzyme was superior to that of the free one. Effect of calcium and heavy metal ions was studied on immobilized enzyme activity. The immobilized enzyme showed little variation in activity with pH and retained 50% activity after nine cycles. The Michaelis constant K_m of the free and immobilized enzyme was estimated to be 2.7 and 4.2×10^{-3} m*M*, respectively, indicating no conformational changes during entrapment. © 2000 John Wiley & Sons, Inc. J Appl Polym Sci 77: 2996–3002, 2000

Key words: *in situ* entrapment; α-chymotryspin; acrylamide; 2-hydroxethyl methacrylate; copolymers; copolymerization; crosslinking; enzyme

INTRODUCTION

Since the recovery and the reusability of the free enzymes is limited, attention has been paid to enzyme immobilization.[1] Various methods have been adapted for immobilization. In proteases, immobilization protects the enzyme from autolysis. α-Chymotrypsin (E. C. 3.4.21.1), one of the widely used proteases, catalyzes the hydrolysis of peptide bonds where the acyl donor component is an aromatic amino acid. This protease also catalyzes the synthesis of peptide bonds with the same specificity.

The immobilization of α-chymotrypsin has been studied by different workers. Tao and Fu-rusaki[2] have immobilized α-chymotrypsin on synthetic porous carriers by covalent bonding. Noritomi et al.[3] have immobilized α-chymotrypsin through noncovalent binding for ester synthesis in organic solvent. Zaborsky[4] immobilized α-chymotrypsin, which could retain 50% of activity at 50°C after 1 h. Clark and Bailey[5] have reported 0.8 mg/g of α-chymotrypsin loading on polypropylene (PP) grafted with methyl methyacrylate. Cicek and Tuncel[6] immobilized α-chymotrypsin by entrapment onto poly(isopropylacrylamide-co-2-hydroxyethyl methacrylate) polymer gel for the application in a batch reactor. 2-Hydroxyethyl methacrylate (HEMA)-based copolymers were also used for the immobilization of glucoamylase[7] and entrapment of β-glucosidase.[8,9] Heras and Acosta[10] immobilized α-chymotrypsin from different sources on

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chitin via glutaraldehyde coupling. Kurukawa et al.[11] have examined properties of free and absorbed α-chymotrypsin in calcium alginate gel coated fibers. One of the factors responsible for poor activity of immobilized enzymes has been reported to be harsh conditions used for immobilization.

In conventional entrapment procedures enzyme activity is lost due to the free radicals generated during polymerization.[12] In addition, the need to keep the average pore size of the gel high enough to prevent excessive diffusion limitation and the broad distribution of pore size of the gel inevitably results in leakage of the entrapped enzyme.

Hence, to achieve the balance between various properties such as optimum stiffness and internal mass transfer resistance of the gel for the reactor studies we have used the crosslinked copolymer of 2-hydroxyethyl methacrylate (HEMA) and acrylamide (AAm) for the entrapment of α-chymotrypsin. By varying the time of addition of enzyme to the polymerizing gel, attempts are made to minimize the deactivation effect of free radicals generated during polymerization. The entrapment of α-chymotrypsin with respect to the time of addition of enzyme during polymerization has been examined. Entrapped enzyme activity (EEA) with respect to pH, thermal stability, storage stability, and stability in organic solvents was also examined. The Michaelis constant K_m and reusability of the immobilized enzyme were also investigated. Effect of activators and inhibitors on enzyme activity was also determined.

EXPERIMENTAL

Materials

α-Chymotrypsin from bovine pancreas, (39 U/mg), N-benzoyl-L-tyrosine ethyl ester (L-BTEE), N-N′-methylene bis acrylamide (bis AAm), and casein from bovine milk with 23,600 molecular weight, ~90% protein content, 0.2% lactose, and <3% moisture content were purchased from Sigma Chemical Co., Ltd. USA. Hydrogen peroxide 30% (w/w) and HEMA were purchased from Merck (India). Ascorbic acid and acrylamide (AAm) were from Spectrochem Pvt. Co., India. All other reagents used were of analytical grade.

