IMMOBILIZATION OF PROTEOLYTIC ENZYMES

CHAPTER 4

CONTENTS

4.1 Proteolytic Enzymes

The primary importance of the proteolytic enzymes is in the hydrolysis of a peptide bond. However, their use is not limited in peptide bond cleaving reactions but they can also catalyze the reverse reaction of peptide bond synthesis. In many instances it has also been shown that transfer reactions may also be catalyzed (transpeptidation).

Proteolytic enzymes are universally distributed in living cells. They are found not only in the gastrointestinal tract of animals but also in all animal tissues, liver, spleen, kidney, brain endocrine glands and other tissues have all proved to be rich sources of such enzymes. In addition such enzymes have been found in plant tissues and microorganisms as well.

In 1942 Bergman [1] classified proteolytic enzymes on the basis of substrates used by the proteases. All proteolytic enzymes attack peptide bonds and the specificity depends not on the chain length of the substrate but on the nature of the amino acid side chains. The activity of proteolytic enzymes also depends on the presence of thiol groups and bound metal ions such as magnesium, cobalt, manganese, zinc and iron. The enzyme activity can be inhibited due to the presence of diisopropylphospho fluorinate or similar organophosphorous compounds. Classification of proteolytic enzymes on the basis of their action on synthetic substrates has been reviewed earlier by Smith [2] and Green and Neurath [3]. However, the widely used classifications is as follows:

- **Exopeptidases** (peptidases) : These enzymes hydrolyze peptide bonds adjacent to terminal α-amino or terminal α-carboxy groups. In case of dipeptide, both groups must be adjacent to the sensitive bond. These enzymes are called aminopeptidases, carboxypeptidases, and dipeptidases respectively.

- **Endopeptidases** : This type of proteolytic enzymes attack centrally located peptide bonds as well as terminal peptide bonds. This class includes the well-known gastrointestinal proteinases, pepsin, trypsin, and chymotrypsin, many plant enzymes such as papain and ficin, intracellular animal enzymes the cathepsins and many bacterial and fungal enzymes such as substlisin.

4.2 Properties of Pepsin, Trypsin and Chymotrypsin

Pepsin, trypsin and chymotrypsin are proteolytic enzymes whose action on the substrate is usually through the hydrolysis of peptide bonds. Among them, trypsin and chymotrypsin are closely related enzymes and their extraction and techniques of assay

have been described in two comprehensive reviews[4,5]. The role of these enzymes in the body activity is to hydrolyze peptide bonds during the intestinal digestion of proteins. They belong to the large class of amide bonds or more specifically secondary amide or peptide bonds splitting enzymes. But they can also split carboxylic or phenolic ester bonds. At the beginning of enzymology enzymes were not very well characterized chemically and were defined through their biological activity. Two enzymes present in the pancreatic secretion and acting on proteins at alkaline pH were therefore named trypsin and chymotrypsin. When Northrop *et al*[6] purified these enzymes from animal source and could get them in crystalline form, it was observed that trypsin preferentially catalyzes the hydrolysis of peptide bonds between the carboxyl groups of arginine or lysine and amino groups of another amino acid residue. Whereas, specificity for this type of reaction is more restrictive than that of chymotrypsin for its substrates. Trypsin also acts as an esterase and as an amidase. The molecular weight of trypsin and chymotrypsin from light scattering, sedimentation, viscosity measurements and chemical analysis was observed to be ~23,800 and ~25,000 respectively[7].

The enzyme pepsin occurs in the gastric juice of all mammals. The chief cells of the gastric mucosa secrete the inactive precursor pepsinogen which is converted into active pepsin by cleavage of peptide bond[8]. Pepsin is an endopeptidase proteolytic enzyme as mentioned earlier which shows low substrate specificity. Peptide bonds between aromatic amino acids are attacked readily by pepsin. Pepsin also acts as an esterase like trypsin.

