CHAPTER - 2

IMMOBILIZATION OF CHYMOTRYPSIN

CONTENTS

2.1 Properties of chymotrypsin

Chymotrypsin (E.C. 3.4.21.1) belongs to a class of enzymes known as *serine proteases*. Chymotrypsin is a digestive enzyme found in the small intestine, where it catalyzes the hydrolysis of certain peptide bonds in proteins. Several known forms of chymotrypsin are: α-, β-, γ-, π- and δ-chymotrypsin. α-Chymotrypsin is converted to δ-chymotrypsin just by keeping the solution for several hours at high pH, and slow reconversion is possible on storage at pH 4 for months.

α-Chymotrypsin hydrolyzes esters and amides of aromatic amino acids as well as proteins and peptides. In the case of proteins, peptide bonds involving the carboxyl groups of aromatic amino acids such as phenylalanine and tyrosine are readily hydrolyzed. The activity of enzyme depends on the presence of thiol group and bound metal ions such as magnesium, cobalt, manganese, zinc and iron. A review by Desnuelle[1] is quite informative for activation processes of chymotrypsin and its properties.

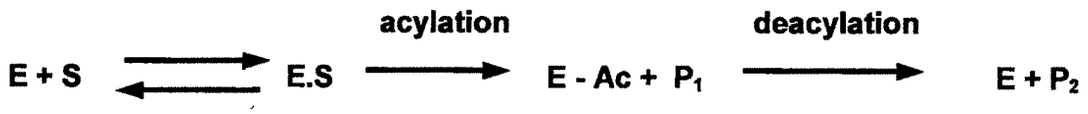
Chymotrypsin is prepared as a by-product during the fractionation of trypsin from an acid extract of mammalian, usually bovine and pancreas. The fractionation isolates the enzyme mainly as the inactive pre-enzyme, chymotrypsinogen, which is activated by trypsin. Molecular weight of α-chymotrypsin is ~25,000 daltons. Chymotrypsin will clot milk but not blood in contrast to trypsin.

Maximum activity of α-chymotrypsin is observed at pH 8 - 9, while at pH 7, ~ 50% of activity is exhibited, at optimum temperature 25 - 35 °C. The enzyme is stable for days in solution at pH 3 and for years as a dry powder when stored refrigerated. The stability of α-chymotrypsin can be increased in the presence of calcium ions. Chymotrypsin is less stable than trypsin in acid.

The enzyme activity is inhibited by, heavy metals, the natural trypsin inhibitors,[2] an inhibitor from potato,[3] and organophosphorous compounds. Gel filtration of chymotrypsin removes autolysis products and other contaminants.[4] The enzyme is also inhibited by α_1-anti-trypsin and α_2-macroglobulin present in human serum. Inhibitory action of α_1-anti chymotrypsin is also reported.

Chymotrypsin is of interest in its use in organic chemistry, food science, analytical chemistry, cosmetics and medicines. In organic chemistry it is of interest for both the synthesis of both oligopeptide and ester, for transesterification and for hydrolysis of esters. It is also useful in peptide synthesis in water miscible solvents. In food science it is useful in hydrolysis of milk and soyabean protein and in the treatment of cheese whey in the dairy industry. It is also useful in meat tenderization. In cosmetics it is used to evaluate skin elasticity. While, in pure form it is used in medicine. In some countries, α-chymotrypsin, because of its antiinflammatory action is prescribed to be taken orally against infections, broken bones, etc. Chymotrypsin has in general a lower toxicity than trypsin. Doses upto 50 mg kg^{-1} body weight on alternate days for 4 weeks failed to show pathological changes in the organs of experimental animals, and an increase in dose of up to 200 mg kg^{-1} body weight produced local necrosis, but no animal death was reported.

The enzyme catalyzed hydrolytic reaction has been shown to occur in at least three kinetically distinguishable steps. The first of these consists of a very fast, diffusion-controlled formation of a non-covalent enzyme-substrate (E.S) complex followed by an acylation step. In the latter, the acyl group (Ac) of the substrate becomes covalently attached to a serine alcohol of the active site of the enzyme with the concomitant release of the amine of an amide substrate as the first product (P_1); then the enzyme intermediate is hydrolyzed by water, thus regenerating the free enzyme and releasing the carboxylic acid and the second product (P_2) :



Various substrates available for the determination of the activity are natural proteins like haemoglobin and casein, and synthetic substrates such as N-acetyl-L-tyrosine ethyl ester, N-benzoyl-L-tyrosine ethyl ester (L-BTEE), N-carbobenzoxy-L-tyrosine-p-nitrophenyl ester, N-acetyl-phenyl-alanine-p-nitroanilide, N-succinyl-L-phenyl-alanine-p-nitroanilide and glutaryl-L-phenyl-alanine-p-nitroanilide. The hydrolysis of these substrates can be measured by spectrophotometrically or titrimetrically.

2.2 Literature survey

The resistance of catalytically active protein structure towards storage, higher temperatures, reusability and other denaturing influences is one of the most important criteria in the application of enzymes. The binding of enzymes to polymeric carriers has proved to be an effective way to stabilize native enzyme structure.[5-7] Numerous synthetic and natural polymeric matrices have been used for the immobilization of chymotrypsin by various coupling methods.

Chymotrypsin immobilized on natural supports

Natural carriers extensively used for enzyme immobilization are polysaccharide derivatives such as cellulose, dextran, agarose, chitin and chitosan. Some are available commercially under trade names such as sephadex, sepharose, bio-gel etc.

Initially immobilization was achieved by just adsorbing or ionically binding it onto water insoluble solid supports. In 1916 Nelson and Griffin[8] found that an enzyme in water-insoluble form showed catalytic activity. They reported that invertase extracted from yeast was adsorbed on charcoal, and the adsorbed enzyme showed the same activity as native enzyme. This is considered as the first report on immobilization. Among the natural supports cellulose and its derivatives are widely used for the immobilization of chymotrypsin.

➤ **Cellulose and its derivatives**

Immobilization of an enzyme by ionic binding method was first reported by Mitz[9] in 1956. He prepared immobilized catalase by passing a catalase solution through a column packed with DEAE-cellulose. When hydrogen peroxide solution was passed through the column, no hydrogen peroxide was detected in the effluent. However, Brandenberger[10] used pearl cellulose as cation exchanger to immobilize trypsin and chymotrypsin. Three years latter in 1959, Mitz and Schlueter[11] bound chymotrypsin ionically to cationic exchangers such as CMC, cellulose citrate and cellulose phosphate. They observed the final specific activity to be 0.310, 0.238 and 0.306 $\mu M\ min^{-1}\ g^{-1}$ of protein in comparison with the initial value of 0.311 $\mu M\ min^{-1}\ g^{-1}$ of protein.

Latter in 1961 Mitz and Summaria[12] immobilized α-chymotrypsin onto CMC using sodium nitrate by means of covalent binding. α-Chymotrypsin bound to this CMC-azide was more stable to heat than free α-chymotrypsin and had unchanged pH optima. α-Chymotrypsin adsorbed on cellulose was reported by Lilly et. al.[13] The stability of adsorbed enzyme was reported to be enhanced. Free chymotrypsin was reported to be completely inactivated in 20 min at 50 °C, while immobilized chymotrypsin was stable at 50 °C but was inactivated at 60 °C. Patchornik[14] reported use of bromoacetyl cellulose prepared by reacting cellulose with bromoacetic acid in dioxane for the immobilization of chymotrypsin.

p-Aminobenzyl cellulose is also most commonly used for the immobilization of enzymes. This carrier was first used by Campbell et. al.[15] for bovine serum albumin. Chymotrypsin was also covalently bound to p-aminobenzyl cellulose activated with cyanuric chloride in Schweitzer reagent by Surinov and Manoilov.[16] Coupling of chymotrypsin was carried out in phosphate buffer of pH 8 at 4 °C for 10 - 12 h. The immobilized protein was observed to retain 60% activity.

Kay and Crook[17] bound α-chymotrypsin to cellulose using chloro-s-triazine coupling reagent. They could get only 1 - 2% yield by weight of enzyme with retention of only 25% of its initial activity. Similarly Kay and Lilly[18] covalently bound α-chymotrypsin to cellulose, DEAE-cellulose, CMC, sephadex and sepharose using 2-amino-4,6-dichloro-s-triazine activating reagent. The results showed that the overall reaction rate is restricted by the rate of diffusion of substrate into and product out of, the immobilized enzyme derivatives.

Takami and Ando[19] observed change in the optimum pH of free and bound chymotrypsin. The optimum pH was observed to be higher by 0.5 units in the case of trypsin and chymotrypsin covalently bound to CMC. The immobilized enzymes were observed to be more resistant to urea upto 1 M concentration. However, Martinek et. al.[20] could achieve retention of enzyme activity upto 4 M urea concentration when α-chymotrypsin was bound to p-aminobenzyl cellulose.

Activation of primary amino and hydroxyl groups of sephadex, cellulose, starch, p-aminophenoxy hydroxypropyl sephadex and aminoethyl sephadex by glutaraldehyde and CNBr was carried out by Axen et. al.[21] This is considered as the first report for the CNBr activation method. They observed that coupling of α-chymotrypsin was lower on sephadex than that on cellulose. However, at room temperature the coupled products were stable over a wide range of pH. Similar type of stability over pH range 2 - 9 was observed by Green and Crutchfield,[22]

when α-chymotrypsin was immobilized on agarose by CNBr activating agent. Axen and coworkers[23,24] covalently attached α-chymotrypsin to agarose cross-linked with epichlorohydrin. Immobilization carried out at pH 6.5 for 6 h was reported to yield a polymer bound enzyme 40 mg g^{-1} agarose. They also observed activity of the bound α-chymotrypsin against casein to be 40% that of the free enzyme. Bennett et. al.[25,26] reported that CNBr activated sepharose could bind α-chymotrypsin which was observed to be stable for 7 months and could give complete hydrolysis of polypeptides and proteins.

The esters formed by the introduction of trans-2,3-cyclic carbonate into cellulose through the reaction with ethylchloro formate in anhydrous organic solvents could exhibit suitable reactivity for the covalent coupling of enzymic protein under mild aqueous conditions. Kennedy[27] used this method to immobilize α-chymotrypsin. He observed that in comparison to free enzyme immobilized chymotrypsin could retain 26% and 65% of its activity when high and low molecular weight substrates casein and tyrosine ethylester were used respectively. α-Chymotrypsin bound to macroporous cellulose and the caseinolytic activity of the immobilized enzyme was reported to be improved by swelling the matrix in dimethyl sulphoxide before coupling with the enzyme. This improvement was attributed to an increase in the porosity of the insoluble support during the preswelling process, enabling macromolecules to diffuse into the matrix.

Enzyme loading upto 55% was achieved by Skachova and Kucera[28] during covalent coupling of chymotrypsin to pearl cellulose activated with p-benzoquinone. Hydrolysis of ethyl (R)-2-(benzyloxy carbonylamino)-3-sulfamoyl propionate to its respective free acid and ethanol by free and α-chymotrypsin immobilized on dialdehyde cellulose was reported by Aleksiev et. al.[29,30]

Cik and Srokova[31] immobilized α-chymotrypsin onto gel of o-hydroxyethyl cellulose by the photochemical generation of nitrene radicals caused by the photolysis of an azido group of bi-functional reagent 4,4'-bis-azidostilbene-2,2'-disodium sulphate.

➤ **Chitin and chitosan**

Immobilization of α-chymotrypsin has also been reported on chitin derived from various sources, through glutaraldehyde activation method. Stanley et. al.[32] immobilized α-chymotrypsin on chitin from shrimp and crab. They observed that α-chymotrypsin bound to shrimp chitin appeared to be more sensitive than α-chymotrypsin bound to crab chitin and hence enzyme coupled on crab chitin was studied intensively. The pH optima of the immobilized α-chymotrypsin was shifted by ~1 pH unit toward alkaline pH.

However, Muzzarelli et. al.[33] immobilized α-chymotrypsin on chitin and chitosan without any intermediate reagent. The activity of immobilized α-chymotrypsin on chitosan was observed to be much higher than α-chymotrypsin immobilized on chitin. Further, they used α-chymotrypsin immobilized on chitosan at optimum pH for the large scale transformation of substrates without decrease in the activity or enzyme losses.

Heras and Acosta[34] immobilized α-chymotrypsin on chitin from squills, lobsters and prawns by means of glutaraldehyde activation. Hydrolase and peptide synthetase activities were determined by them in aqueous and organic solvents respectively. α-Chymotrypsin immobilized on chitin from prawns was observed to show maximum synthatase activity.

➤ **Sehparose**

Heras et. al.[35] could achieve high coupling yield on immobilization of α-chymotrypsin on sepharose after tosylation of free hydroxyl groups. It was further used for the examination of hydrolytic and synthetic activity in different organic media at substrate concentrations ranging from 10 to 70%.

Martin et. al.[36,37] also carried out immobilization of α-chymotrypsin on sepharose activated through tosylation of hydroxyl groups, by using p-tolyl sulphonyl chloride (PTS). The system was found to be suitable for peptide synthesis. The hydrolase and peptide synthetase activities of the immobilized enzymes were studied in aqueous and organic media respectively. Peptide synthetase activity was found to be related to the procedure used for activating the support. A 50 - 70% yield in the synthesis of dipeptide was reported in 70% butenediol at pH 7 and 37 °C, whereas 54% when 70 : 30 butendiol : water was used at pH 9.0 and 37 °C.

Chymotrypsin immobilized on synthetic supports

Various synthetic polymers have been used for the immobilization of enzymes by different techniques. Copolymers containing amino acid groups were used for the immobilization of papain for the first time by Katachalski et. al.[38] in 1960. Later in 1963 they immobilized α-chymotrypsin[39,40] to chain-shaped polymeric links such as polypeptides, polyamides and polyethylene glycols. The polymeric links were, in turn bound to water insoluble carriers such as copolymers of amino acids or their functional derivatives. N,N'-hexamethylene bis(carbamoyllysine) was synthesized by Ozawa[41] treating the copper complex of lysine with hexaethylene diisocyanate. This was further used for the coupling of chymotrypsin. The immobilized enzyme was reported to retain 30% of its activity.

Glutaraldehyde is most commonly employed as the cross-linking reagent, and many enzymes have been immobilized through the formation of Schiff bases between the free amino groups of carriers and of enzyme protein. Poly(AAm) beads activated via glutaraldehyde were used for the immobilization of α-chymotrypsin by Weston and Avrameas.[42] They reported 55% retention of enzyme activity. The thermal and storage stability of the immobilized enzyme was observed to be increased. Synthetic resins prepared by acid anhydride, aldehyde and isothiocyanate were used for the immobilization of α-chymotrypsin by Bruemmer et. al.[43] Surprisingly they observed that relative to free enzyme immobilized enzyme lost specific activity when bound to resin, when esterase activity with low molecular weight substrate was measured.

Many cation and anion exchangers have also been used for the immobilization of enzymes. Polyanionic and polycationic derivatives of chymotrypsin were prepared by coupling chymotrypsin to an ethylene-maleic acid copolymer and by partial succinylation or acetylation by Goldstein.[44,45] The pH activity profiles of polyanionic derivatives of the chymotrypsin were shifted toward more alkaline pH values as compared to the free enzyme. Conversely the pH activity profiles of polycationic derivatives were shifted towards more acidic pH values. Zaborsky[46] used imidoester containing polymers for immobilization of α-chymotrypsin. The enzyme was reported to retain 50% of its activity at 50 °C after 1 h.

Later Goldstein and coworkers[47] synthesized polyiosnitrile-nylon by reacting partially hydrolyzed nylon with acetaldehyde and 1,6 diisocyano hexane. α-Chymotrypsin was bound to this carrier through the free amino groups. The immobilized enzyme retained 60% of its activity while, the pH activity profiles of immobilized α-chymotrypsin was broad and shifted more towards alkaline side.

Martinek et. al.[48,49] examined the thermal stability of chymotrypsin in polymethacrylate gel cross-linked with bis-AAm. The rate of thermal inactivation

was nearly constant at 60 °C. However, when chymotrypsin was first reacted with acylol chloride and the resulting modified enzyme was copolymerized with sodium methacrylate monomer and bis-AAm as cross-linking agent, immobilized chymotrypsin was ~1000 fold more stable than native chymotrypsin at 60 °C.

Cheng and Lai[50] immobilized α-chymotrypsin on copolymer such as styrene-maleic anhydride using CNBr, glutaraldehyde and diazo coupling methods. They observed that all the immobilized enzymes showed no more than 6% activity loss for over 10 months of storage at 4 °C.

Sumida et al.[51] prepared polyamide microcapsules containing carboxyl groups in their membrane using an interfacial polymerization technique. Chymotrypsin was further covalently immobilized to the surface. The Michaelis constants K_m was reported to be changed inappreciably, whereas the V_{max} showed a considerable reduction after immobilization. They also observed a slight shift in optimum pH to the alkaline side due to negative charges present on the surface.

Luthra et. al.[52] used microgels of acrylic acid, ethylene glycol dimethacrylate and HEMA for immobilization of α-chymotrypsin. The enzyme was conjugated to carboxy groups with a water soluble carbodiimide. The Michaelis Menten parameters of the conjugated enzyme vs. L-BTEE were almost identical for the native and immobilized enzyme except at low pH.

Free and α-chymotrypsin immobilized on PVA were used by Kise et. al.[53,54] for the synthesis of N-acetyl-L-tyrosine glycinamide. Free and immobilized enzymes respectively showed maximum yields of 35% and 50% in ethanol and 21% and 84% in acetonitrile at 30 °C in 24 h.

Itamer et. al.[55] entrapped α-chymotrypsin in AAm copolymers which contained photoisomerizable components. The cross-linked systems were used for the hydrolysis of low molecular weight substrates.

Graft copolymers have been extensively used for enzyme immobilization.[56,57] Graft copolymers provide substrates with desired physical and chemical characteristics and grafted chains containing active groups are useful for enzyme coupling.[58] Clark and Bailey[59] have reported 0.8 mg g^{-1} of α-chymotrypsin loading on polypropylene grafted with methylmethacrylate.

It has been shown that the systems based on graft polymers containing polyethylene-g-co-HEMA provided very promising supports for the enzyme immobilization. Gil et. al.[60] immobilized α-chymotrypsin onto partially hydrolyzed poly(ethylene-g-co-HEMA) using different activating agents like p-benzoquinone, PTS, epichlorohydrine and cyanuric chloride. However, retention of the activity was only 2.2, 4.0, 0 and 4.1% respectively. Similarly Alcantara et. al.[61,62] used the same support for the immobilization of α-chymotrypsin. They reported enzyme loading of 33 - 87 mg g^{-1} of copolymer. Further, they used these immobilized enzymes for the synthesis of dipeptide.