Methodology

Entrapment of α-Chymotrypsin During Copolymerization

Entrapment of α-chymotrypsin in HEMA/AAm copolymer gel was done by modifying the reported method.[13] AAm 14 g (200 m*M*), HEMA 15 g (100 m*M*), bis AAm 0.6 g (4 m*M*), and ascorbic acid 0.15 g (1 m*M*) were dissolved in 60 g water in three-necked reaction kettle equipped with mechanical stirrer, thermometer, and nitrogen inlet. The reaction mixture was stirred at constant temperature 293 ± 1 K in nitrogen atmosphere for about 30 min to obtain homogeneous solution. The polymerization was initiated by addition of 0.25 mL 30% hydrogen peroxide (2.4 m*M*) into a reaction mixture. A 10 mL buffer solution of pH 8 containing 400 U of enzyme was added after 5 min of initiation time. At this stage, the solution was stirred more vigorously to obtain uniform enzyme entrapment in the polymer matrix. After 1 h, the gel obtained was taken out and washed twice with cold water and filtered. The gel was smashed when wet to obtain coarse particles and dried at room temperature for constant weight and sieved for 400–250 μ size. The entrapped α-chymotrypsin was stored at 4°C until further use. Approximately 90% of active enzyme was observed to be entrapped in the polymer network. The assay of entrapped α-chymotrypsin activity was done as per the literature method.[14] Percentage of active enzyme was calculated from the total initial activity of the enzyme before polymerization and the total activity of the enzyme after entrapment.

Assay of α-Chymotrypsin Activity

The activity of free and immobilized enzyme was determined spectroscopically as per the Bergmeyer method[14] using low and high molecular weight substrates.

Variation in the absorbance of N-benzoyl-L-tyrosine in undissociated and dissociated form is taken into consideration while developing the assay procedure for α-chymotrypsin. L-BTEE of low molecular weight was used as a substrate whose enzymatic hydrolysis was studied through extinction changes. The rate of hydrolysis of L-BTEE was monitored by incubating a reaction mixture containing 1.4 mL (1.1 m*M*) L-BTEE in 50% (w/w) methanol, 1.5 mL (80 m*M*) Tris buffer of pH 7.8 (containing 50 m*M* Ca^{2+}), and 0.1 mL free enzyme solution (0.1 mg/mL) or equivalent amount of immobilized enzyme, for 2 min at 25°C and

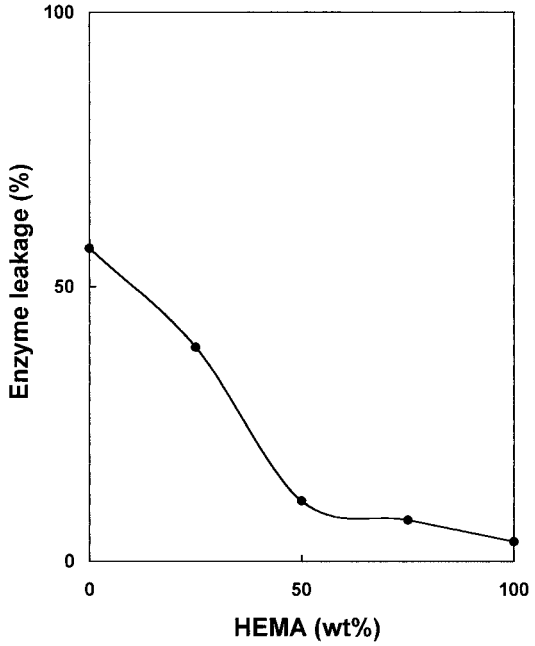

Figure 1 Effect of copolymer composition on leakage of entrapped enzyme.

measuring the absorbance of the solution at 256 nm.