There is a great interest in use of proteolytic enzymes in food industry for the hydrolysis of proteins. The major reasons for this interest are solubilization, texturization and increased digestibility of proteins. Soluble proteolytic enzymes have been used for several years for the hydrolysis of casein and soybean protein. However, hydrolysis of proteins must be economically feasible because many proteolytic enzymes are very costly. This difficulty can be solved by using immobilized proteases. For the better retention of enzyme activity and recycling during processes, enzymes have been immobilized on various matrices. A brief survey about the immobilization of pepsin, trypsin and chymotrypsin reported so far is given below:

4.3 Literature Survey for the Immobilization of Pepsin, Trypsin and α-chymotrypsin

A large variety of natural and synthetic polymeric matrices have been used for the immobilization of pepsin, trypsin and α-chymotrypsin. The immobilization of **PEPSIN** on both organic and inorganic supports has been widely investigated[9-18]. Manecke[11] and

Manecke and Singer[12] have reported copolymer of methacrylic acid and m-fluoro dinitro anilide derivatives for pepsin immobilization. Glass derivatives are widely used as support material for the immobilization of pepsin. In 1971 Line *et al*[14] used alkyl amine porous glass for the immobilization of pepsin. The physico-chemical properties of the immobilized pepsin were thoroughly studied by using hemoglobin as substrate. The operational stability of the immobilized pepsin was observed to be for 4 weeks.

Cheryan *et al*[17,18] have reported covalent binding of pepsin to alkylamine porous glass through modified procedure of Line *et al*[14]. They used Woodward reagents or glutaraldehyde as the coupling reagents. The immobilized systems were used for the study of clotting of milk using fluidized bed reactor. Effect of pH, temperature and dilution on the rate of coagulation was studied by passing skimmed milk through a column containing pepsin bound to porous glass[18]. Among the proteolytic enzymes investigated, immobilized pepsin and calf rennet were reported to retain sufficient activity towards skimmed milk[17]. All other enzymes lost their activity on exposure to skimmed milk. The inactivation rates were observed to be lower at higher substrate pH and were least affected by reactor temperature. Attempts to regenerate the immobilized pepsin were partially successful. Porous glass derivatives were also used for the immobilization of pepsin by Ferrier *et al*[19].

Goldstein [20] used 1:1 copolymer of ethylene maleic anhydride and 1,6 diaminohexane (EMA : HMD) for the immobilization of pepsin and trypsin through the carboxyl groups activation using dicyclohexyl carbodiimide. The retention of immobilized enzyme activity was about 4% for trypsin and 20% for pepsin. Systems showed good thermal stability.

Pepsin, trypsin and other enzymes were immobilized by Beddows et al[21] using p-amino carbanilated cellulose derivatives and methylol derivative and diazo and glutaraldehyde activation. They have reported high coupling extent for pepsin as well as trypsin.

Another support, chitosan, was used for pepsin immobilization through glutaraldehyde coupling[22]. 95% of the enzyme activity was retained even after repeated use for four times. The immobilized pepsin retained ~90% of its activity after storing the xerogels in a refrigerator at 5°C for one month at pH 7.6 and at 40°C for three days.

In 1981, Abdel-Hay *et al*[23] carried out radiation induced graft copolymerization of meta and para-fluorostyrene onto nylon. The product was further nitrated and used for

the coupling of various enzymes and proteins. They observed that high amounts of proteins were coupled but activities of the coupled proteins were relatively low. The enzymatic activity of the pepsin bound to the activated fluoro styrene copolymer was negligible.

Puvanakrishnan and Boss[24,25] investigated various coupling processes for immobilization of pepsin and trypsin on sand. The optimum temperature for enzyme activity was shown to be 40-45° C. K_m values for immobilized enzymes were observed to be higher than those for free forms. The enzymes showed stability at 4° C for 60 days.

Gorgani and Karimian [26] have reported immobilization of bovine serum albumin, hemoglobin, glutamate dehydrogenase and pepsin on Triton-X-100 substituted Sepharose-4B. They have observed that binding of these protein is independent of pH and NaCl concentration. Use of immobilized proteins in continuous operations was also discussed.

Poly(vinyl pyridine) as a support for the immobilization of pepsin was reported by Lastra and Ortega [27]. The enzyme binding capacity, pH, and thermal stability of the immobilized pepsin were discussed in detail.

Pepsin was immobilized onto porous synthetic adsorbant such as polystyrene, polymethacrylic acid and esters or porous glass beads by Higeta Shoyu Co. [28]. The bound material was used for the milk coagulation. Curd forming time for 10% skimmed milk passed through the column was observed to be 8 min 4 sec.