Tao and Furusaki[63] have immobilized α-chymotrypsin on porous carriers synthesized by suspension polymerization of acrolein with vinyl acetate and divinylbenzene in the presence of pore-creating agent through covalent bonding. Same support was modified and used by Guoliang and Furusaki[64] for immobilization of α-chymotrypsin using different coupling methods. A polymer containing amino groups was obtained by further reaction of it with p-nitroaniline. The resulting nitro product was further reduced to form amino group. The glutaraldehyde or diazo method was used for the activation before coupling the enzyme. Improvement in physico-chemical properties of immobilized α-chymotrypsin was reported.

Poly(AAm) beads and celite were used for immobilization of α-chymotrypsin by Triantatyllou et. al.[65] Poly(AAm) with a high degree of cross-linking lead to high catalytic rates when used as immobilization matrix. They also observed that derivatization of the polymer with carboxyl and tertiary amino groups prior to immobilization caused no significant changes in α-chymotrypsin behaviour.

Tuncel and coworkers[66,67] immobilized α-chymotrypsin via physical entrapment within large, uniformly spherical and thermally reversible polymeric beads prepared from poly[N-isopropyl(AAm)] and poly[isopropyl(AAm)-co-HEMA], copolymer gels for the application in a batch reactor. They observed that due to the thermoresponsive character of the carrier gel, the maximum activity was achieved at 30 °C, while the free enzyme exhibited maximum activity at 40 °C. The Lineweaver-Burk plot indicated that the enzyme-gel system had a reasonably higher K_m value relative to that of free enzyme due to the internal mass transfer resistance against the substrate diffusion.

Shimomura et. al.[68] investigated enzymic properties of α-chymotrypsin, covalently immobilized on magnetic particles via graft polymerization of acrylic acid. Graft polymerization was carried out in a redox system consisting of mercapto groups introduced onto the surfaces of magnetic particles. The amount of α-chymotrypsin immobilized per g of magnetite was 13 - 17 mg and the activity of the immobilized α-chymotrypsin at 37 °C, pH 8 was 70% of the native enzyme. They also observed that due to immobilization, optimum pH for α-chymotrypsin activity shifted to slightly higher value, whereas optimum temperature did not change. In water at 37 °C, immobilized α-chymotrypsin retained 93% of its original activity over a period of 25 days, though the native enzyme was completely deactivated within 5 days.

Recently Vankova et. al.[69] prepared immobilized α-chymotrypsin by coupling it to periodate oxidized poly(AAm-co-allyl-α-D-galactoside or ally β-lactoside) copolymers. Specific activity of α-chymotrypsin linked to the copolymer was 59.1 and 314.4% for α-D-galactosyl and β-lactosyl residues respectively. While Chen and Su[70] synthesized latex particles composed of styrene, N-isopropylacrylamide and N-acryloxy succinimide by surfactant-free emulsion polymerization for the immobilization of α-chymotrypsin.

Chymotrypsin immobilized on inorganic supports

Many inorganic supports have also been used for the immobilization of enzymes. Robinson et. al.[71] used magnetic particles such as iron oxide activated with amino silane reagent and cellulose-iron oxide composite activated with CNBr for the immobilization of α-chymotrypsin. α-Chymotrypsin was immobilized on several magnetic particulate materials by Munro et. al.[72] The rate of loss of activity was greatly reduced by coating the magnetic particles with a thin layer of bovine serum albumin before immobilizing the enzyme on them.

Tanizawa and Bender[73] immobilized α-chymotrypsin onto porous glass. The kinetic behaviour of immobilized enzyme proved to be the same as that of the free enzyme. While Cheryan et. al.[74] used porous alkylamino glass for the immobilization of α-chymotrypsin and other enzymes. They also used these immobilized enzymes into fluidized bed reactor. The inactivation rates were lower at high substrate pH and were insignificantly affected by reactor temperature.

Recently Iborra et. al.[75] prepared a dynamic membrane reactor with immobilized α-chymotrypsin for continuous synthesis of Kyotorphin (dipeptide) using water : DMSO 60 : 40, v/v and hexane : ethanol : water 57 : 40 : 3 by volume as reaction

media. α-Chymotrypsin was covalently bound to α-alumina ultrafilteration membrane coated with an inert protein using glutaraldehyde.

Chymotrypsin immobilized by cross-linking (without support)

Immobilization of α-chymotrypsin has also been achieved through formation of intermolecular cross-linkages between the enzyme molecules by using bi - or multifunctional reagents. Glutarldehyde, hexaethylene diisocyanate or hexaethylene diisothiocyanate are employed for the immobilization of enzymes. In 1964, Quiocho and Richards[76] treated carboxy peptidase A with glutarldehyde and obtained the immobilized enzyme by intermolecular cross-linkage. This is the oldest report on the cross-linking method.

Jansen et. al.[77] observed increase in the rate of inactivation of α-chymotrypsin due to cross-linking with glutarldehyde with increasing pH. The formation of insoluble but active α-chymotrypsin was efficient at pH 6.2. However, Tomimatsu et. al.[78] reported considerable loss of activity (60-70%) during the cross-linking of α-chymotrypsin with glutarldehyde. Hsu and Mohapatra[79] also cross-linked α-chymotrypsin with glutarldehyde and studied the effect of glutaraldehye and enzyme concentration on the immobilized enzyme activity. They observed a large drop in the enzymatic activity during the first few minutes of the reaction when the particle formation rate was small but the activity remained almost constant when the particle formation rate was very large.

Miscellaneous

Krysteva et. al.[80] used an inexpensive carrier lignin for the immobilization of chymotrypsin by covalent attachment through formaldehyde. They used haemoglobin and L-BTEE as high and low molecular weight substrates for the

activity measurements. The pH activity curves for immobilized chymotrypsin showed optimum activity at pH 7.8 and 9.3 respectively for haemoglobin and L-BTEE substrates. Draganova et. al.[81] prepared aminolignin by ammonolysis of chlorolignin with ammonium carbonate for the coupling of α-chymotrypsin through diazonium salt. They reported 80 - 90% of enzyme loading at 4 °C and 24 h coupling time. The immobilized α-chymotrypsin could give quantitative hydrolysis of N-benzyloxy carboxyl-L-cysteic acid s-amide ethyl ester to its respective acid and amide.

Recently[82] α-chymotrypsin was immobilized on the outer surface of the polymerized liposomes activated through carbodiimide linkages. Coupling was rapid and nearly complete at a weight ratio of enzyme to the polymer of < 0.12. The immobilized enzyme showed favourable activity for both low and high molecular weight substrates. They reported 90% activity for L-BTEE and 59% for casein upto an enzyme coupling of 380 mg g^{-1} of polymer.

Haensler et. al.[83] immobilized α-chymotrypsin on different supports such as silica, Enzacryl AA, CMC and chitin using different activators and covalent binding techniques. They used immobilized α-chymotrypsin for peptide synthesis in frozen aqueous reaction mixtures. This is believed to be the first report on the application of an immobilized enzyme in frozen state.

More recently, Kurukawa et. al.[84] prepared a new composite gel fiber by the gel formation of cellulose acetate and titanium isopropoxide. However, they observed that the optimum temperature for immobilized α-chymotrypsin is lower than that for the native enzyme. This indicates that the active site of the enzyme is affected by immobilization.

Work undertaken

Immobilization of biologically active species is industrially important due to ease of product separation, reuse of enzyme and no unfavorable side reactions. Choice of support and method of immobilization play crucial role in governing enzymatic reactions. Naturally occurring polymers such as chitin and chitosan offer certain advantages due to low bulk density, coarse porous structure, no toxicity, biocompability and possibility of immobilization either by adsorption or by chemical reaction. They are insoluble in water, alkali and organic solvents. Their solubility in dilute organic acids allows them to be suitable for their use as matrix for enzyme or cell immobilization.[85] Various enzymes have been coupled to chitosan by different techniques. It is currently employed in chromatography, chemical analysis, ultrafilteration and in food and pharmaceutical industries.[86] Hence we have used it for the immobilization of enzymes. It has an added advantage of biodegradability.

However, naturally occuring polymers lack in conformational stability and resistance to internal mass transfer. Whereas synthetic polymers such as poly(AAm), poly(HEMA), graft polymers and cross-linked copolymers offer stiffness as well as internal mass transfer resistance.

The aim of present work is to stabilize α-chymotrypsin by immobilization and to characterize the stabilities of the immobilized forms. We have investigated the stabilization of α-chymotrypsin towards the hydrolysis of low molecular weight (313.4) substrate L-BTEE and high molecular weight (23,600) substrate casein. We have explored basically two techniques : *insitu* entrapment of α-chymotrypsin (ENT-CT) into poly(AAm-co-HEMA) and covalent binding of α-chymotrypsin (CB-CT) to the beads of natural polysaccharide chitosan.

We have synthesized copolymer of AAm and HEMA in our laboratory by free radical polymerization at low temperature, and have prepared uniform spherical beads of chitosan, for the immobilization of α-chymotrypsin.

2.3 Experimental

➤ Chemicals used

α-Chymotrypsin (from *bovine pancreas* of strength 39 U/mg) Albumin, bovine, 96-99% (nitrogen - 15 6%, water - 5 9%) N-Benzoyl-L-tyrosine-ethyl ester N,N'-Methylene-bis-acrylamide (bis-AAm) Casein (molecular wt 23,600, 90% protein, 0 2% lactose, < 3% moisture)	:	Sigma Chemical Co., Ltd., USA
Tris-(hydroxymethyl)-aminoethane	:	Fluka, Germany
2-Hydroxyethyl methacrylate Hydrogen peroxide (30% w/w) Calcium chloride	:	E.Merck, Mumbai, India
Folin's reagent di-Potassium hydrogen orthophosphate Potassium dihydrogen orthophosphate Potassium sodium (+) tartrate	:	Glaxo, Mumbai, India

Methanol	:	Ranbaxy Lab. Ltd., Mumbai,
Acetic acid (glacial)	:	India
Acetonitrile	:	
Acrylamide	:	Spectrochem Pvt. Co., Mumbai,
Ascorbic acid	:	India
Chitosan [α-(1-4) 2-amino-2-deoxy β-D-glucan]	:	Central Institute of Fisheries Technology (CIFT), Cochin, India
Glutaraldehyde	:	
Dimethyl formamide	:	S.D.Fine Chemicals, Mumbai,
Dimethyl sulphoxide	:	India
Tetrahydrofuran	:	
Trichloro Acetic acid	:	Loba Chemical Co., Mumbai,
Metal chlorides (Cu, Ba, Sr)	:	India

All chemicals used were of Analytical grade and solvents were distilled before use. Double distilled deionized water was used throughout the work.

All the experiments were done in triplicate to ensure repeatability.

2.3.1 Preparation of organic polymeric supports

➤ **Synthesis of poly(acrylamide-co-hydroxyethyl methacrylate)**

AAm 14 g (200 mM), HEMA 15 g (100 mM), bis AAm 0.6 g (4 mM) and ascorbic acid 0.15 g (1 mM) were dissolved in 60 g water in three-neck 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 adding 0.25 mL of 30% hydrogen peroxide (2.4 mM) into a reaction mixture. 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.

➤ **Preparation of chitosan [α-(1-4) 2-amino-2-deoxy β-D-glucan] beads**

Following the procedure reported by Sun and Payne[87] chitosan beads were prepared by dissolving 4 g chitosan powder into 100 mL of 2% (v/v) acetic acid solution. The viscous chitosan solution was stirred overnight at 30 °C and then centrifuged at 7000 rpm for 15 min to remove undissolved chitosan and debris. The solution was added dropwise through a syringe into 10 N sodium hydroxide solution at room temperature. The beads formed were washed thoroughly with double distilled deionized water, until washings showed no colour with phenolphthalein. Beads were stored in double distilled deionized water at room temperature. The average diameter of the wet beads was ~ 2 mm.

2.3.2 Immobilization of α-chymotrypsin

Two different methods were used for the immobilization of α-chymotrypsin. Glutaraldehyde activated chitosan beads were used for covalent coupling, while poly(AAm-co-HEMA) was used for the *insitu* entrapment. The methods are described here briefly.

(a) *Insitu* entrapment of α-chymotrypsin

Entrapment of α-chymotrypsin in AAm/HEMA copolymer gel was done by modifying the earlier reported method.[88] As discussed in **section 2.3.1**, free radical copolymerization was initiated by adding hydrogen peroxide into a

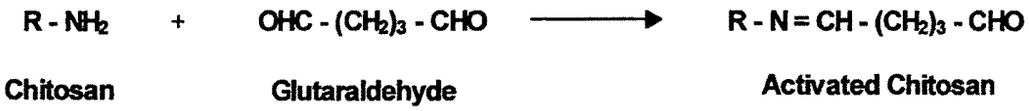
reaction mixture. But the enzyme was added after 5 min of initiation to minimize the deactivation effect of free radicals generated during polymerization.

(b) Covalent coupling of α-chymotrypsin

Covalent bonding involves the chemical conversion of a functional group of the polymer through multifunctional group reagent, prior to the coupling of the enzyme.

➤ Activation step

Semidry chitosan beads were activated with various concentrations of glutaraldehyde at room temperature for the activation of free amino groups. Excess of glutaraldehyde was washed with water and 100 mM borate buffer of pH 8.



The activation of chitosan by glutaraldehyde was confirmed through FTIR analysis. **Fig. 2.1** exhibits IR spectra of chitosan and activated chitosan. Free amino groups of chitosan react with glutaraldehyde and forms Schiff's base (CH = N - R) as shown in the above reaction. The spectra of activated chitosan exhibited additional band for Schiff's base formation at 1644 cm^{-1}. The other bands observed were at 1390 cm^{-1}, at 1078 cm^{-1} and at 1029 cm^{-1} for imines, aldehyde and amino overlap respectively.

➤ Coupling step

The activated chitosan beads were further used for the immobilization of α-chymotrypsin in 100 mM borate buffers of different pH at 4 °C. A low

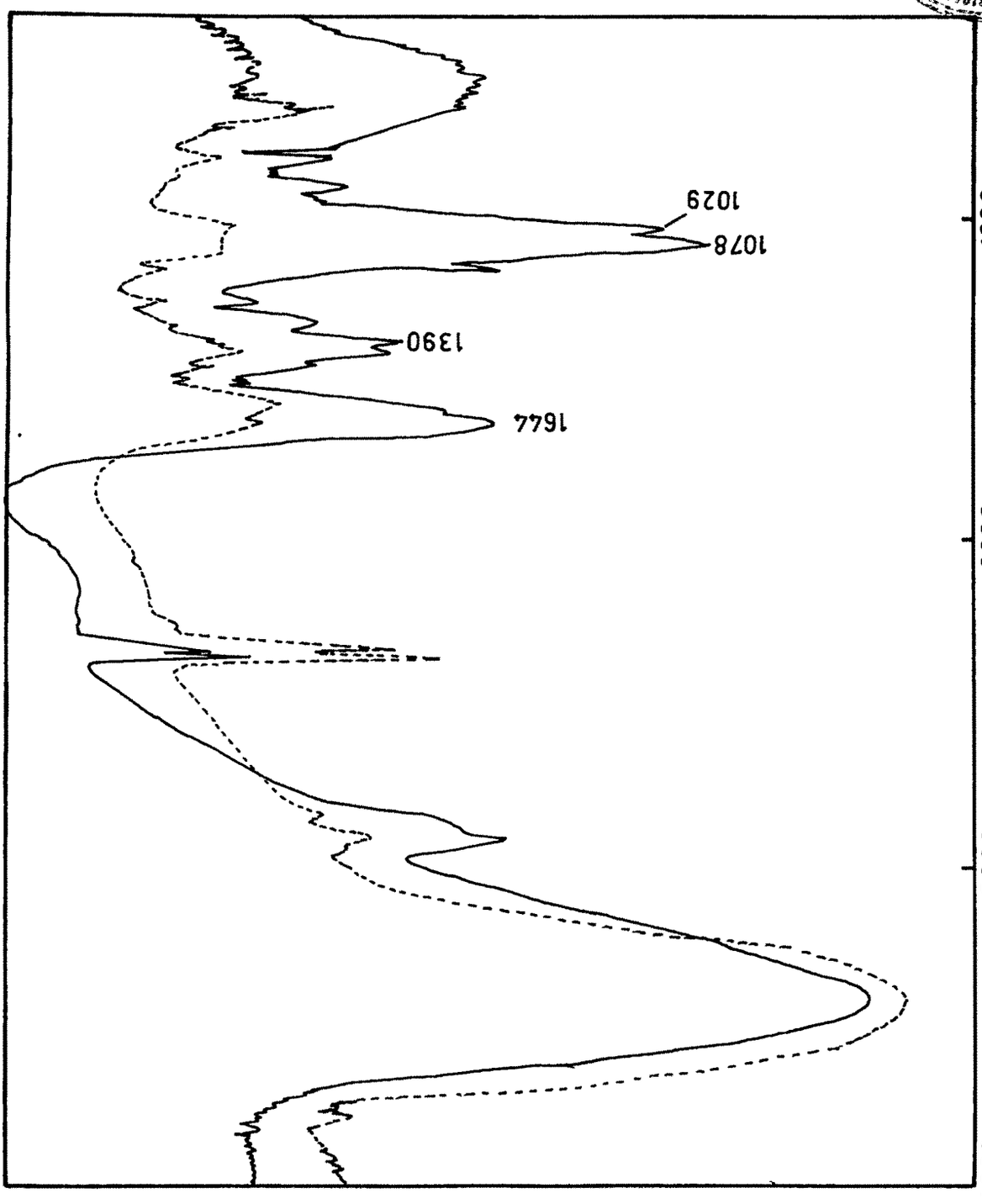
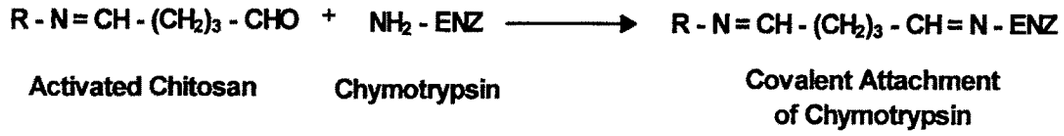


Fig. 2.1
Chitosan (------), Activated chitosan (——)

temperature shaker bath Julabo SW1 was used for the reaction. The amount of protein bound to the support was determined, using Lowry assay[89] procedure and the difference in protein concentration before and after immobilization.



2.3.3 Assay of α-chymotrypsin activity

The activity of free and immobilized enzyme was determined spectroscopically as per the Bergmeyer method[90] using low and high molecular weight substrates.

➤ **Measurement of enzyme activity with casein as substrate**

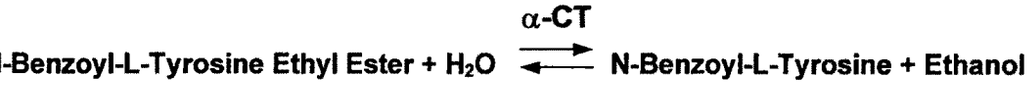
Casein is hydrolyzed by chymotrypsin with the formation of hydrolysis products whose tyrosine and cytophan content can be measured spectrophotometrically after precipitation of the residual substrate.