Casein a high molecular weight substrate was hydrolyzed by α-chymotrypsin and tyrosine content of the hydrolyzed product was measured spectrophotometrically at 280 nm after precipitation of the residual substrate. A typical reaction mixture consisting 0.9 mL (100 m*M*) borate buffer of pH 8, 0.1 mL free enzyme solution containing 4 U α-chymotrypsin or equivalent amount of immobilized enzyme and 1 mL of 1% casein solution containing 5 m*M* Ca^{2+} was incubated for 20 min at 35°C, followed by termination of the reaction with 3 mL of 5% trichloroacetic acid. The absorbance of the supernatant liquid was measured at 280 nm.

RESULTS AND DISCUSSION

Use of poly(HEMA) or poly(AAm) gels for the enzyme entrapment is known. However, crosslinked poly(AAm) does not have dimensional stability, whereas crosslinked poly(HEMA) imparts stiffness also contributes to larger internal mass transfer resistance. Hence to achieve balanced properties, HEMA/AAm copolymers with different compositions were used for the entrapment of α-chymotrypsin. The results obtained for the leaching of entrapped enzyme after 2 h at pH 8 are given in Figure 1. It is observed that the crosslinked poly(AAm) shows more than 50% leaching of enzyme whereas the introduction of HEMA into poly(AAm) gel showed decrease in extent of leaching. At and above 1:1 ratio of HEMA:AAm leaching was constant and was only 2–3%. Hence 1:1 ratio of copolymer was selected for further studies.

Effect of Enzyme Addition Time on EEA

Activity of enzyme entrapped during copolymerization is affected by the free radicals present in the system. Hence, effect of addition of enzyme during various stages of polymerization was examined. The results are given in Figure 2. It was observed that simultaneous addition of enzyme and free radical initiator inactivated the entrapped enzyme completely. As the time of addition of enzyme during polymerization increases, the activity of entrapped enzyme increases and at 5 min interval between addition of initiator and enzyme 98% of enzyme activity is retained when casein was used as substrate. This can be attributed to the decreasing concentration of free radicals left in the reaction mixture. As a result of 70% polymerization, in 5 min, the reaction mass was converted into a viscous gel and hence addition of enzyme after 5 min of polymerization was not practical.

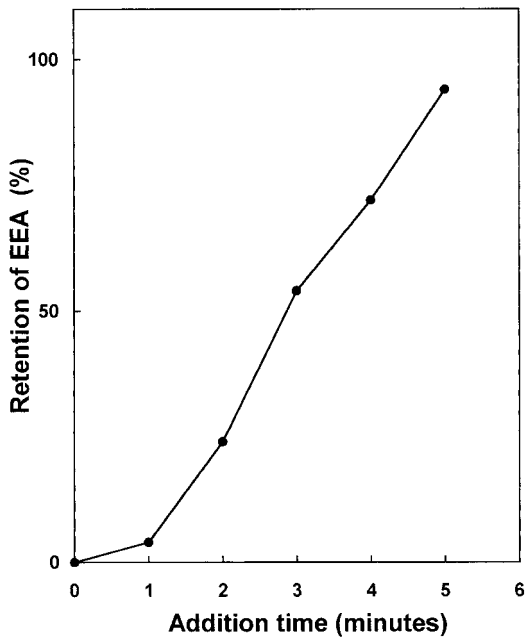

Figure 2 Effect of enzyme addition time on EEA.