Dumitriu *et al* [29] used N-[4-carboxy phenyl carbamoyl methyl] cellulose as a support for pepsin immobilization. The coupling extent was observed to be influenced by enzyme/support and activator/support ratios, pH, and coupling duration. The Michaelis constant (K_m) for the immobilized enzyme was reported to be lower than that for the free form. They reported, high catalytic activity at low pH and low activator support ratios but at long reaction times and high enzyme support ratios. After three repeated runs immobilized pepsin showed 25% decrease in the initial enzyme activity.

Recently, Ivanov and Turkova [30] used porous amino alkyl derivatized inorganic supports for immobilization of pepsin by using covalent coupling methods involving carbodiimide coupling reagent. Porous glass based catalyst showed 92% retention of pepsin activity. Storage stability of the immobilized enzyme at 8°C and pH 4.5 was observed for 1 month.

Another type of support succenilated chitosan was used by Gustovo and Jacqueline[31] for the immobilization of pepsin. They have reported preparation of insoluble active pepsin through covalent binding of the enzyme to the support chitosan through amide bond formation using carbodiimide condensing reagent. The enzyme retained 80% of its activity and showed greater storage stability than the soluble enzyme.

Numerous synthetic and natural polymeric matrices have been used for the immobilization of **TRYPSIN** and **CHYMOTRYPSIN** [32-43] . However, many of these polymeric support systems have resulted in considerable reduction of enzyme activity as well as its binding capacity on immobilization. Hence a review of selected works is given here.

Graft copolymers have been extensively used for enzyme immobilization [44,45] . Graft copolymers provide substrates with desired physical and chemical characteristics and grafted chains containing active groups are useful for enzyme coupling[46]. A polyethylene grafted with acrylic acid was used for the immobilization of enzymes including trypsin [46] . Because of low enzyme activity obtained with carbodiimide activation the copolymer was activated with EEDQ (N-ethoxy carbonyl 2-ethoxy 1,2 dihydro quinoline) in organic solvents. Several organic solvents such as acetone, tetrahydrofuran, benzene and benzene-ethanol were investigated for the preparation of the activated polyacrylic acid intermediate. The activated systems showed high extent of trypsin binding to the support but the relative activities were not very high.

The graft copolymerization of acrylic acid onto woollen substrates was successfully carried out using photo chemical initiation procedures by Barker et al[47] using 4-(sulphomethyl)benzyl sodium salt initiator. The grafted copolymer was used for the immobilization of trypsin and other enzymes. The results obtained from their study showed poor retention of trypsin activity.

In another article Abdel-Hay *et al* [48] reported the use of acrylic acid-nylon copolymer for immobilization of enzymes. Large amounts of coupled proteins were reported when carbodiimides (CMC and EDAC) were used as coupling agents. However, when the enzymes ß-galactosidase and trypsin were immobilized, the retention of activity was relatively low.

Cohenford and coworkers [49] reported crosslinking of trypsin to human serum albumin (HSA) with glutaraldehyde to form soluble and insoluble copolymers. The physical and kinetic parameters of trypsin and trypsin-HSA systems were compared.

Trypsin retained only 24% of its enzyme activity after heating for 5 min at 60° C. The trypsin-HSA system showed shifts in pH optimum, increase in activation energy and a broadening of pH stability profile.

Trypsin was immobilized onto algenic acid-poly (glycidylmethacrylate) graft copolymer (AAGMA) by Raghuram Reddy *et al*[50]. The resulting immobilized enzyme showed 65% of the soluble enzymatic activity. The optimum temperature was shifted by 5° C to a higher value. The optimum pH of the immobilized trypsin was also shifted by 0.5 units toward the alkaline side when compared to that of soluble enzyme. The pH and thermal stability of immobilized system was better than that of free enzyme.

Immobilization of trypsin to p-benzoquinone activated poly(vinyl alcohol) gel was reported to contain 0.4 mg protein/mL gel having a 70% residual protease activity and 95% residual esterase activity[51]. It was also observed that the apparent Michaelis menten constant of the enzyme showed decrease for hemoglobin substrate but not with BAEE substrate.

Beddows *et al*[52] discussed the immobilization of trypsin, glucose oxidase, acid and alkaline phosphatase enzymes and cells of *Bacillus stearothermophilus* onto substrates poly(maleic anhydride/styrene)-co-polyethylene and poly(maleic anhydride/vinyl acetate) co-polyethylene. The copolymer was hydrolysed to release the hydroxyl groups from poly(vinyl acetate) component of the grafted chains. Thereafter, p-benzoquinone and p-tolyl sulphonyl chloride activation methods were used for the immobilization of trypsin. Activation with PTS gave good coupling of protein but percentage retention of trypsin activity was low.