A typical reaction mixture consisting 0.9 mL (100 mM) 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 mM 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 after precipitation of the residual substrate.

The absorbance of the solution or the supernatant at 280 nm were plotted against the enzyme concentration to evaluate the enzymatic activity.

➤ **Measurement of enzyme activity with N-benzoyl-L-tyrosine ethyl ester (L-BTEE) as substrate**

N-benzoyl-L-tyrosine with an undissociated carboxyl group absorbs less between 250 and 260 nm than N-benzoyl-L-tyrosine with a dissociated carboxyl group. N-benzoyl-L-tyrosine ethyl ester behaves like undissociated N-benzoyl-L-tyrosine, so that hydrolysis of the ester can be followed by measurements of the extinction changes. By plotting the extinction against time a kinetic plot is obtained from which the initial rate of the reaction is determined.



The rate of hydrolysis of L-BTEE was monitored by incubating a reaction mixture containing 1.4 mL (1.1 mM) L-BTEE in 50% (w/w) methanol, 1.5 mL (80 mM) tris buffer of pH 7.8 (containing 50 mM Ca^{2+}), and 0.1 mL free enzyme solution (0.1 mg mL^{-1}) or equivalent amount of immobilized enzyme, for 2 min at 25 °C and measuring the absorbance of the solution at 256 nm. One unit enzyme activity is considered as the quantity required for the hydrolysis of 1.0 μmole of L-BTEE per min at pH 7.8 at 25 °C.

The activity of the immobilized enzyme is expressed as the relative activity in percent based on that of free enzyme.

2.3.4 Selection of AAm : HEMA ratio for *insitu* entrapment

To achieve the balance between various properties such as optimum stiffness, internal mass transfer resistance and excessive diffusion limitation, entrapment of enzyme was carried out in homopolymers of AAm and HEMA, and their copolymers with different compositions. Leakage of enzyme from the polymer after 2 h in buffer of pH 8 was determined.

2.3.5 Optimization of *insitu* entrapment

Entrapment of an enzyme is generally performed by polymerizing an aqueous solution containing monomer(s) and the enzyme in the presence of cross-linking agent. This technique was first used by Bernfeld et. al.[91] in 1963, who entrapped trypsin, papain, amylase and ribonuclease in a gel lattice of poly(AAm) in the presence of bis-AAm cross-linking agent and the polymerization was initiated by potassium persulphate and accelerated by β-dimethylamino propionitrile. However, in conventional entrapment procedures enzyme activity is lost due to the free radicals generated during polymerization. Hence, to minimize this effect and to achieve maximum entrapped enzyme activity (EEA), enzyme addition time and cross-linking extents were varied.

(a) Effect of enzyme addition time

By varying the time of addition of enzyme to the polymerizing gel, attempts were made to minimize the deactivation effect of free radicals generated during polymerization. The entrapment of α-chymotrypsin and retention of its activity with respect to the time of addition of enzyme during polymerization was examined.

(b) Effect of cross-linking

The effect of cross-linking on enzyme loading and retention of its activity was examined by using different concentrations of cross-linking agent bis-AAm during polymerization of AAm, HEMA and AAm/HEMA mixture. This was also studied on the basis of swelling of homopolymer as well as copolymer.

2.3.6 Optimization of covalent coupling conditions

Various coupling conditions were optimized for the better retention of activity and stability of immobilized α-chymotrypsin using 50 mg of semidry chitosan beads. Protein coupled per g of support was calculated by measuring enzyme activity by standard procedure.

(a) Effect of glutaraldehyde concentration

Activation of free amino groups of chitosan was carried out by using 50 mg of beads and 0.0 to 0 2% (v/v) glutaraldehyde solution. Beads were activated for 60 min and then washed with water and 100 mM phosphate buffer of pH 8. Activated beads were further coupled with 100 μg enzyme for 16 h and protein coupled was found from the supernatant liquid as well as beads.

(b) Effect of cross-linking time

Effect of cross-linking time for activation of chitosan beads (50 mg) was examined by varying time intervals ranging from 15 min to 90 min. Glutaraldehyde activated chitosan beads were washed with water and borate buffer of pH 8 and were equilibrated with 100 μg enzyme for 16 h coupling time. The coupled protein and its activity was measured as described earlier.

(c) Effect of pH of coupling medium

Effect of pH of coupling medium on the extent of enzyme immobilization was studied by coupling 100 μg of α-chymotrypsin with 50 mg of activated chitosan beads at pH 3 - 10. The pH of the coupling medium was maintained by using 100 mM potassium phosphate buffer. The coupling was carried out for 16 h.

(d) Effect of enzyme concentration

Effect of enzyme concentration on the loading of enzyme was studied by using 12.5 to 250 μg (0.5 to 8.0 U) of α-chymotrypsin and 50 mg of activated chitosan beads. The coupling was carried out at pH 8 for 16 h. The protein coupled and active protein was calculated as described earlier.

(e) Effect of coupling time

The optimum time required for the coupling of enzyme was determined by measuring the amount of protein coupled on the support at various time intervals. 100 μg enzyme in pH 8 buffer was added to the activated chitosan beads and coupled protein was measured at various time intervals ranging from 30 min to 16 h.

2.3.7 Comparative account of free and immobilized α-chymotrypsin

Enzymes, like people, are affected by the surroundings in which they find themselves. Surrounding an enzyme with a high concentration of insoluble polymer is similar to locking a man in a room. Just what the enzyme or the man can do and how efficiently it or he can do it, will depend entirely on what is allowed into or out of the environment and what physical constraints are present. The polymer matrix to which the enzyme is attached may prevent substrate from passing to the enzyme or may prevent sufficient movement for it to perform its task. Thus, the change in enzymatic activity is considered to be caused by physical and chemical properties of the carriers and methods used for the immobilization.

Hence, free and immobilized enzyme activity with respect to pH, thermal stability, storage stability and stability in water miscible organic solvents was compared. Kinetic behaviour and reusability of the immobilized enzyme were also investigated. Effect of activators and inhibitors on enzymatic activity was also determined.

(a) pH activity profile

The activity of the free and immobilized α-chymotrypsin was measured by incubating free and immobilized enzyme in the buffer of different pH using casein as a substrate at 35 °C for 20 min

(b) Thermal stability

The catalytic activity of enzyme increases with elevation of temperature as in the case of usual chemical catalysts. But as enzyme consists of protein and which is generally unstable to heat, the enzymatic reaction can not be practically carried out at high temperature. If the heat stability of an enzyme is enhanced by immobilization the potential utilization of such enzyme will be extensive.

Free and immobilized enzymes were placed in the optimum pH buffer and incubated at different temperatures for different time intervals. Immediately after cooling to 10 °C, the activity of the enzyme was determined as described earlier. The thermodeactivation constant (K_d) was calculated by using following equation.[92]

$$\ln A_t = \ln A_o - K_d (t)$$

where 'A_o' is the initial activity and 'A_t' is the activity after heat treatment for 't' minutes.

(c) Storage stability

Free and immobilized α-chymotrypsin were stored at room temperature (30 °C) in borate buffer of pH 8 and the activity of the enzymes was determined periodically.

(d) Determination of kinetic constants

Kinetic parameters K_m and V_{max} are usually applied to an enzyme in order to describe its catalytic characteristics. K_m and V_{max} are the constants of a rate equation derived to describe enzyme activity in dilute solution. Under such conditions the equation relating the reaction velocity(V) and the substrate concentration (S) describes a rectangular hyperbole. When the reciprocals of V and S are plotted against each other the result is a straight line graph, the intercepts of which give the reciprocal values of K_m and V_{max} **(Fig. 2.2)**. This plot is known as Lineweaver-Burk plot.

The rate of 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. The Michaelis constant K_m and maximum reaction rate V_{max} were determined from the Lineweaver-Burk plots of 1/s vs. 1/v for free and immobilized enzymes. K_m and V_{max} values were also calculated by keeping substrate concentration fixed and varying the concentration of free and immobilized enzyme.

(e) Reusability of immobilized α-chymotrypsin

Conventionally enzymatic reactions have been carried out in batch processes by incubating a mixture of substrate and soluble enzyme. It is technically impossible to recover the active enzyme from the product mixture and hence it is

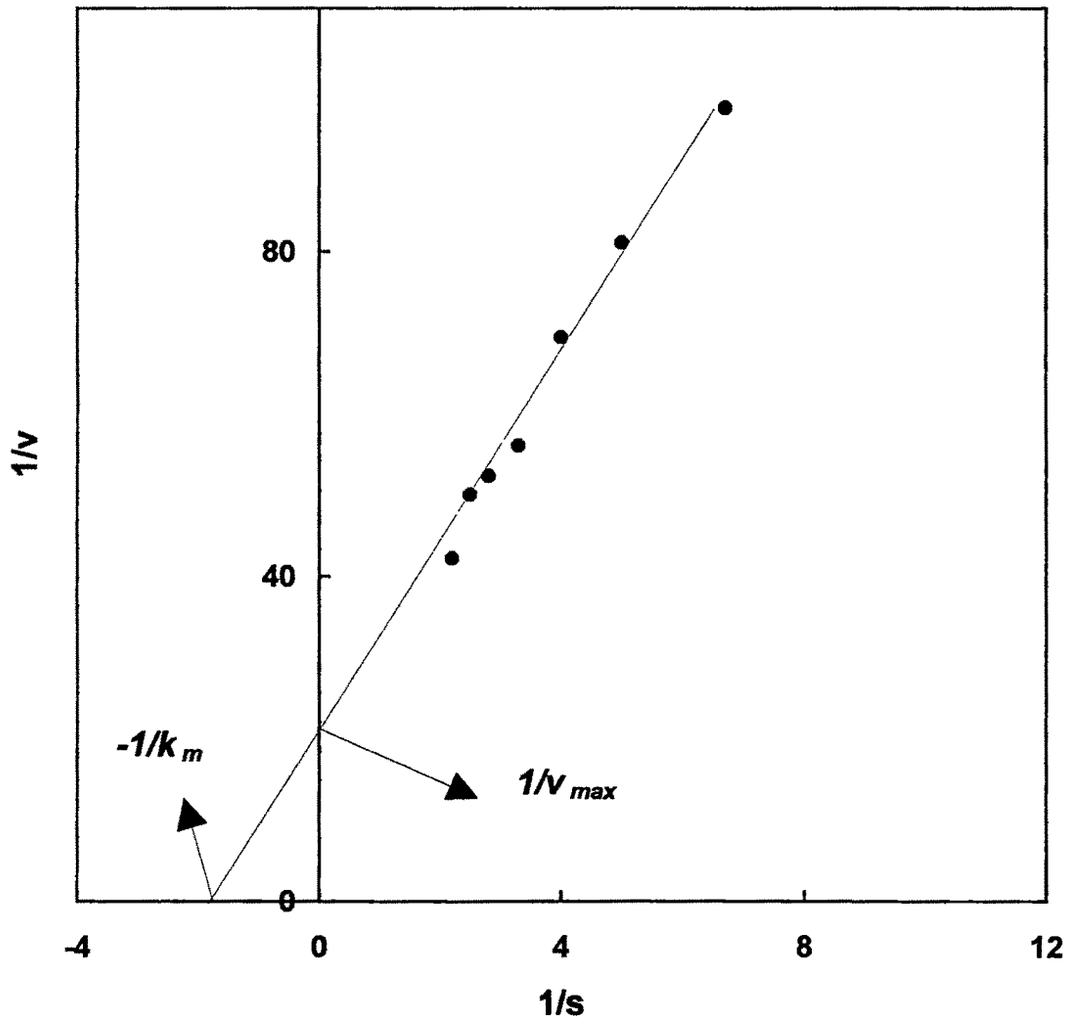


Fig. 2.2 Lineweaver-Burk Plot

irrecoverable loss and one can not reuse expensive enzyme. This can be overcome by using immobilized enzyme.

To evaluate reusability of the immobilized α-chymotrypsin it was washed with water and buffer after each use and then suspended again in a fresh reaction mixture to measure the enzymatic activity. This procedure was repeated till enzyme lost 50% of its activity. Reusability of immobilized α-chymotrypsin was examined by using casein as substrate. Leakage of the enzyme, if any was determined by measuring the enzyme activity in the washings.

(f) Effect of water miscible organic solvents

Stabilities towards various organic solvents are important for the application of immobilized enzymes. If enzymatic reactions can be carried out in organic solvents, it could greatly increase the utility of immobilized enzymes as catalysts for organic synthesis.

Enzyme stability towards solvents was determined by measuring the activity of the free and immobilized α-chymotrypsin in the different concentrations of water miscible solvents such as dimethyl formamide, dimethyl sulphoxide, acetonitrile, methanol, and tetrahydrofuran and using casein as substrate.

2.3.8 Effect of metal ions on enzyme activity

(a) Effect of alkaline earth metal ions

In addition to the nature of the solvent and the water content it has been reported that the activity of enzymes can be dramatically altered by small amounts of additives. Recent studies have shown that activity of enzyme in organic solvents can be greatly increased by organic additives such as amides

or amines. Kise and Yamamoto[93] observed that catalytic activity of chymotrypsin for transesterification in ethanol was increased by addition of tertiary amines. They reported the results using triethylamine, tri-n-propylamine, tri-n-bytylamine and tri-n-octylamine as additives.

However, effect of metal salts on enzyme activity in organic solvents has rarely been investigated. Recently, it was found that calcium ion greatly increases the catalytic activity of α-chymotrypsin for esterification, transesterification and hydrolysis in ethanol by Kise and Sasaki.[94] They observed that reaction rate of transesterification of N-acetyl-L-tyrosine methyl ester to N-acetyl-L-tyrosine ethyl ester was increased by addition of $CaCl_2$. However, hydrolytic activity of casein in organic solvents has not been studied extensively. Hence the reaction rate was measured in the presence of several divalent metal chlorides of Mg, Ca, Sr and Ba, at 10% methanol concentration.

(b) Metal inhibition

It is known that heavy metal ions are inhibitors to α-chymotrypsin. Hence activity of free and immobilized enzymes was assayed in the presence of different concentrations of copper and nickel ions. Similar type of study was also carried out by Guoliang and Furusaki.[72]

2.3.9 Hydrolysis of casein in packed bed reactor

To examine the potential of immobilized enzyme at industrial level it is necessary to compare the efficiency of free enzyme (at batch scale) with efficiency of immobilized enzyme either at batch or at a continuous reactor scale. However, we have carried out hydrolysis of casein in packed bed reactor using immobilized enzyme and in a batch reactor using free enzyme.

The schematic diagram of the packed bed reactor used for the study of continuous hydrolysis of casein is given in **Fig. 2.3**. The compact column of 1.2 x 20 cm dimensions containing α-chymotrypsin immobilized on chitosan beads was prepared by placing porous frit at the bottom of the reactor. The reactor was maintained at constant controlled temperature by circulating water through outer jacket. The packed column bed was washed with borate buffer of pH 8 before operation. The substrate was passed through the column by means of peristaltic pump. The extent of hydrolysis was measured at different concentrations of casein and flow velocities. The efficiency of the reactor was measured from the residual casein after precipitation by trichloro acetic acid by standard assay procedure.

(a) Optimization of column conditions

➢ **Effect of temperature**

Effect of column temperature on the continuous hydrolysis of casein by immobilized α-chymotrypsin was studied by varying the reactor temperatures from 30 to 50 °C. From the results column efficiency for the hydrolysis of casein was calculated.

➢ **Effect of flow rate**

Flow rate of substrate in packed bed reactor is a critical parameter which affects the product conversion. By varying the substrate flow rate in the immobilized enzyme reactor the residence time of substrate varies and hence affects the product conversion. Hydrolysis of casein was studied by varying the flow velocities from 12 mL h^{-1} to 36 mL h^{-1}, at different concentrations of substrate ranging from 0.125 mM to 1 mM.

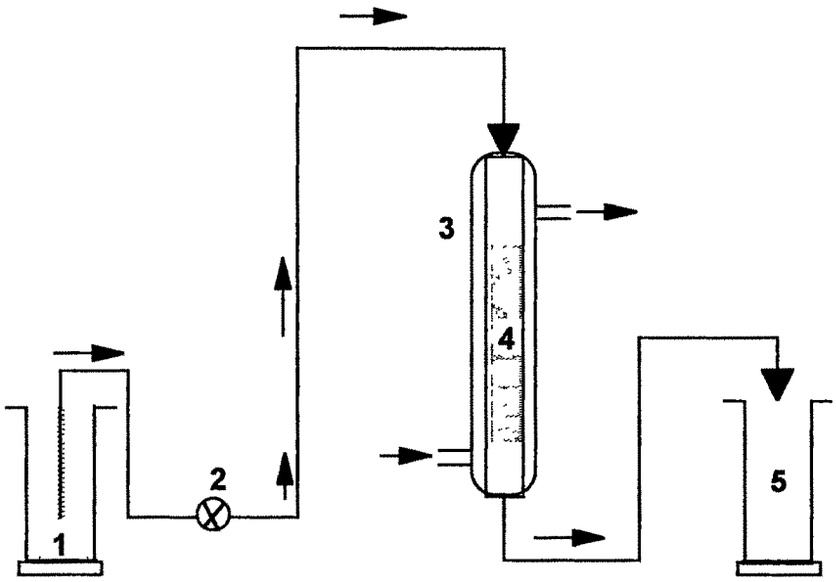


Fig. 2.3 Schematic diagram of continuous flow packed bed Bioreactor

1. **Substrate**
2. **Peristaltic pump**
3. **Bioreactor**
4. **Immobilized enzyme**
5. **Product**

➤ **Operational stability**

The operational stability of immobilized enzymes is an important factor in the success or failure of industrialization of immobilized system. This operational stability directly affects the cost of the enzyme and of repackaging or regeneration of deteriorated enzyme column.

The operational stability of the immobilized α-chymotrypsin was determined by operating the reactor continuously at constant temperature (35 °C) and 24 mL h^{-1} flow rate for varying time intervals and measuring the residual activity periodically.

2.4 Results and Discussion

The catalytic efficiency of enzyme depends to a very large extent on the choice of the support used for immobilization and the method used for immobilization. Hence we have tried to optimize AAm : HEMA ratio to achieve maximum enzyme entrapment.