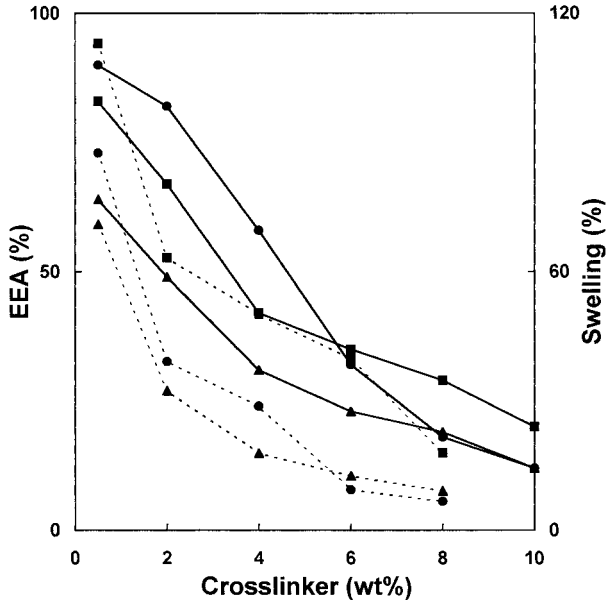

Figure 3 Effect of polymer crosslinking on enzyme activity. Poly(AAm) (■), poly(HEMA) (▲), and poly(AAm-co-HEMA) (●). (——) EEA,(- - - -) % swelling.

Effect of Crosslinking on EEA

The effect of crosslinking on entrapped enzyme activity was studied by using 0.5–10% bis AAm crosslinker during polymerization of AAm, HEMA, and AAm–HEMA mixture. This can also be explained on the basis of equilibrium swelling study. The results obtained are given in Figure 3. Enzyme activity was tested using casein substrate. EEA was observed to decrease with increasing crosslinking for homo- as well as copolymer gel. However, loss of activity was relatively lower in case of AAm–HEMA copolymers. The observed trend can be attributed to the variation in internal mass transfer resistance. This can be supported from the data observed in equilibrium swelling studies (Figure 3). Diffusional limitations were also reported by Oste et al.[15] in the entrapment of α-chymotrypsin in highly crosslinked poly(AAm) gel.

Comparative Account of Free and Entrapped Enzyme

The thermal stability of the enzyme is one of the most important criteria for its application. Free and entrapped enzymes in 50 m*M* borate buffer of pH 8 were incubated for 1 h at different temperatures. The enzyme activity was measured at different time intervals after cooling the enzyme to 10°C and following the procedure described earlier for casein substrate. Results obtained in triplicate are illustrated in Figure 4. It was observed that entrapped enzyme shows better thermal stability at all temperatures and times. However, considerable reduction in activity for both free and entrapped enzyme was observed at and above 60°C. The thermal stability of covalently bound α-chymotrypsin has been reported to be better than that of entrapped enzyme by Tao and Furusaki.[2]

Enzymes being heat sensitive catalysts generally need low-temperature storage. Immobilization of enzyme can overcome this constraint and it can be stored in some cases at room temperature without much loss in enzyme activity. This is very important for the commercial applications of enzymes at reactor scales. Hence storage stability of the entrapped and free enzyme was determined at 30°C for various time intervals. The immobilized and free enzymes were stored in 50 m*M* borate buffer of pH 8. The residual activity of the enzymes at different intervals was estimated using casein substrate and the results are given in Figure 5. It was observed that at room temperature free enzyme looses its activity very rapidly, whereas entrapped enzyme looses it less rapidly. Even after 30 days storage entrapped enzyme retained more than 50% of its activity. Dry storage of enzyme showed very high stability and no loss in activity, indicating that in buffer en-

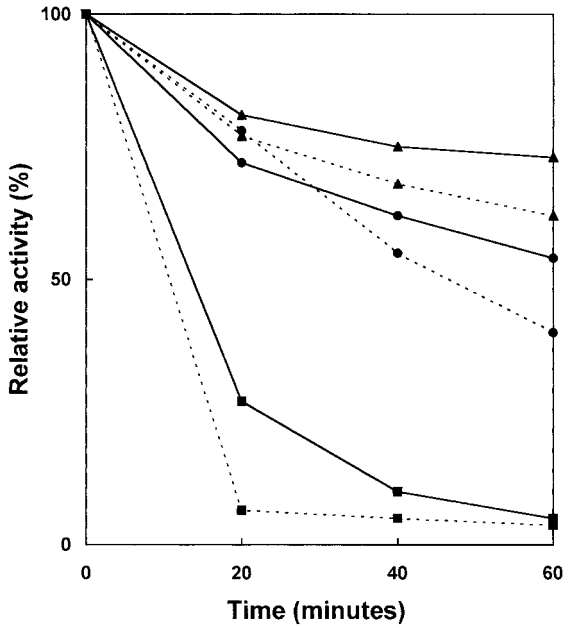

Figure 4 Thermal stability of enzymes. Free α-chymotrypsin (- - - -), Immobilized α-chymotrypsin (——). Incubation time 1 h at 40°C (♦), 50°C (●), 60°C (■).