Ulbrich *et al*[53] reported covalent binding of trypsin, chymotrypsin and α-amylase onto silica derivatives; polystyrene and polyacrylamide. Various activation methods were used for the immobilization purpose. Various kinetic parameters of the immobilized systems were discussed. Thermoinactivation of the immobilized systems was thoroughly investigated.

Immobilization of trypsin using glutaraldehyde as a crosslinking reagent on insoluble chromium-polygalacturonate was reported by Ortega and Ortiga [54]. The comparison of immobilized and free enzyme towards optimum pH for enzyme activity, thermostability and activators such as $CaCl_2$ solution (10-20 mM) was done. The IME system packed into a column and fed with a continuous flow of denatured casein of pH

7 was assessed over 30 days and showed no apparent decrease of proteolytic enzyme activity.

Recently, Masko and coworkers [55] studied trypsin immobilization on carbon fibres differing in the texture porosity and surface properties. The efficiency of trypsin binding and its catalytic functions showed dependence on the texture and porosity of the carbon fibre material used.

Covalent binding of trypsin onto poly(2-hydroxy ethyl methacrylate)/polystyrene composite microspheres by cyanogen bromide method has been reported by Okubo *et al* [56].

Recently Beddows *et al* [57] reported the immobilization of trypsin and other enzymes onto hydrolyzed poly(2-hydroxy ethyl methacrylate)-g-co-polyethylene using hydroxyl and carboxyl groups activating agents. Emphasis was given on the immobilized trypsin system which involved examination of variation in (i) the extent of hydrolysis of graft copolymer, (ii) the concentration of activating agent and (iii) the temperature of coupling. With the trypsin system, an increase in carbodiimide concentration gave an increase in the amount of protein immobilized but a marked decrease in the retention of enzyme activity. Comparison of the behaviour of the free with the immobilized enzyme showed that the immobilized system has an extended pH profile and good stability. The kinetic factors were examined further and the role of the graft copolymer chains in the immobilized system was discussed.

Devi *et al* [58] reported the immobilization of trypsin and glucose oxidase onto natural rubber-g-co-HEMA system. Optimization of immobilization conditions involving trypsin was studied with respect to the pH and type of crosslinking agent. They observed that the immobilized enzyme had superior stability over a wider pH range when compared to the free trypsin. The retention of activity demonstrated by the immobilized trypsin was significant.

A large number of supports have been used for the immobilization of α-chymotrypsin and their uses for peptide synthesis were reported [59-63].

Heras and coworkers [64] studied the immobilization of α-chymotrypsin on solid support and the influence of organic solvents on IME activity. Sepharose-chitin activated by different chemical methods was used as supports. Higher retention of activity in the presence of organic solvents was also observed.

Martin *et al*[65] reported, immobilization of *α*-chymotrypsin on sepharose activated through tosylation of CH_2OH groups by using different procedures. 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 by p-tolyl sulphonyl chloride. A 50-70% yield in the synthesis of dipeptide was [Tyr-Leu] obtained by using 70% butene diol at pH 7 and 37° C.

Luthra *et al* [66] reported micro gels as support for immobilization of *α*-chymotrypsin. The enzyme was conjugated to carboxy bearing micro gels with a water soluble carbodiimide. The Michaelis Menten parameters of the conjugated enzyme vs N-benzoyl-L-tyrosine ethyl ester were almost identical for the native and immobilized enzyme except at low pH.

Recently, acrylamide copolymers were reported for chymotrypsin immobilization[67]. These copolymers contained photoisomerizable compounds and hence the resulting enzyme-copolymer assemblies revealed photo switchable on-off biocatalytic activities.

Immobilization of chymotrypsin on chitin from various sources ~~through~~ by the glutaraldehyde activation method was reported by Heras and Acosta[68]. Hydrolase and peptide synthetase activities were determined in aqueous and organic solvents, respectively. Chymotrypsin immobilized on chitin from prawn was observed to be most active immobilized enzyme derivative based on its synthetase activity.

Immobilization of chymotrypsin on Acrolein-vinyl acetate-divinyl benzene copolymer was reported by Guoliang and Shintaro[69]. A polymer containing amino group was obtained by further reaction with p-nitroaniline. The resulting nitro products was further reduced to form an amino group. The glutaraldehyde or diazo method was used for the activation before coupling with the enzyme. An improvement in the physico-chemical properties of immobilized chymotrypsin was reported.