2.4.1 Selection of AAm/HEMA ratio for entrapment

Use of poly(HEMA) or poly(AAm) gels for the enzyme entrapment is well studied process. Poly(HEMA) is non toxic, biocompatible and hydrophilic in nature. It is very inert towards microbial contamination and resistant to attack by many chemicals. Cross-linked poly(HEMA) has adequate mechanical strength and it also contributes to resistance to internal mass transfer. On the other side poly(AAm) is more hydrophobic and is generally employed for the immobilization of enzymes by adsorption and requires high degree of cross-linking. Triantatyllou et. al.[70] prepared different cross-linked poly(AAm) gels using bis-AAm cross-linking agent for the immobilization of α-chymotrypsin. They

reported 5.9 U g^{-1} enzyme activity with 47% cross-linking in comparison to 0.5 U g^{-1} enzyme activity with 10% cross-linked poly(AAm).

Hence the ratio of HEMA to AAm is critical for the structure stability and desired mass transfer. Therefore 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 **Fig. 2.4.** 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.

2.4.2 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 **Fig. 2.5.** 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 90% 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 with the progress of polymerization. In 5 min ~70% polymerization **(Fig. 2.6)** takes place converting the reaction mass into a viscous gel and hence addition of enzyme after 5 min of polymerization was not practical. The copolymerization reaction was terminated by adding methanol in the reaction mixture.

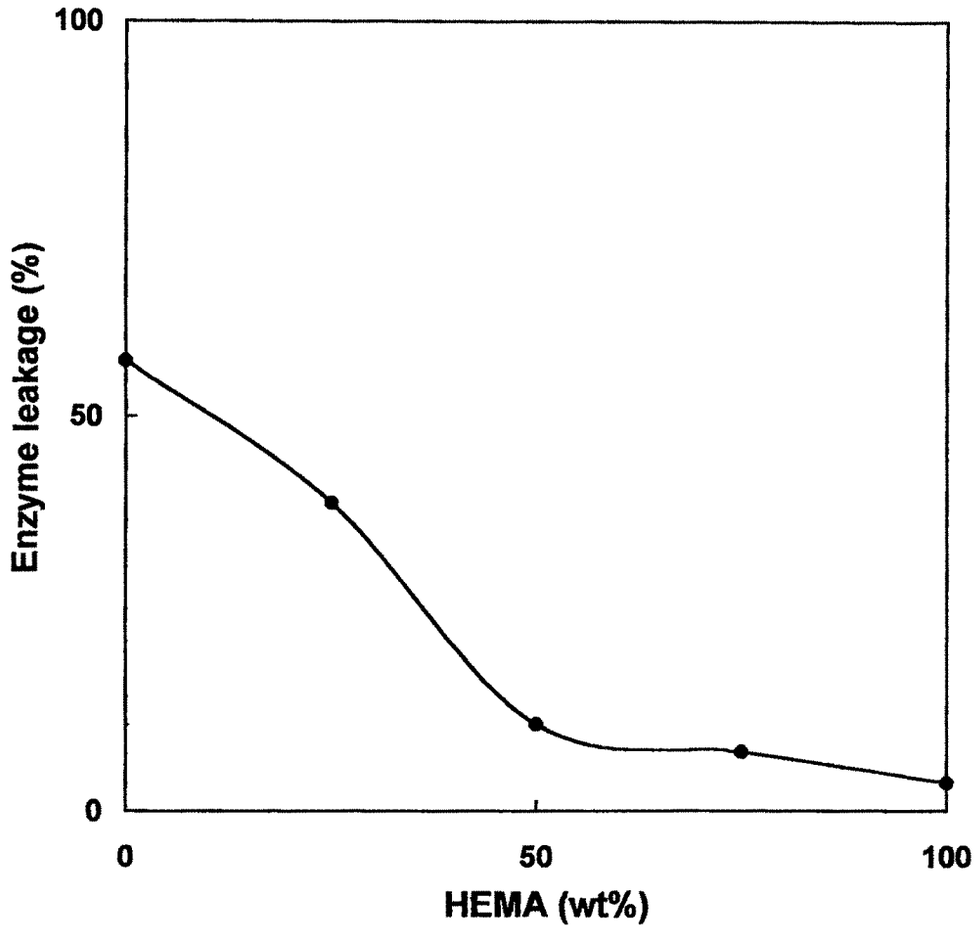


Fig. 2.4 Effect of copolymer composition on leakage of entrapped α-chymotrypsin at pH 8 after 2 h

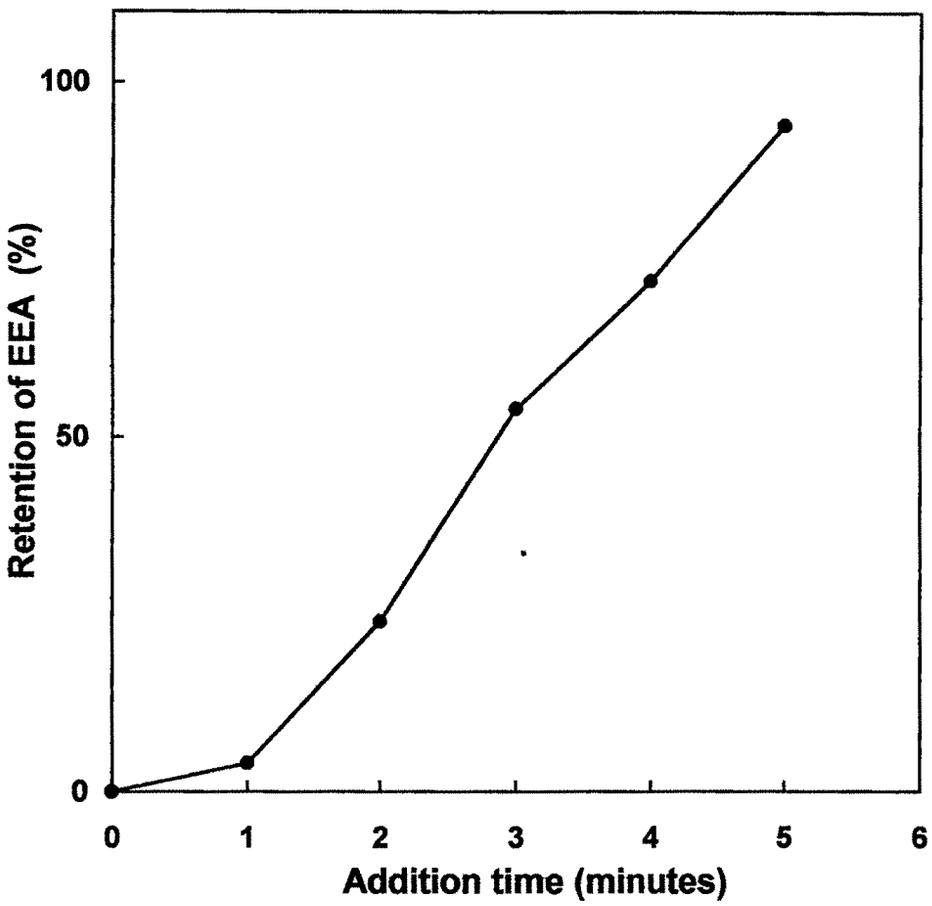


Fig. 2.5 Effect of enzyme addition time on entrapped enzyme activity

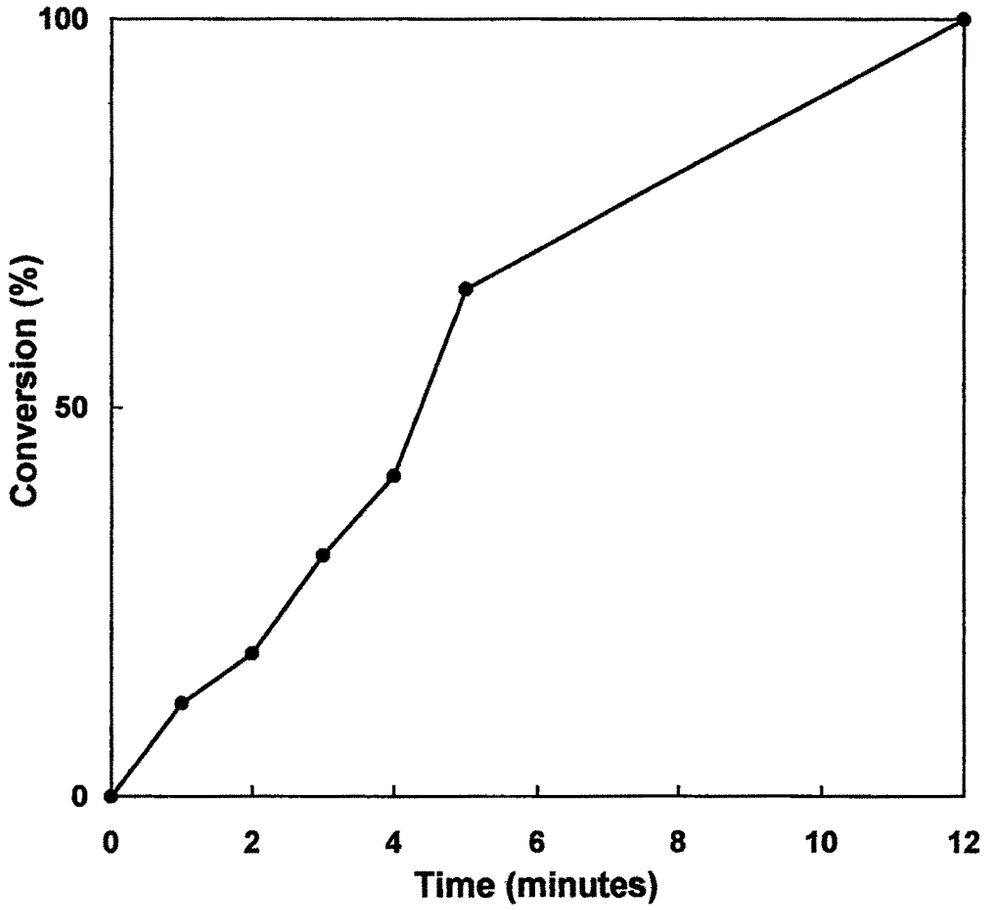


Fig. 2.6 Effect of time on polymer conversion

2.4.3 Effect of cross-linking on EEA

Effect of cross-linking on EEA was studied by using 0.5 to 10% bis-AAm cross-linker during polymerization of AAm, HEMA and AAm/HEMA mixture. The results obtained are given in **Fig. 2.7**. Enzyme activity was tested using casein substrate. EEA was observed to decrease with increasing cross-linking for homo as well as copolymer gel. However, loss of activity was relatively lower in case of AAm/HEMA copolymers.

When no cross-linking agent was used EEA could not be examined due to solubility of polymer in water. When cross-linker concentration was varied it was observed that upto 2% concentration EEA remained almost unaffected, but above 2% cross-linking drastic decrease in EEA was observed. 50% reduction in EEA was observed when cross-linking extent was 6% and above. This can be attributed to increased three dimension network inhibiting the diffusion of substrate and the product to and from the enzyme site.

This can be supported from the data observed in swelling studies. From the **Fig. 2.7** it is observed that at lower cross-linking the swelling of all the polymers is high and diffusion of substrate into the gel becomes easy and hence EEA is high. However, at very low cross-linking the copolymers did not have stiffness but with 2% cross-linking copolymer of AAm/HEMA had adequate swelling capacity to diffuse the substrate and hence the EEA is very high. Above 2% cross-linking the % swelling is very low except for poly(AAm) and hence diffusion of substrate into polymer network is restricted. Such type of diffusional limitations were also reported by Oste et. al.[95] in the entrapment of α-chymotrypsin in highly cross-linked poly(AAm) gel.

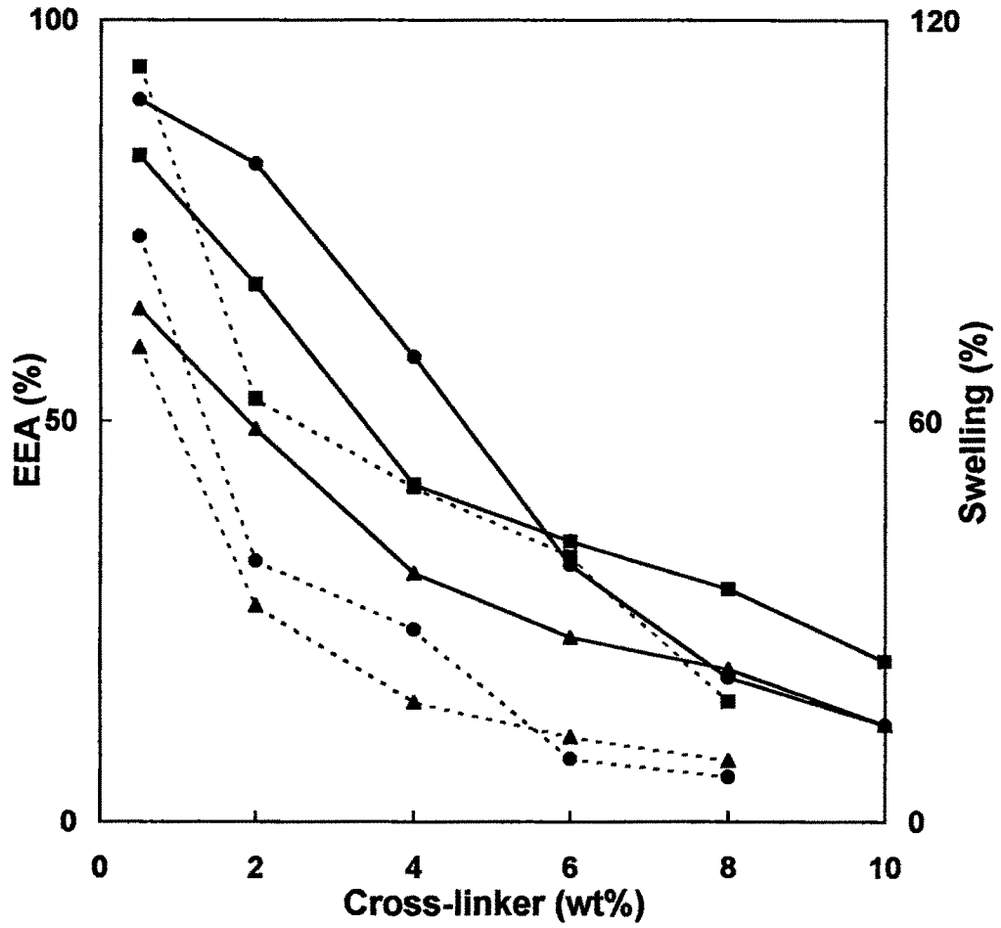


Fig. 2.7 Effect of polymer cross-linking on α-chymotrypsin activity and % swelling

poly (AAm) (■), poly (HEMA) (▲), poly (AAm-co-HEMA) (●)
Swelling (----), EEA (—)

2.4.4 *Insitu* entrapment of α-chymotrypsin

Thus, *insitu* entrapment of α-chymotrypsin was done with 2% cross-linker and by addition of 10 mL buffer solution of pH 8 containing 400 U of enzyme after 5 min of initiation time. At this stage the solution was stirred more vigorously to obtain uniform enzyme entrapment in the polymer matrix. The entrapped α-chymotrypsin was stored at 4 °C till further use. The assay of entrapped α-chymotrypsin activity was done as per the literature method using casein substrate. 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. Approximately 90% of enzyme activity was observed to be retained after entrapment.

2.4.5 Comparative account of free and entrapped enzyme

(a) pH activity profile

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 various pH using casein as substrate. It was observed from the results given in **Fig. 2.8** 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.

(b) Thermal stability

The thermal stability of the enzyme is one of the most important criteria for its application. Free and entrapped enzymes in 50 mM 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

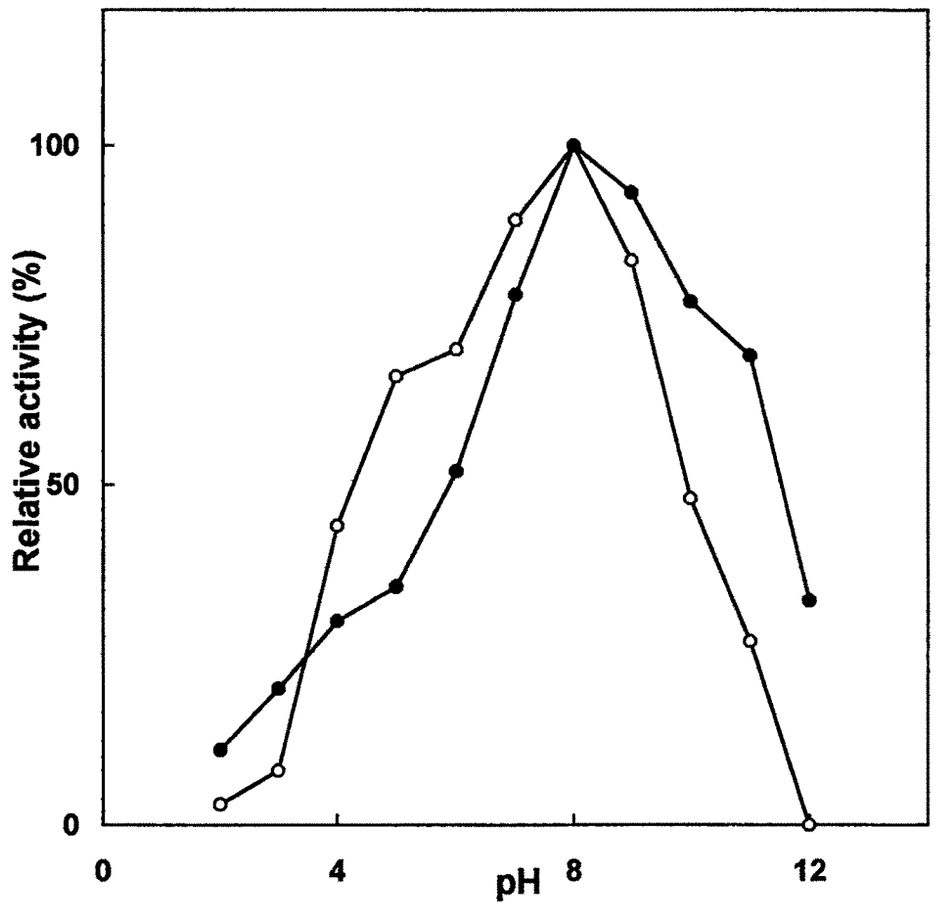


Fig. 2.8 pH activity profile for free (O) and immobilized (●) α-chymotrypsin using casein as substrate

procedure described earlier for casein substrate. Results obtained in triplicate are illustrated in **Fig. 2.9**. 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.

(c) Storage stability

Enzymes being heat sensitive catalysts generally need low temperature storage. Immobilization of enzyme can overcome this constrain 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 mM 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 **Fig. 2.10**.

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 entrapped 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. **(Fig. 2.11)** Extent of leaching was observed to go upto 15% after 10 days.