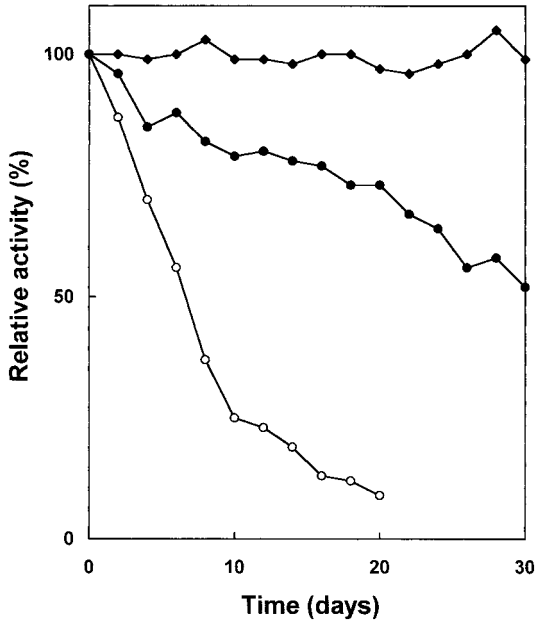

Figure 5 Storage stability of enzymes at 30°C. Free α-chymotrypsin in pH 8 buffer (○), entrapped α-chymotrypsin in pH 8 buffer (●), and dry entrapped α-chymotrypsin (♦).

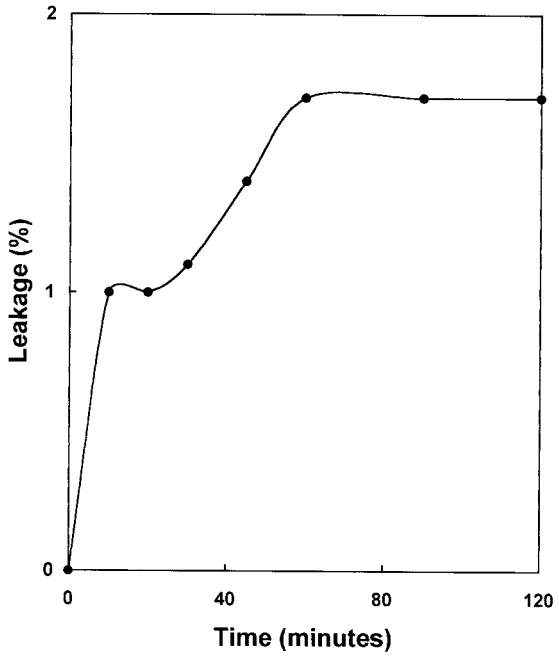

Figure 6 Leaching of entrapped enzyme.

trapped enzyme slowly leaches due to immobilization of enzyme through physical entrapment and not through chemical bonding. A limited study for leaching of enzyme in buffer was carried out at pH 8 and ~2% leaching was observed in supernatant buffer after 2 h time (Figure 6). Extent of leaching went up to 15% after 10 days.

Free enzymes suffer from a major drawback of nonreusability. This is an advantage for immobilized enzymes. The reusability of enzyme was examined by using the same enzyme repeatedly with fresh aliquot of L-BTEE substrate. It was observed that entrapped enzyme showed gradual decrease in its activity with increased number of cycles. However, 50% activity was retained after nine repeated cycles (Figure 7) This can be compared with the results obtained in immobilization of α-chymotrypsin through adsorption[16] where 35% residual activity was retained after nine repeated cycles. In both the cases immobilization is through physical process and not through chemical bonding.

Due to conformational changes there is a possibility of change in the pH of enzyme activity on immobilization. The activity of free and entrapped α-chymotrypsin was measured at different pH using casein as substrate. It was observed from the results given in Figure 8 that maximum activity was exhibited at pH 8 by free as well as entrapped α-chymotrypsin, indicating no changes in conformation of the enzyme during entrapment.