Chymotrypsin was covalently immobilized on polymerized liposome namely phosphatidyl ethanol amine support ~~through~~ by the carbodiimide crosslinking method[70]. Immobilized enzyme showed good temperature stability, pH stability, operational stability and good retention of activity than free enzyme.

We are using the previously reported polymeric supports derived from salicylic acid-resorcinol-formaldehyde (SRF), poly(vinyl alcohol) and Amberlite-IRA-400 for the immobilization of pepsin, trypsin and chymotrypsin. The immobilized pepsin was

thoroughly characterized and further used for the continuous coagulation of milk using fluidized bed reactor. The details are discussed in following sections.

4.4 Experimental

Chemicals and materials used in this study are listed below:

► Chemicals used

Pepsin (EC.3.4.23.1) [from porcine stomach mucosa of strength 3380 U/mg]	Sigma Chemical Co,USA
Trypsin (EC.3.4.21.4) [from bovine pancreas Type XII-S-of 7500 U/mg strength]	Sisco Research Lab, India
Chymotrypsin (EC.3.4.21.1) [from bovine pancreas of strength 52 U/mg]	Sigma Chemical Co, USA
Tyrosine	Sigma Chemical Co, USA
N-Benzoyl L-Tyrosine ethyl ester (BTEE)	Sigma Chemical Co, USA
Hemoglobin	Loba, India
N-Benzoyl-D.L. arginine p-nitroanilide (BAPNA)	E. Merck, India
Poly(vinyl alcohol) [~77,000 mol. wt. 98% mole hydrolysis]	Loba, India
Amberlite-IRA-400	Rohm & Hass Co, USA
Phenol reagent [1 : 2 (v/v) dilution]	BDH, India
Trichloro acetic acid	BDH, India
Tris-Buffer	Sigma Chemical Co, USA
Dimethyl Formamide	Qualigens, India

All other solvents and reagents used were of A.R. grade and distilled deionised water was used throughout the study. Fresh skimmed milk of pH 6-6.7 was procured from Baroda Dairy Plant, Vadodara, India.

4.4.1 Polymeric supports

Salicylic acid-resorcinol-formaldehyde resin (SRF) in the form of 60-100 mesh size was synthesized in our laboratory as reported in Section 2.4.1(a) Other two supports poly(vinyl alcohol) with ~77,000 molecular weight and 98-99% hydrolysis mole and Amberlite IRA-

400 were obtained from Loba (India) and Rohm & Hass Co (USA) respectively and were used for the immobilization of proteolytic enzymes without any further modification.

4.4.2 Immobilization of pepsin, trypsin and chymotrypsin

Activation of the polymeric supports SRF, IRA-400, and poly(vinyl alcohol) was done through hydroxyl groups by using p-benzoquinone, p-tolyl sulphonyl chloride and cyanuric chloride spacers. The experimental methods for the activation processes are reported in Section 2.4.2. The activated supports were used for the immobilization of pepsin (0.5 mg ml^{-1}), trypsin (4 mg ml^{-1}) and chymotrypsin (4 mg ml^{-1}), in a buffer of suitable pH at 277°K and 18 h reaction time. Controlled temperature shaker bath (INSREF) was used for the reaction to reduce the external diffusion barrier. The immobilized protein content was determined by Lowry assay procedure as reported earlier in Section 2.4.2(e) and the activity of the coupled protein was measured as described in Section 4.4.3. The effect of concentration of enzymes, concentration of crosslinking agents, coupling time and pH on the extent of coupled protein and retention of its activity was studied ~~through~~ by appropriate procedures as reported in Section 2.4.5.

4.4.3 Assays of enzyme activity

(a) Assays of pepsin activity

Pepsin activity was determined by using hemoglobin as substrate as described in the literature [71,72]. The enzymatic reaction is shown below:

$$\text{Hemoglobin} + H_2O \xrightarrow{\textit{Pepsin}} \text{Tyrosine} + \text{Tryptophan}$$

The enzymatic hydrolysis of hemoglobin gives rise to these products which are soluble in trichloroacetic acid and the product tyrosine was measured spectrophotometrically by using phenol reagent of Folin and Ciocalteau [71] as per the procedure given below.