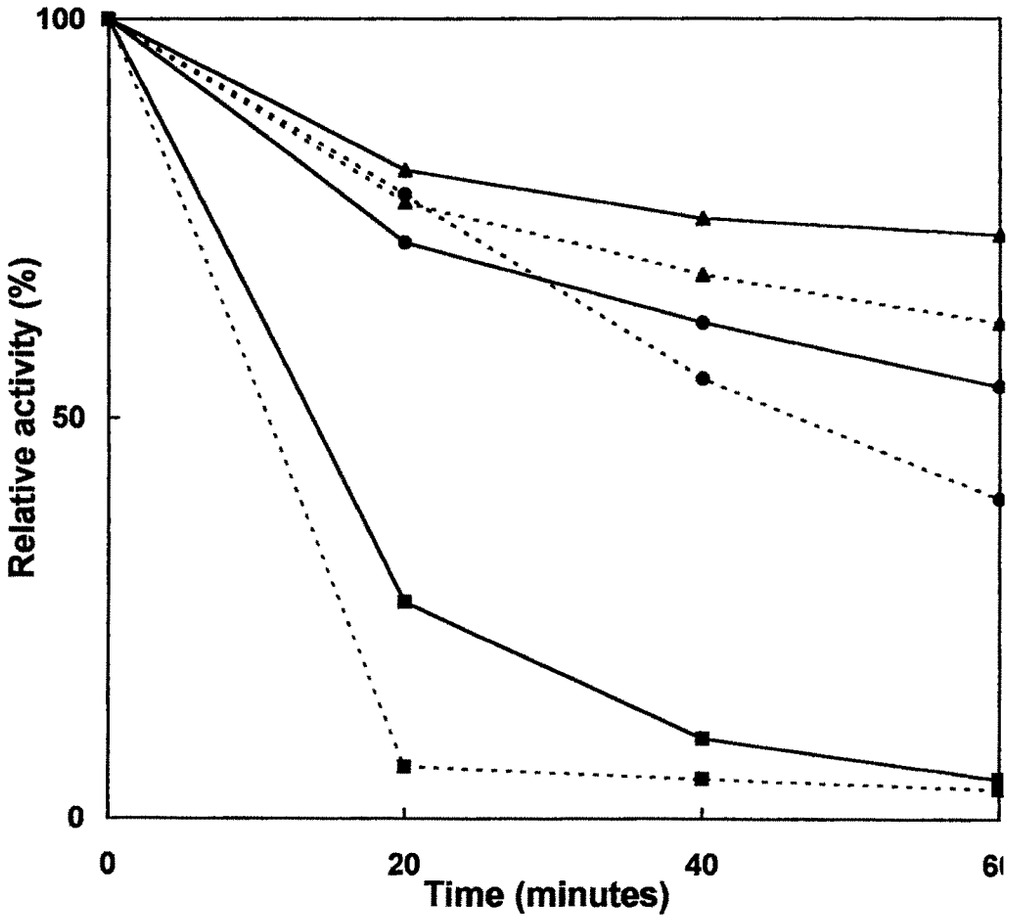


Fig. 2.9 Thermal deactivation of free (----) and entrapped (—) α-chymotrypsin at

40 °C (▲), 50 °C (●), 60 °C (■)

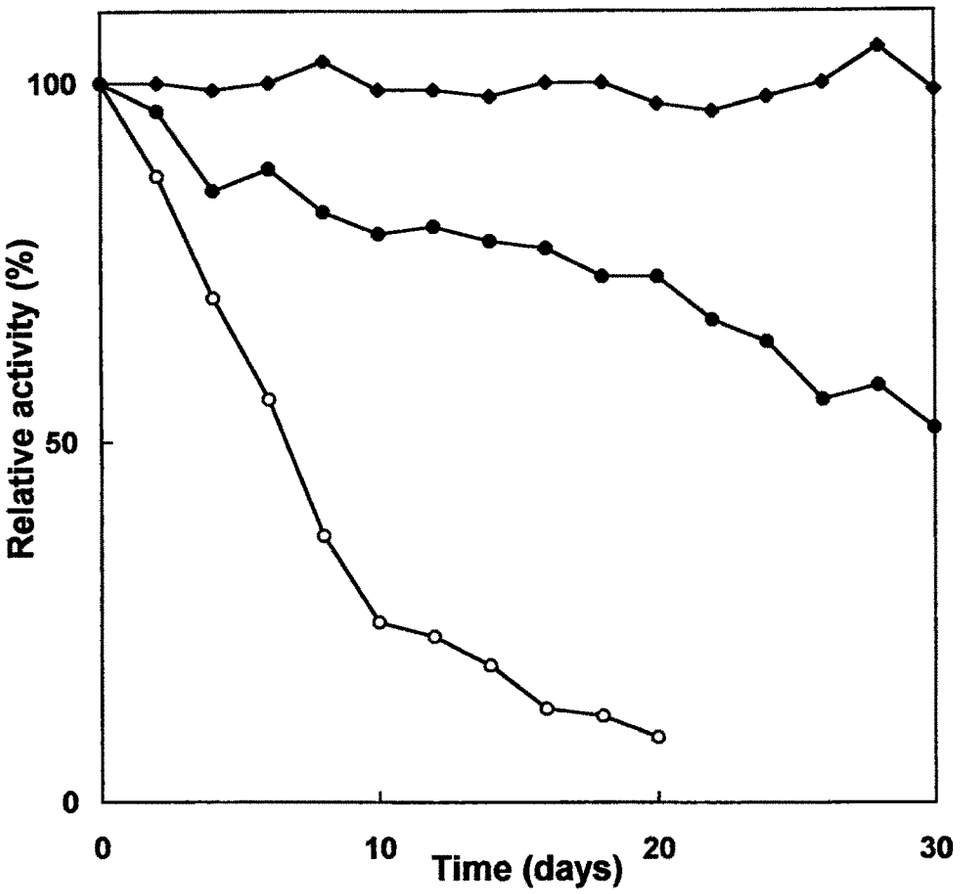


Fig. 2.10 Storage stability of α-chymotrypsin at 30 °C

In pH 8 buffer, free (O), entrapped (●)

without buffer(dry) entrapped (◆)

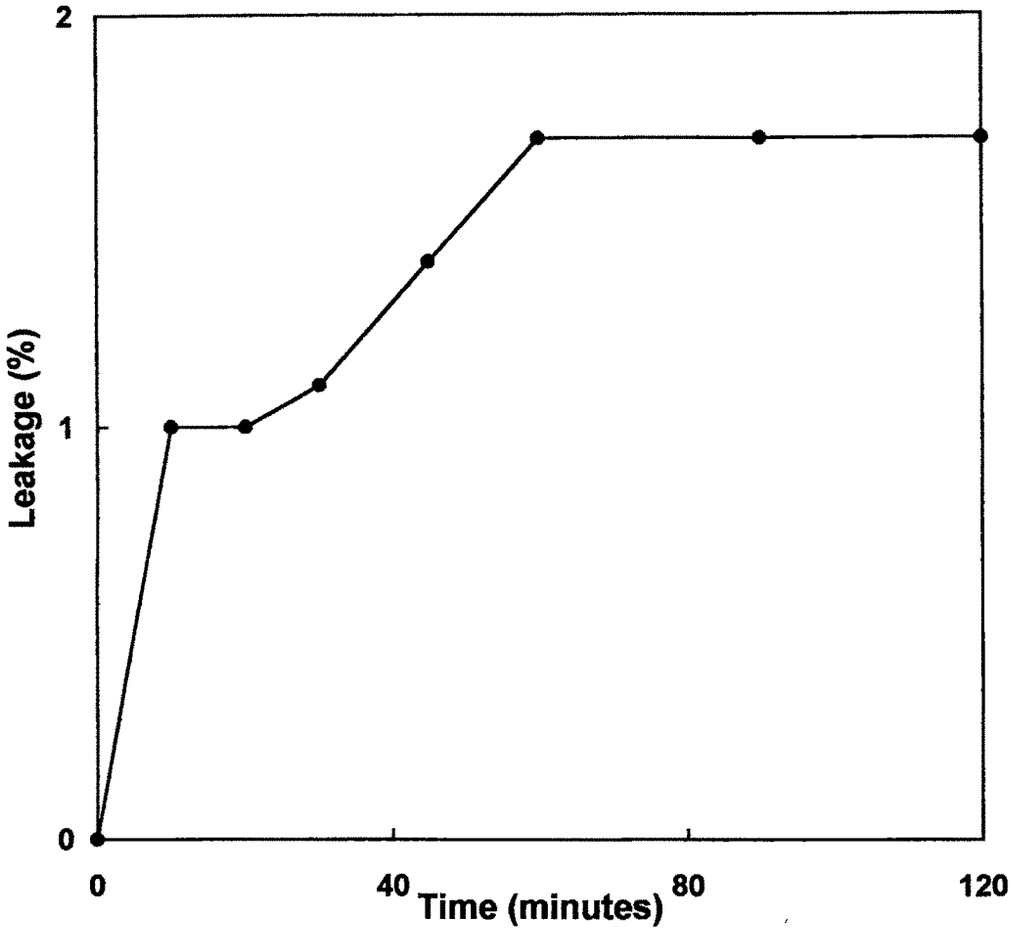


Fig. 2.11 Leaching of entrapped enzyme in borate buffer of pH 8

(d) 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 substrates from 0.6 x 10^{-3} to 4.23 x 10^{-3} mM and 0.2 to 2.0 mM respectively. For the study 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 2.1**. 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 x 10^{-3} mM 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.

(e) Reusability of entrapped enzyme

Free enzymes suffer from a major drawback of non reusability. 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 **(Fig. 2.12)**. This can be compared with the results reported in immobilization of α-chymotrypsin through adsorption,[96] where 35% residual

Table - 2.1

Kinetic parameters for free and entrapped α-chymotrypsin

Substrate	K_m (mM)		V_{max} (mM min^{-1})	
	Free	Entrapped	Free	Entrapped
Casein*	2.7×10^{-3}	4.2×10^{-3}	5.95×10^{-2}	4.84×10^{-2}
L-BTEE**	38.46	31.25	13.74	16.2

* at 35 °C, pH 8.0 for 20 minutes

** at 25 °C, pH 7.8 for 2 minutes

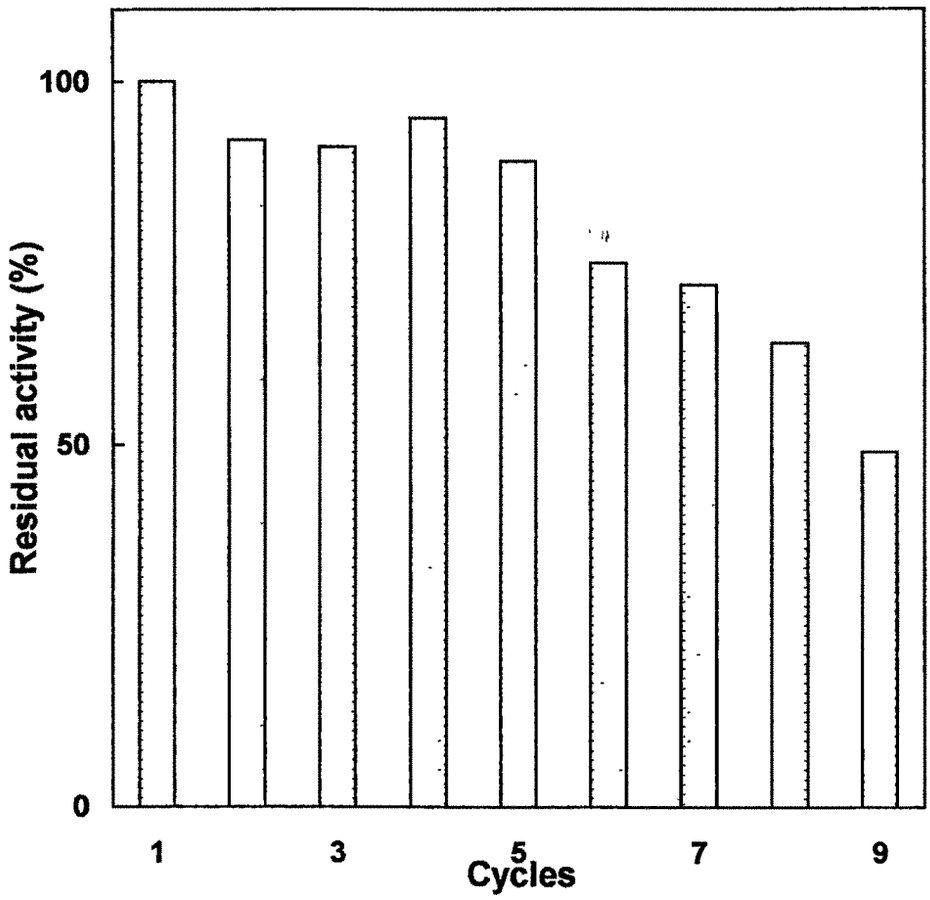


Fig. 2.12 Reusability of entrapped α-chymotrypsin at 35 °C and pH 8

activity was retained after nine repeated cycles. It should be noted that in both the cases immobilization is through physical process and not through chemical bonding. Hence the present system shows better prospectus for commercial applications.

(f) Effect of water miscible solvents on casein hydrolysis

Many enzymatic reactions are carried out in organic media. Hence retention of enzyme activity in organic solvents such as DMF, DMSO, MeOH, CH_3CN and 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 2.2)** 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.

2.4.6 Effect of metals on EEA

(a) Effect of alkaline earth metals on EEA

Various additives such as amides, amines and calcium in organic solvents have shown activating effect on the α-chymotrypsin activity. Hence hydrolysis of casein in 10% methanol in the presence of several metal chlorides of Ca, Mg, Sr and Ba was carried out. **(Fig. 2.13)** The concentration of these metal salts was in the range of 0 mM to 10 mM. By addition of about 2.5 mM $CaCl_2$, the reaction rate was observed to increase by 20% in both free and immobilized enzymes. Chlorides of Mg, Sr and Ba also accelerated the reaction, but the magnitude of the increase in rate was much smaller than that by $CaCl_2$. When different

Table - 2.2

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
CH_3CN	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

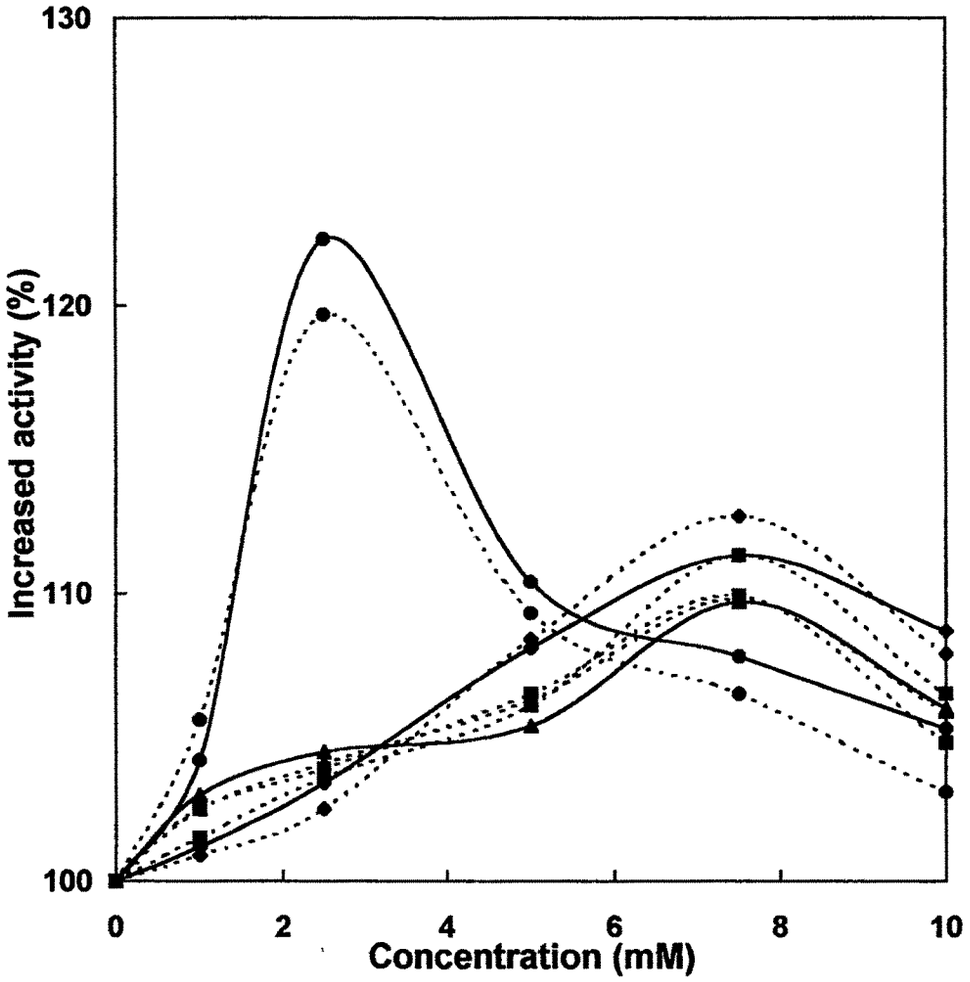


Fig. 2.13 Effect of divalent metal ions on α-chymotrypsin

Free (----), Entrapped (——)

Ca^{2+}(●), Mg^{2+}(▲), Ba^{2+}(■), Sr^{2+}(◆)

calcium salts were used the rate of hydrolysis remained unaltered indicating no effect of anions on hydrolysis of casein. Similar types of results were reported by Kise and coworkers.[93,94] They observed that by addition of about 50 μM $CaCl_2$ in ethanol, the reaction rate increased about 4 fold. They also observed that about 10 fold reaction rate increased by addition of tertiary amines for transesterification by α-chymotrypsin in ethanol. However, the product yield after 24 h reactions are very low as compared to that for the reactions without amines.

In serine proteases like chymotrypsin, the Ca^{2+} ion is not essential for enzymatic activity, but plays more of structural role. On addition of such specific ions, they may interact with various ligands from the protein molecule itself and in doing so help enzyme to maintain the three dimensional structure which is essential for activity. Some times metal ions act as a Lewis acid and assist the enzyme in the catalytic process and hence activity enhances.

(b) Effect of heavy metals on EEA

It is known that heavy metal ions are inhibitors to α-chymotrypsin. Hence activity of the free and entrapped enzyme was assayed in the presence of different concentrations (0 - 2 mM) of copper and nickel ions. From the results in **Fig. 2.14** 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.