Effect of Solvents on Casein Hydrolysis

Many enzymatic reactions are carried out in organic media. Hence retention of enzyme activity in organic solvents such as dimethyl formamide

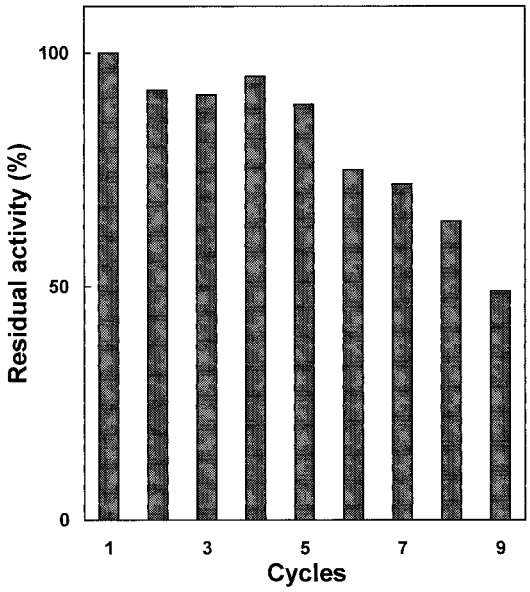

Figure 7 Reusability of entrapped enzyme.

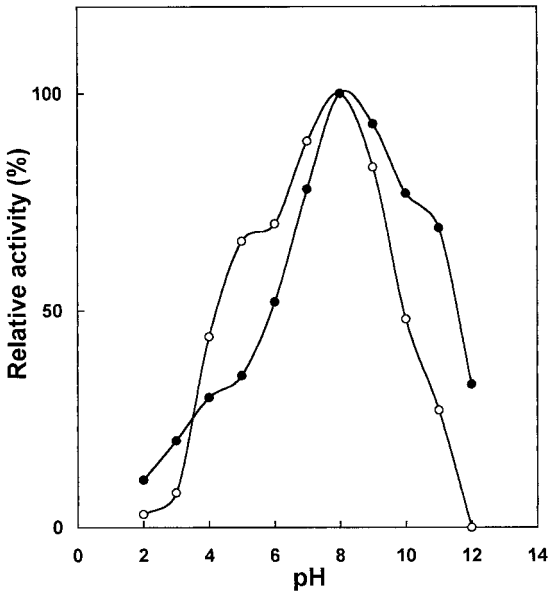

Figure 8 Effect of pH on enzyme activity with casein as substrate. Free α-chymotrypsin (○) and immobilized α-chymotrypsin (●).

(DMF), DMSO, MeOH, CH_3CN, and tetrahydrofuran (THF) using free and immobilized α-chymotrypsin was examined. The strength of organic solvents is expressed as the percentage based on the total reaction volume. From the results (Table I) overall improved stability of enzyme was observed for immobilized enzymes with respect to various organic solvents of different strengths. This may be just because enzyme is entrapped within a polymeric matrix which acts as a semipermeable protective membrane. This is a promising observation particularly for the enzymatic reactions in nonaqueous media.

Michaelis Constant K_m

The rate of the hydrolysis of casein and L-BTEE was determined by taking the fixed concentration of free and equivalent amount of immobilized enzyme and varying the concentration of substrate from 0.6 to 4.23×10^{-3} m*M* and 0.2 to 2.0 m*M*, respectively. For the study, a gel matrix produced by addition of enzyme after 5 min polymerization was used. The apparent Michaelis constant K_m and maximum reaction rate V_{max} were determined from the Lineweaver–Burk plots of 1/s vs 1/v for the free and immobilized enzymes. The results are given in Table II. The similar values of K_m and V_{max} observed for free and entrapped enzyme indicate no conformational changes in the enzyme during entrapment. However, higher K_m and V_{max} values for both free and entrapped enzyme obtained with L-BTEE suggest less mass transfer resistance to the substrate due to lower molecular weight than casein.