This can be explained as when the metal ion binds to the enzyme near or at the end of active site, prevents the substrate from binding properly. The inhibitor and the substrate are thus competing for the active site of the enzyme and hence enzyme activity reduces. If the metal ion does not bind at or near the active site

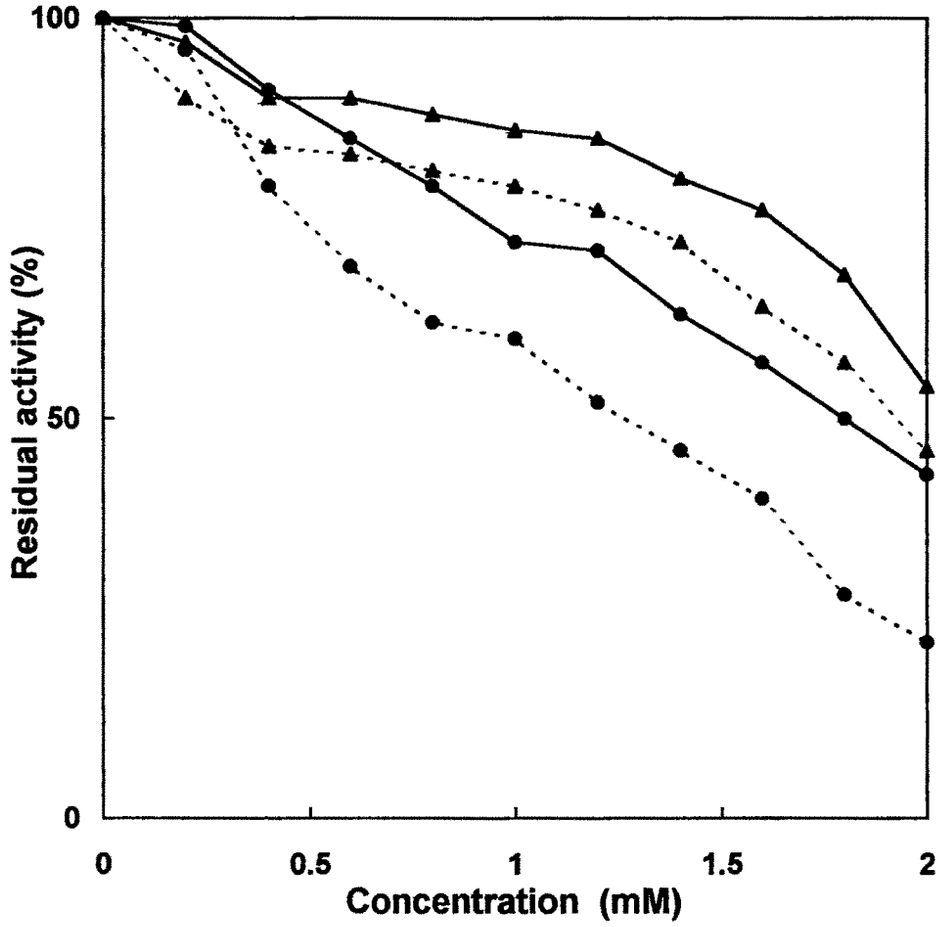


Fig. 2.14 Effect of heavy metal ions on α-chymotrypsin

Free (----), Entrapped (—), Cu^{2+} (●), Ni^{2+} (▲)

but causes a change in the shape of the active site on binding, causing the reduction in the enzyme activity.

2.4.7 Optimization of conditions for covalent coupling

Various coupling conditions optimized for the immobilization of α-chymotrypsin through covalent binding are described here.

(a) Effect of concentration of cross-linking agent

For the exhibition of maximum activity immobilized enzyme needs to be freely flowing in the substrate solution and should not adhere to the support used for immobilization. For this purpose various cross-linkers discussed earlier in **section 2.3.6** are used which can keep only one end of the enzyme attached to the support and remaining part of the enzyme can be in substrate solution. Hence the amount of cross-linking reagent used in the reaction significantly influences the amount of coupled enzyme and its activity. The results obtained in the study of effect of glutaraldehyde concentration for the activation of chitosan beads are given in **Fig. 2.15**. Maximum coupling of α-chymotrypsin 40 U g^{-1} of support is achieved with 0.02% glutaraldehyde concentration. Further increase in the concentration of glutaraldehyde decreases coupled protein to 6 U g^{-1}. The observed decrease in enzyme activity may be due to denaturation of enzyme with higher concentration of activator. Higher concentration of glutaraldehyde renders chitosan beads char.[97]

(b) Effect of cross-linking time

Cross-linking time is another important condition for achieving maximum coupling of enzyme. Cross-linking time was varied from 15 min to 90 min and

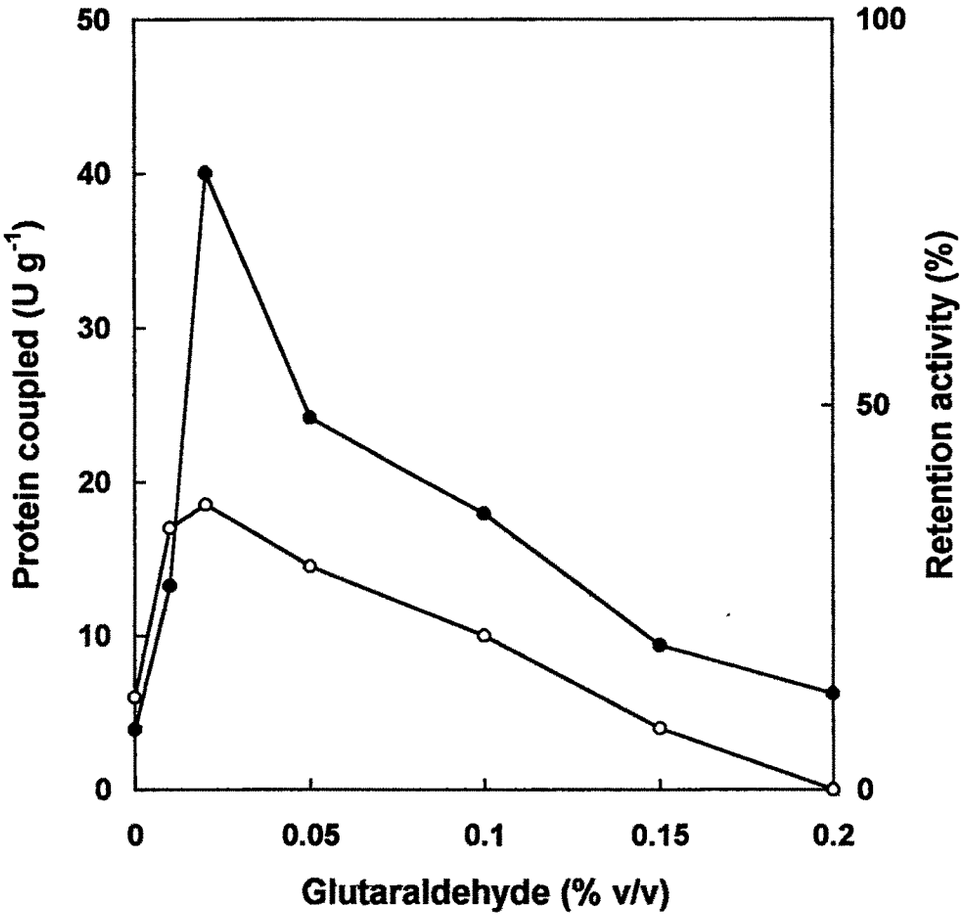


Fig. 2.15 Effect of glutaraldehyde concentration on immobilization of α-chymotrypsin :

Support 50 mg, Cross-linking time 60 mins, Enzyme concentration 100 μg, pH 8, Incubation time 16 h.

Protein coupled (●), Retention activity (O)

enzyme was coupled by incubating it at pH 8 for 16 h at 30 °C. The results obtained are given in **Fig. 2.16**. From the results it is observed that maximum coupling of enzyme is achieved when the cross-linking time was 45 min.

(c) Effect of pH

The effect of pH of the coupling medium on the extent of immobilization of α-chymotrypsin was studied in the pH range 3 to 10 using 100 mM potassium phosphate buffer. From the results **(Fig. 2.17)**, it was observed that with increasing pH of coupling medium protein coupling increases upto pH 8. At high and low pH it decrease to 13 U and 3 U g^{-1} of support respectively.

(d) Effect of concentration of enzyme

Commercial utility of the immobilized enzyme will depend upon the loading capacity of the enzyme on the support. Hence variation in enzyme from 10 U to 160 U per g of support was done during immobilization. The maximum loading of the enzyme was achieved when 40 U g^{-1} of protein was used. **(Fig. 2.18)** Use of higher enzyme concentration did not alter the extent of immobilization indicating the limit of activation and hence coupling extent.

(e) Effect of coupling time

The effect of coupling time on the extent of immobilization of α-chymotrypsin was studied for various time intervals of 30 min to 15 h. The results are given in **Fig. 2.19**. It was observed that within 6 h almost quantitative coupling of α-chymotrypsin takes place indicating the fast coupling reaction with the activated support. Further increasing coupling time could not increase coupled protein. This indicates that within 6 h the amount (59 U g^{-1}) of enzyme bound on the surface of chitosan beads has reached a saturated point.

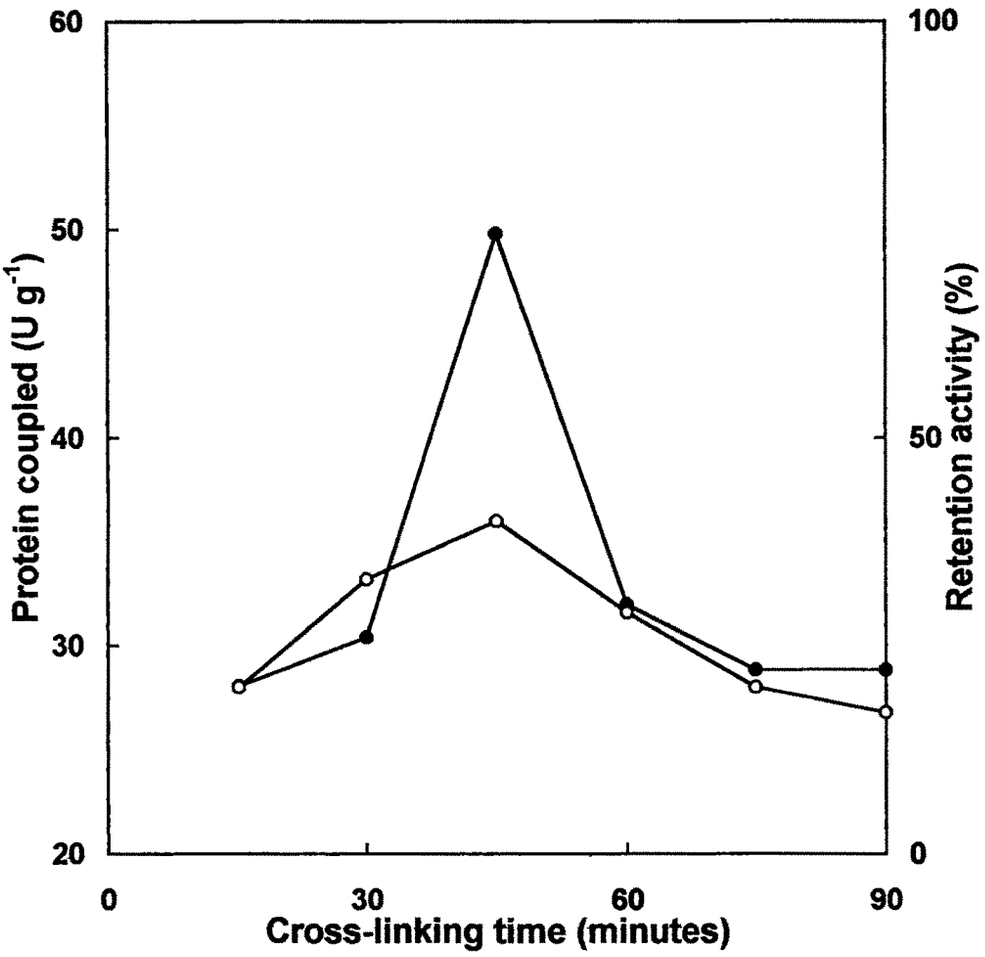


Fig. 2.16 Effect of cross-linking time on immobilization :

Support 50 mg, Glutaraldehyde conentration 0.02%, Enzyme concentration 100 μg, pH 8, Incubatin time 16 h

Protein coupled (●), Retention activity (O)

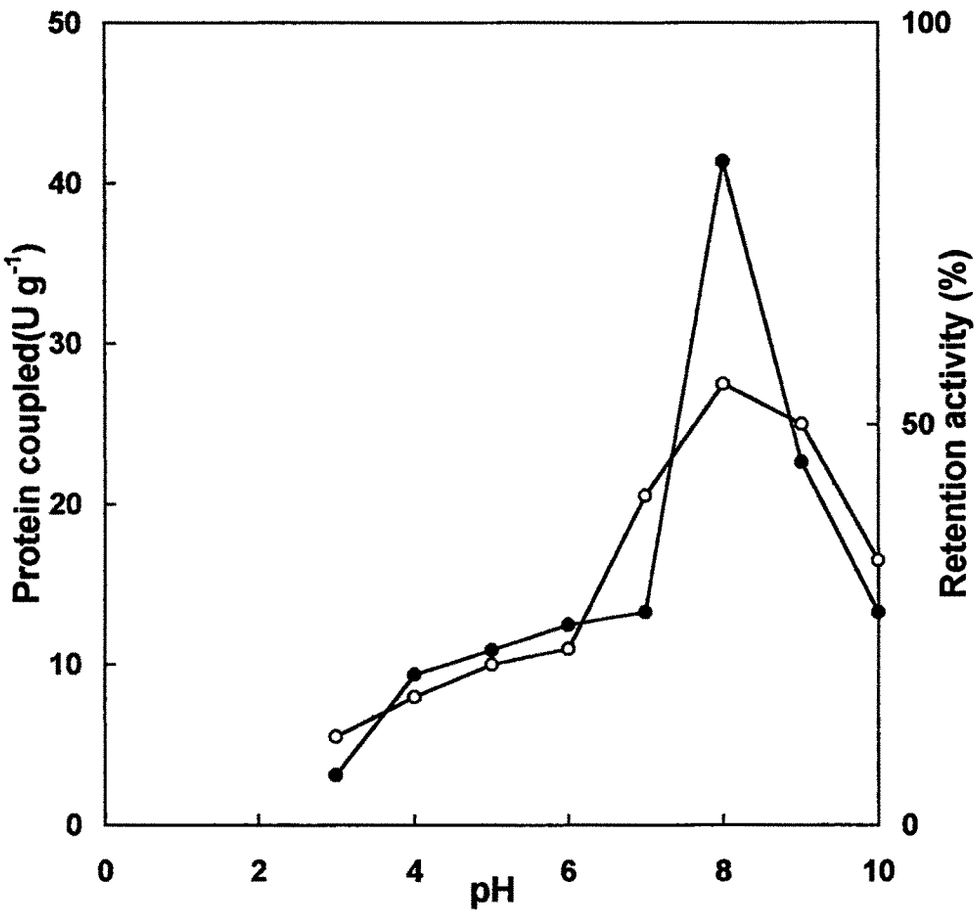


Fig. 2.17 Effect of pH of the medium on immobilization :

Support 50 mg, Glutaraldehyde concentration 0.02%, Cross-linking time 45 min, Enzyme concentration 100 μg, Incubation time 16 h

Protein coupled (●), Retention activity (O)

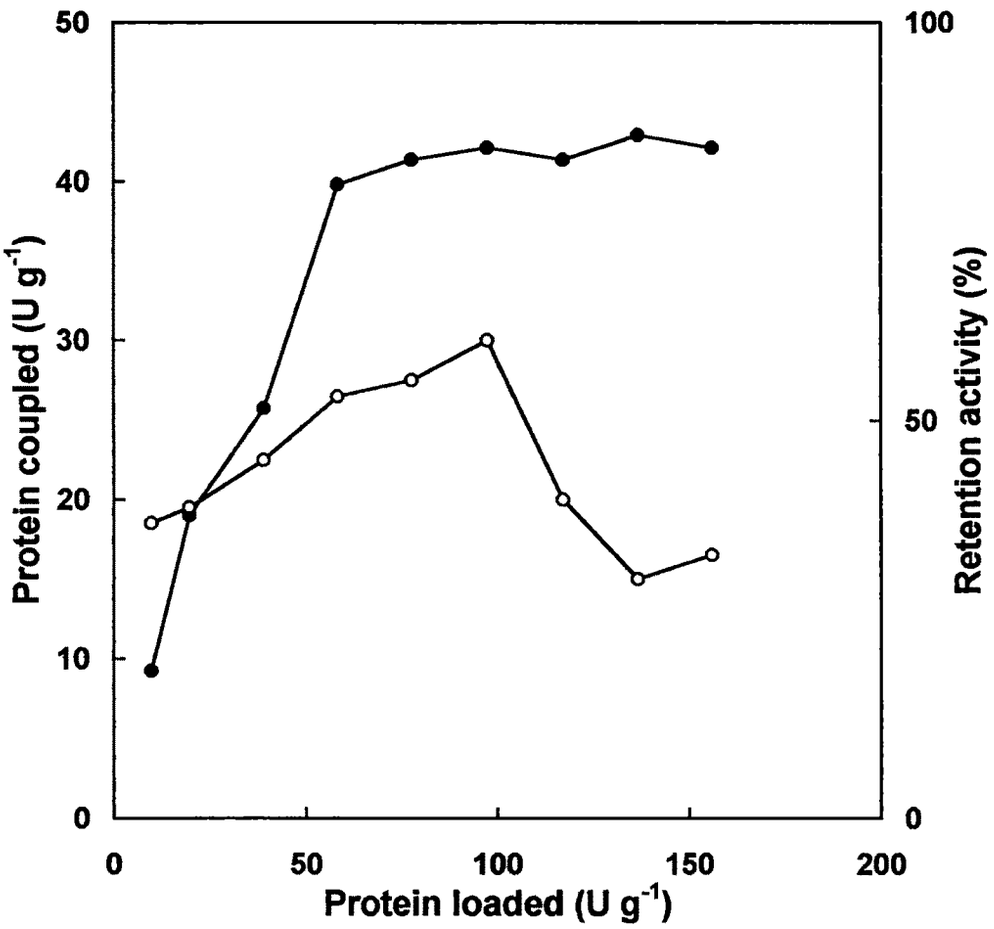


Fig. 2.18 Effect of enzyme concentration on immobilization :

Support 50 mg, Glutaraldehyde concentration 0.02%, Cross-linking time 45 min, pH 8, Incubation time 16 h

Protein coupled (●), Retention activity (O)

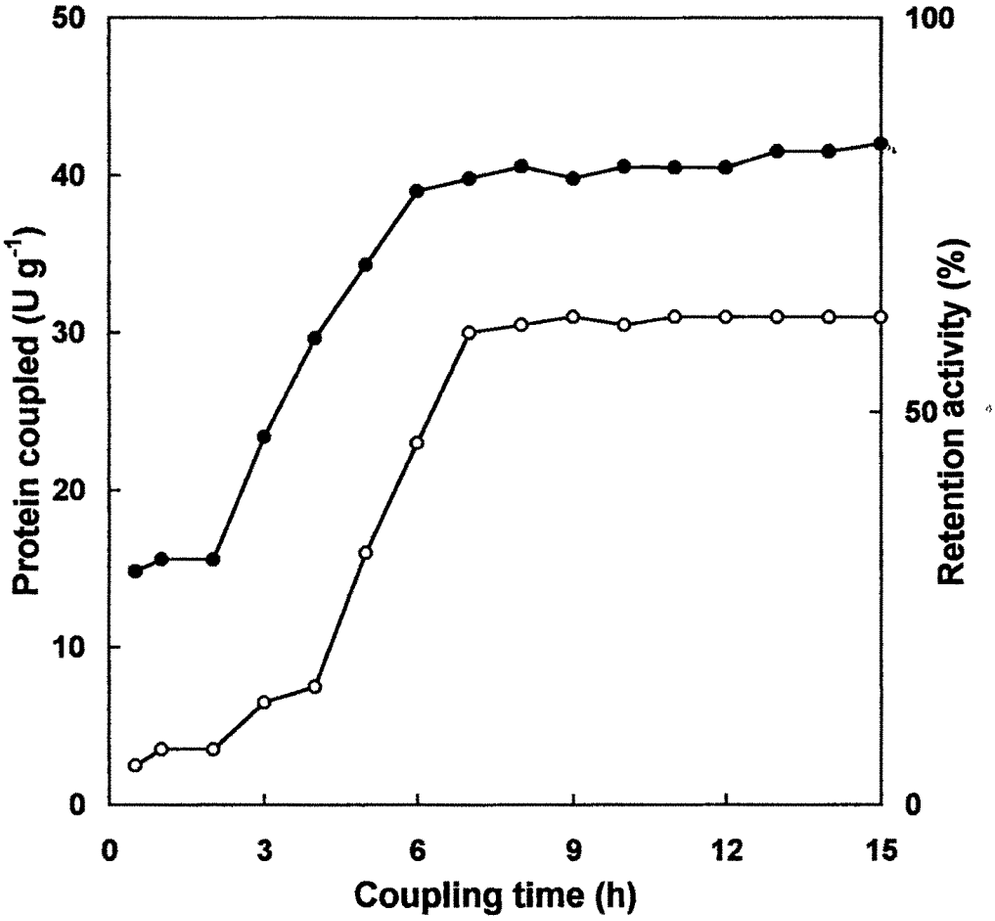


Fig. 2.19 Effect of coupling time on immobilization :

Support 50 mg, Glutaraldehyde concentration 0.02%, Cross-linking time 45 min, pH 8, Enzyme Concentration 100μg

Protein coupled (●), Retention activity (O)

Though the protein coupled to the support was observed to be 59 U g^{-1} only 60% of the enzyme activity was retained. This indicates that either the coupling has taken place through active sites decreasing the enzyme activity or denaturation of enzyme is taking place during the process. Optimized conditions for covalently immobilized α-chymotrypsin onto porous chitosan beads activated with glutaraldehyde are summarized in **Table 2.3**.

2.4.8 Comparative account of free and covalently coupled enzyme

(a) pH activity profile

A change in optimum pH for enzyme activity is generally observed upon immobilization depending on the nature of the support used. This type of change is very useful in understanding the structure-function relationship of enzyme. Therefore, it is necessary to compare the activity of the free and the immobilized enzyme as a function of pH. **Fig. 2.20** illustrates the effect of immobilization on the optimum pH for the enzyme activity. The free enzyme shows maximum activity at pH 8, whereas the optimum pH for the immobilized enzyme was shifted to pH 7 - 8 indicating that polymer matrix behaves as a polycationic to some extent affecting the microenviroment for the enzyme activity. This behaviour is of considerable interest for the use of the enzyme in the food processing industries.[98] Such type of change in pH was not observed in the entrapment of α-chymotrypsin in poly(HEMA-co-AAm) gel.

Goldstein et. al.[99,100] carried out experiments to investigate this pH shift in relation to the electrostatic field produced by a highly charged carrier. The optimum pH of α-chymotrypsin immobilized by using a polyanionic carrier, ethylene-maleic anhydride copolymer, shifted 1 pH unit to the alkaline side, while in α-chymotrypsin immobilized by using a polycationic carrier,

Table - 2.3

Optimized conditions for covalently immobilized α-chymotrypsin on chitosan

Substrate	Casein/BTEE
Glutaraldehyde concentration (mg g^{-1})	16
Crosslinking time (min)	45
Coupling pH	8.0
Coupling time (h)	6
Enzyme loaded (U g^{-1})	59
Retention of activity (%)	60

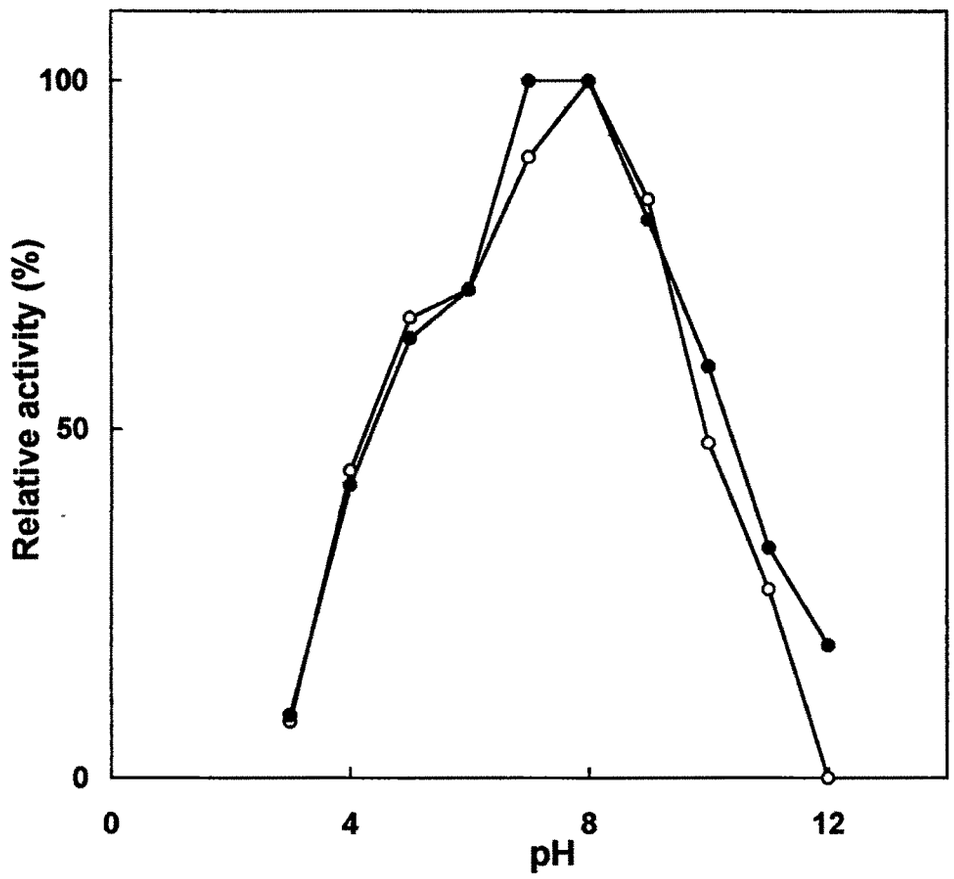


Fig. 2.20 pH activity profile for free (O) and immobilized (●) α-chymotrypsin

polyornithine, a shift of 1.5 pH unit to the acid side was reported. Chitosan being polycationic we have also observed about 1 unit shift in pH towards acidic side.

(b) Storage stability

The storage stability of free and covalently coupled enzyme has been investigated and results are given in **Fig. 2.21**. The immobilized enzyme showed only 10% loss of activity until 30 days of storage, whereas free enzyme loses its activity completely after 25 days of storage at room temperature (30 °C), in buffer of pH 8. Similar type of results were also observed for α-chymotrypsin immobilized by diazo coupling to amino acid copolymer.[101] α-Chymotrypsin entrapped in poly(HEMA-co-AAm) showed 50% retention of activity after 30 days storage in buffer of pH 8, while dry storage of enzyme showed no loss in activity. The more stability of enzyme by covalent attachment in buffer may be due to the strong chemical bond formed between the enzyme and chitosan.

(c) Thermal stability

Free and immobilized α-chymotrypsin were placed in the pH 8 buffer and incubated at different temperatures ranging from 40 to 60 °C, for different time intervals. The activity of the enzyme was then determined as described earlier. From the study the thermodeactivation constants (K_d) was calculated using the equation[92] and results obtained are given in **Table 2.4**. From the data it is observed that rate of deactivation increases with temperature for both free and immobilized α-chymotrypsin. However, it can be seen that rate of deactivation is higher for the free α-chymotrypsin in comparison with covalently bound α-chymotrypsin indicating the improved thermal stability of immobilized systems than free α-chymotrypsin. At 60 °C entrapped α-chymotrypsin retains 30% activity, while covalently bonded α-chymotrypsin retains 45% activity, indicating enzyme is more strongly bound to the support by chemical bonding. The thermal

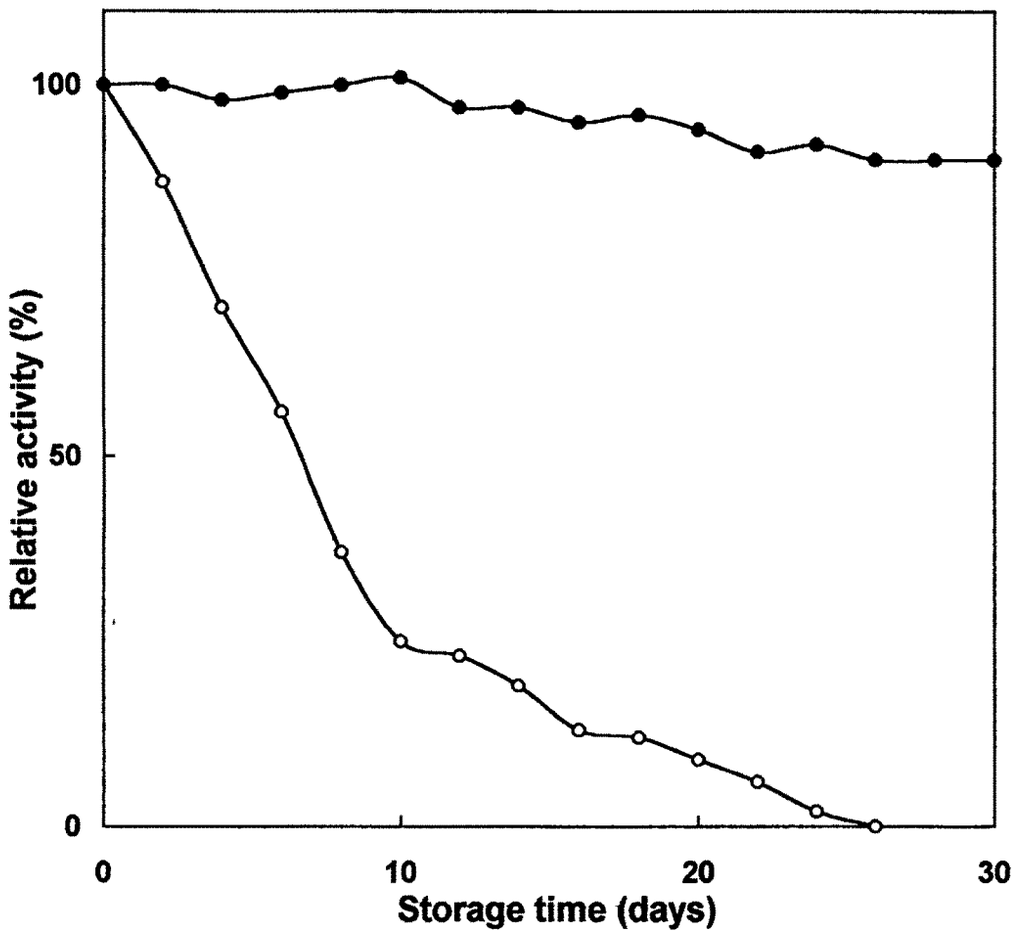


Fig. 2.21 Storage stability of free (O) and immobilized (●) α-chymotrypsin in buffer pH 8, at room temperature (30 °C)

Table - 2.4

Effect of temperature on the deactivation of α-chymotrypsin

Time (min)	Temperature (°C)	Deactivation rate constant ($K_d \times 10^{-2}$)	
		Free	CB-CT
20	40	0.3	0.15
	50	1.2	0.5
	60	3.9	2.8
40	40	0.95	0.4
	50	1.4	0.55
	60	4.6	3.4
60	40	1.1	0.52
	50	1.6	0.62
	60	5.6	3.5

stability of covalently bound α-chymotrypsin has been reported to be better than that of entrapped enzyme by Tao and Furusaki[71] also.

(d) Reusability of immobilized enzyme

Reusability of immobilized enzymes is one of the most important factors affecting the success of industrialization of an immobilized system. Stability of immobilized α-chymotrypsin is very important in application as it is subjected to repeated hydrolysis reaction. **Fig. 2.22** shows the effect of repeated use on the residual activity of immobilized α-chymotrypsin on L-BTEE hydrolysis. The activity was observed to be retained 50% even after six cycles. No leakage of enzyme was observed in the repeated washings during the study.

(e) Michaelis constant K_m

Due to conformational changes of the enzyme occurring during immobilization, the affinity between enzyme and substrate may change. Hence the investigation of kinetic constants for immobilized enzymes is very important. Initial reaction rates were determined at different concentrations of casein ranging from 0.6×10^{-3} to 4.23×10^{-3} mM. **Fig. 2.23** shows Lineweaver-Burk plots for the free and immobilized α-chymotrypsin. The values of the Michaelis constant (K_m) and the maximum reaction velocity (V_{max}) were obtained from the plot of 1/v vs. 1/s and are observed to be 2.71×10^{-3}, 2.5×10^{-3} mM and 5.95×10^{-2} and 9.6×10^{-2} mM min^{-1} for free and immobilized α-chymotrypsin respectively. The apparent K_m value of the immobilized α-chymotrypsin is slightly lower than that of free one. However, as Michaelis constant values remain almost unchanged for free and immobilized systems, it can be stated that enzyme has not undergone any conformational changes during immobilization. α-Chymotrypsin immobilized by CNBr activated sephadex was reported to show ten times higher K_m for acetyl-L-tyrosine ethyl ester than that of native enzyme.[102]

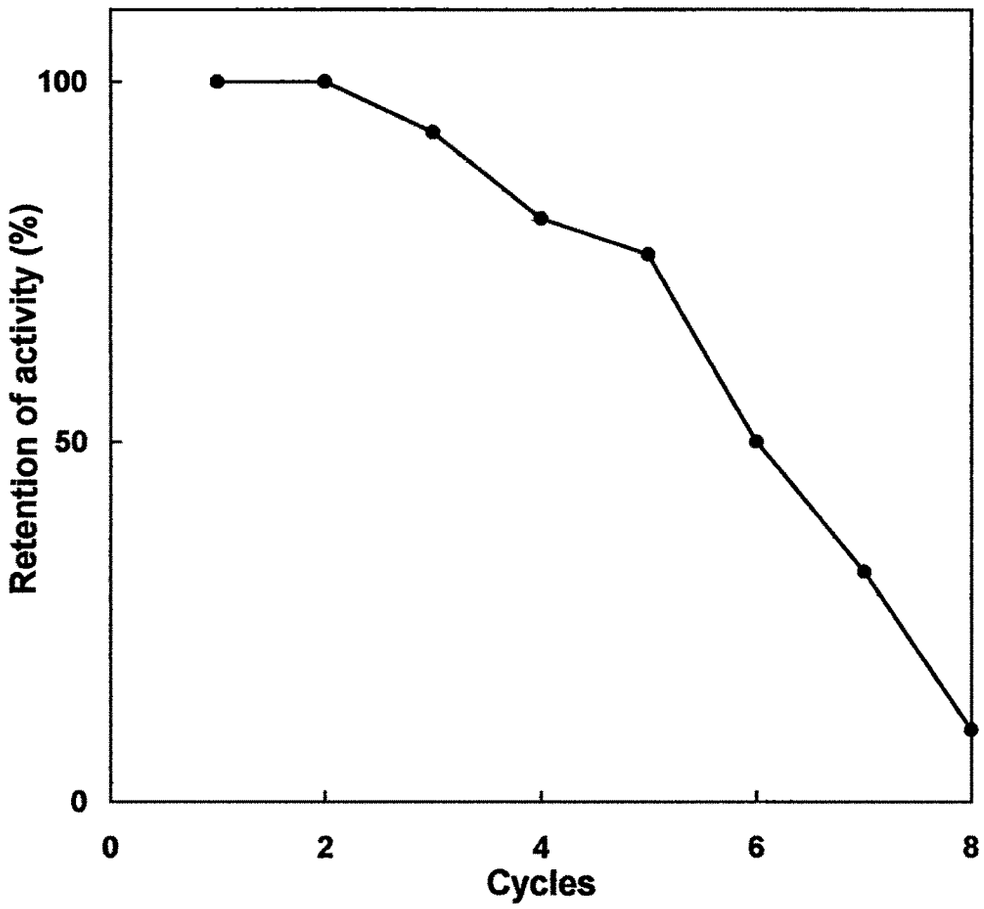


Fig. 2.22 Reusability of immobilized α-chymotrypsin at 30 °C and pH 8

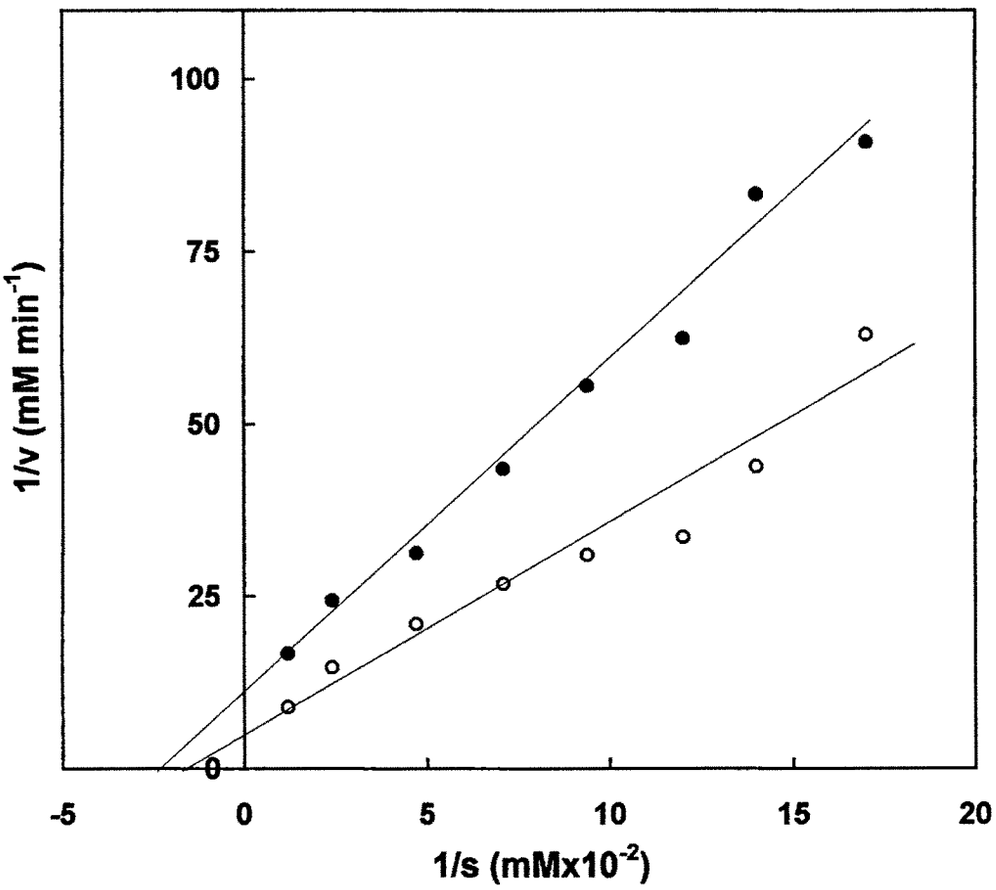


Fig. 2.23 Lineweaver-Burk plots for casein hydrolysis at 35 °C, pH 8 for 20 mins for free (O) and immobilized (●) α-chymotrypsin

(f) Solvents stability

The stability of immobilized enzymes in organic solvents is of significant importance in organic synthesis.[103,104] However, the reported stabilities of immobilized enzymes in such solvents are highly variable and have been found to be dependent upon the nature of the enzyme itself as well as the type of support and solvents.[105] In the present study stability of free and immobilized α-chymotrypsin towards DMSO, MeOH and THF was studied. The strength of solvents is expressed as the percentage based on the total reaction volume. Hydrolysis of casein by these enzyme systems was carried out in 10 to 30% solvents. From the **Fig. 2.24** it is observed that immobilized systems show better retention of activity than free system.