K_m and V_{max} values were also calculated by keeping casein concentration at 1.06×10^{-3} m*M* and varying the concentration of free and entrapped enzyme from 0.4 to 4 U and 25 to 250 mg, respectively. The values obtained were very much similar to those obtained by keeping the enzyme concentration constant and varying casein concentration (Table II).

Effect of Metal Ions on Enzyme Activity

It is known that heavy metal ions are inhibitors to α-chymotrpsin.[17] Hence activity of the free and entrapped enzyme was assayed in the presence of different concentrations (0–2 m*M*) of copper and nickel ions. From the results in Figure 9 it is observed that enzyme activity decreases with increased concentration of copper and nickel ions in case of free as well as entrapped enzyme. However, Cu^{2+} shows more inhibitory effect than Ni^{2+}. Entrapped enzyme has less inhibitory effect than the free enzyme, indicating better potential for its applications.

Various additives such as amides or amines[18–20] and calcium[21] in organic solvents have shown activating effect on the α-chymotrypsin activity. Hence hydrolysis of casein in 10%

Table I Effect of Solvents on Hydrolytic Activity of Casein

Solvent	Strength (% v/v)	Retention of Activity (%)	
		Free	Entrapped
DMF	10	87	94
	20	43	69
	30	18	25
DMSO	10	87	94
	20	68	69
	30	44	56
AN	10	37	56
	20	6	44
	30	0	31
MeOH	10	94	94
	20	56	81
	30	12	56
THF	10	19	50
	20	12	44
	30	6	31

Table II Kinetic Parameters for Free and Entrapped α-Chymotrypsin

	K_m (m*M*)		V_{max} (m*M*/min)	
Substrate	Free	Entrapped	Free	Entrapped
Casein[a]	2.7×10^{-3}	4.2×10^{-3}	5.95×10^{-2}	4.84×10^{-2}
L-BTEE[b]	38.46	31.25	13.74	16.2

[a] At 35°C, pH 8.0 for 20 min.
[b] At 25°C, pH 7.8 for 2 min.

methanol in the presence of 2.5 m*M* of calcium chloride was carried out. It was observed that presence of calcium ion enhances the rate of hydrolysis by 20%.

CONCLUSION

Activity of the entrapped enzyme was observed to be critically dependent on the time of addition of enzyme during polymerization. Entrapped enzyme showed improved storage and thermal stability and showed only 50% loss in activity after nine cycles. No conformational changes in enzyme were observed during entrapment. The rate of the hydrolysis of the substrate was observed to be dependent on the molecular weights of the substrate due to the difference in the internal mass transfer extent. Resistance to heavy metals inhibitory action was observed to be more for the entrapped enzyme.

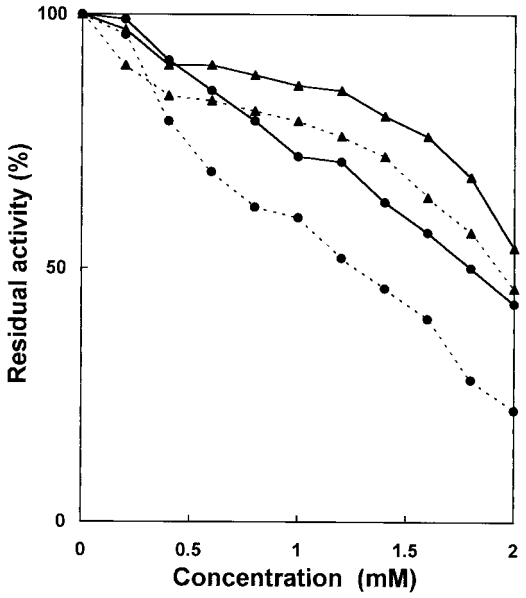

Figure 9 Effect of heavy metal ions. Free α-chymotrypsin (- - - -) and immobilized α-chymotrypsin (——) for Cu^{2+} (■) and Ni^{2+} (♦).

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