2.4.9 Optimization of column conditions

Industrial applications of immobilized enzymes require column studies. Enzymes are packed in various types of column reactors and their performance depends on the conditions used. Hence column conditions were optimized.

➤ Effect of temperature

The results obtained in the hydrolysis of 0.5 mM casein by α-chymotrypsin immobilized onto chitosan in a fixed bed reactor keeping flow rate 24 mL h^{-1}, and reactor temperatures 30, 35, 40 and 50 °C is illustrated in **Fig. 2.25**. It was observed that at 35 and 40 °C maximum % hydrolysis of casein takes place. However, further increase in temperature decreases the extent of hydrolysis.

➤ Effect of flow rate

The effect of substrate flow velocity on the efficiency of the immobilized α-chymotrypsin was studied in the packed bed reactor at 35 °C and pH 8 for the

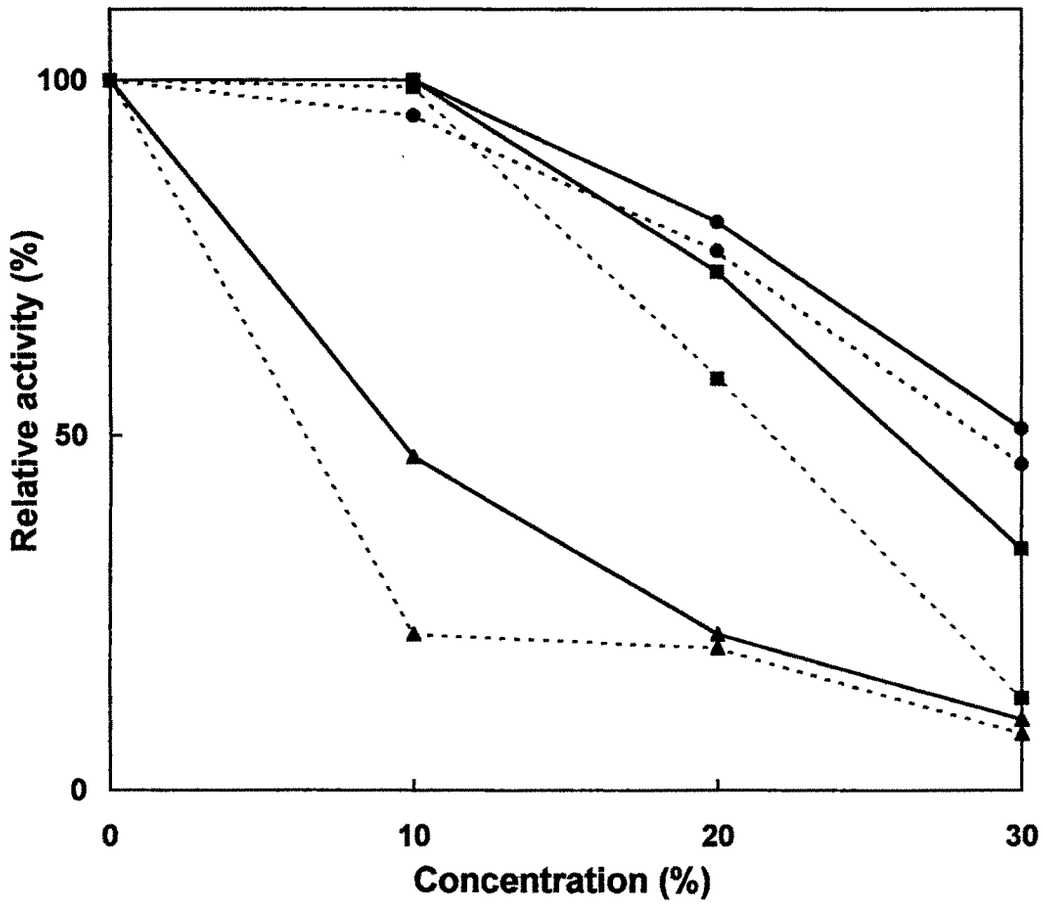


Fig. 2.24 Effect of water miscible solvents on the casein hydrolysis activity of free (----) and immobilized (——) α-chymotrypsin at 35 °C in DMSO (●), MeOH (■), THF (▲)

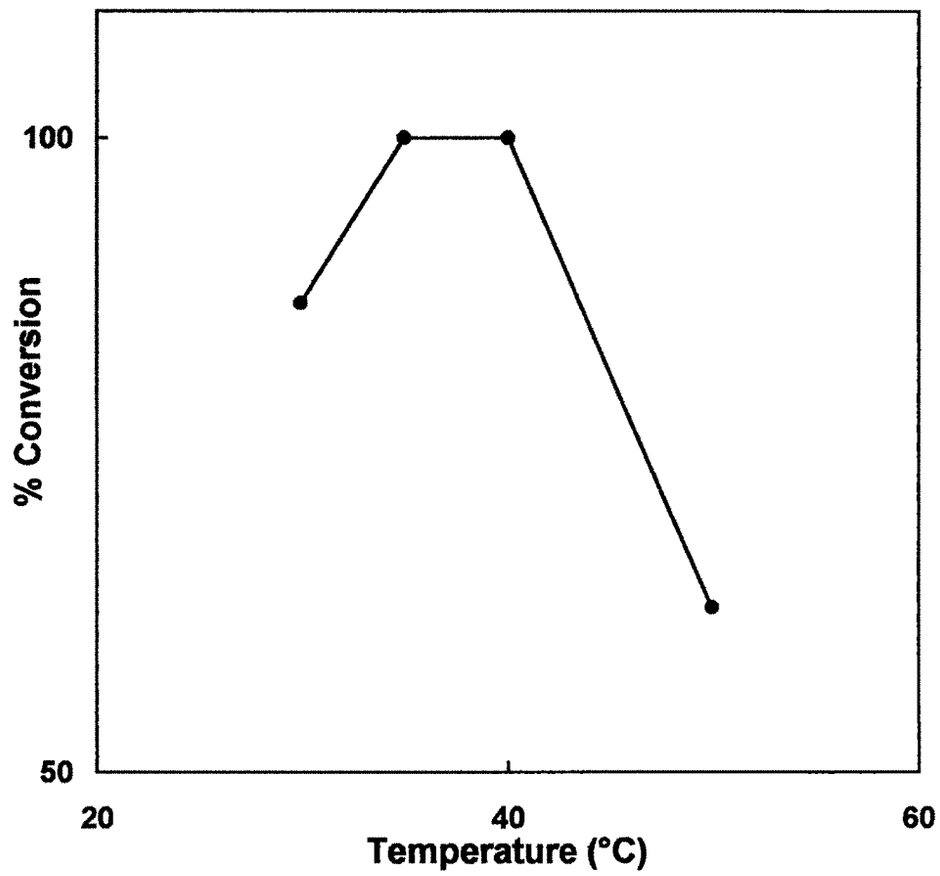


Fig. 2.25 Effect of column temperature on casein hydrolysis at pH 8, flow rate 24 mL h^{-1}

hydrolysis of casein by varying the flow velocities from 12 mL to 36 mL h^{-1}. From the results presented in **Fig. 2.26**. It is observed that with increasing flow velocity and concentration of the substrate hydrolysis decreases. This is because with increasing the flow rate the residence time of substrate with immobilized enzyme in the packed bed reactor decreases and hence the % conversion decreases.

➤ **Operational stability**

Operational stability of the immobilized α-chymotrypsin was determined by operating the reactor continuously at constant temperature 35 °C and 24 mL h^{-1} flow rate for varying time intervals and measuring the residual activity periodically. From the results given in **Fig. 2.27**, it is observed that the percentage hydrolysis of casein after 5 cycles (150 min operation) operation changes a little. However, 50% reduction in activity of immobilized system was observed after repeated use for 12 cycles (330 min operation).

2.5 Summary and Conclusions

1. Using 1:1 AAm/HEMA ratio for coplymerization very high amount (90%) of α-chymotrypsin was immobilized by the entrapment into poly(AAm-co-HEMA) gel. 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 (100% for 70 days) and thermal stability (30% at 60 °C) 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 metal inhibitory action was observed to be more for the entrapped enzyme.

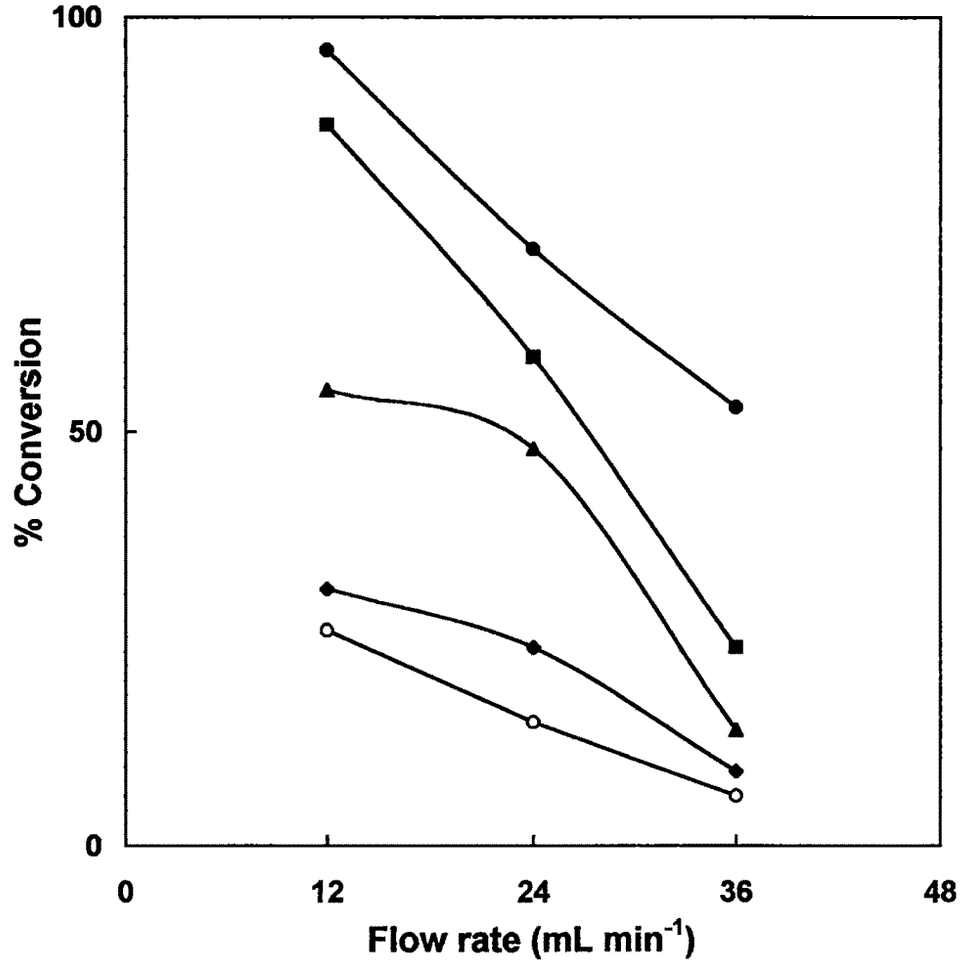


Fig. 2.26 Effect of flow rate on casein hydrolysis at 35 °C, pH 8

0.125 mM (●), 0.25 mM (■), 0.50 mM (▲), 0.75 mM, (◆) 1.0 mM (O)

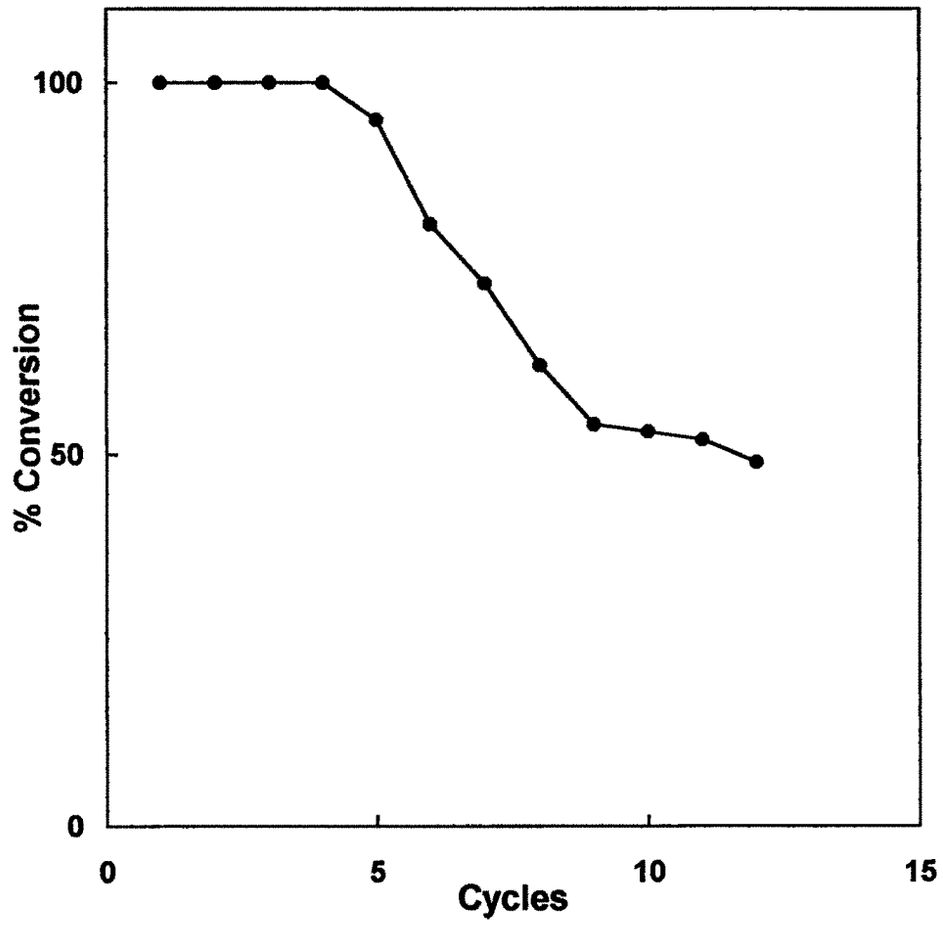


Fig. 2.27 Efficiency of column in hydrolysis of casein at 35 °C, pH 8, flow rate 24 mL h^{-1}

2. α-Chymotrypsin was immobilized on the porous chitosan beads by covalent attachment technique. Maximum coupling of 59 U g^{-1} and retention of 60% enzyme activity critically depend on concentration of crosslinking reagent and enzyme concentration. Better storage, thermal and solvent stabilities were observed for immobilized enzyme compared to free enzyme. Covalently immobilized α-chymotrypsin can be successfully used in packed bed reactor for continuos hydrolysis of casein with 50% operational stability for 12 cycles of 330 min.

3. The results indicated that the immobilized α-chymotrypsin not only had higher relative activity but also had remarkable increase in the stability and good reusability.

4. Thus, α-chymotrypsin is successfully immobilized into poly(AAm-co-HEMA) and onto chitosan by *insitu* entrapment and covalent binding techniques. A mild and reproducible method has been developed for the entrapment of α-chymotrypsin into cross-linked copolymer poly(AAm-co-HEMA) synthesized by free radical copolymerization at low temperature, using bis-AAm as a cross-linking agent. While porous beads of natural polysaccharide chitosan prepared were activated by glutaraldehyde cross-linking agent. The activity of the free and immobilized α-chymotrypsin was determined using L-BTEE and casein as low and high molecular weight substrates, respectively. Under all conditions of stabilities tested, immobilized α-chymotrypsin was observed to be better than free α-chymotrypsin. However, CB-CT has more potential than ENT-CT. These differences between two techniques are dependent on the nature and surface area of the polymeric supports used for the immobilization. The performance of immobilized enzymes with free enzyme was compared. The properties are summarized in **Table 2.5**.

Table - 2.5

Comparison of properties of free and immobilized α-chymotrypsin

Properties	Free enzyme	Covalent binding	*Insitu* entrapment
Optimum pH	8.0	7 - 8	8 - 9
Optimum temperature (°C)	35	35	40
Storage stability at RT (days)	9	23	70
Durability (cycles)	-	9	9
Thermodeactivation constant (K_d) at 60 °C	0.056	0.035	0.041
Michaeli's constant, K_m (mM)	2.71×10^{-3}	2.5×10^{-3}	4.23×10^{-3}
Maximum velocity, V_m (mM min^{-1})	5.95×10^{-2}	9.6×10^{-2}	4.84×10^{-2}
Stability in methanol (%)	58	88	95
Effect of Cu^{+2} inhibitor (%)	21	17	9

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