Procedure:

The activity of free and immobilized pepsin was determined by using hemoglobin as substrate. 2% Hemoglobin was prepared in 0.06 N HCl and 5 mL of 2% hemoglobin concentration was used for the determination of activity of free and immobilized pepsin. The enzyme was incubated with substrate for 30 min at 310°K. The products of the enzymatic hydrolysis were soluble in trichloroacetic acid. Hence 5% (w/v) trichloroacetic acid was added to the reaction mixture and 5 ml of supernatant after 20 min

centrifugation was mixed with 10 mL of 0.5 N NaOH and 3 ml of dilute phenol reagent. The assay of enzyme as liberated tyrosin was done spectrophotometrically at 750 nm using proper calibration plot.

(b) Assay of trypsin activity

The activity of free and immobilized trypsin was determined using Erlanger's method[73] and N-Benzoyl-DL-arginine p-nitroanilide (BAPNA) as substrate at 25°C and pH 7.8. 110 mg BAPNA was dissolved in 25 ml distilled DMF and 0.3 ml of this solution was used for the reaction. The reaction was terminated after 10 min by adding 1 mL of 30% acetic acid. The quantity of liberated p-nitroaniline was estimated spectrophotometically at 405 nm for the activity of the enzyme.

$$N_{\alpha}\text{-Benzoyl-DL-arginine-p-nitroanilide} + H_2O \xrightarrow{Trypsin} \text{N-Benzoyl-L-arginine} + \text{p-nitroaniline}$$

(C) Assay of chymotrypsin activity

The free and immobilized chymotrypsin activity was determined by using BTEE substrate (N-benzoyl-L-tyrosine ethyl ester) at 25° ± 1° C and Tris-buffer of pH 8. Three ml of 0.16 M BTEE solution was used for the activity measurements of chymotrypsin at 30 seconds reaction time, spectrophotometrically at 250 nm as reported by Hummel[74].

$$\text{N-Benzoyl-L-tyrosine Ethyl ester} + H_2O \xrightleftharpoons{Chymotrypsin} \text{N-Benzoyl-L-tyrosine} + \text{Ethanol}$$

4.4.4 Stability studies for free and immobilized enzymes

The pH stability studies were carried out for free and immobilized enzymes by determining the enzyme activity at various pH values as discussed in Section 2.4.6.

The thermal stability of the free and immobilized enzymes was evaluated by measuring the residual activity of the system exposed to various temperatures for 30 min. The energy of activation (ΔE_a) and thermodeactivation constant (K_d) were calculated from temperature data as described in Section 2.4.6.

The Michaelis constant (K_m) and maximum reaction velocity (V_{max}) were calculated from Lineweavers-Burk plots using varying amounts of substrates.

Storage stability of the free and immobilized pepsin was determined by storing the enzyme at 4° C in 0.2 M acetate buffer of pH 4 for various time intervals and measuring the residual activity periodically as mentioned in Section 2.4.6.

Immobilization in the presence of substrate was carried out under optimized conditions to reduce the inactivation of enzyme. The immobilization of pepsin and chymotrypsin was carried out under optimized conditions in the presence of hemoglobin (2%) and BTEE (0.16 M) substrates respectively.

4.4.5 Clotting of milk

The schematic diagram of the fluidized bed reactor used in the study of clotting of milk is illustrated in the Fig 4.1. The dimensions of the reactor were 0.6 x 10 cm. At the bottom of the reactor a porous glass frit was placed and a compact column of pepsin immobilized on SRF was prepared by using 1 g of the material. The flow velocity was maintained at 2 ml min^{-1}. Space time of the reactor was (W/F) 0.5 g ml^{-1} min^{-1}. The reactor was maintained at 15°C controlled temperature by circulating water through the outer jacket. Two other supports poly(vinyl alcohol) and Amberlite-IRA-400 under study exhibited comparatively inferior properties in terms of retention of activity and hence were not used for the study of clotting of milk. Enzymatic treatment of milk was carried out at 15° C for 30 min. In the secondary phase of clotting the temperature was raised to 30 to 50° C and the time required for the coagulation was measured. For the practical applicability of the method dilution of treated milk with fresh skimmed milk was done in 1 : 2 (v/v) ratio prior to the clotting studies.

For the study of effect of pH on clotting of milk the pH of the milk was adjusted with 2 M phosphoric acid and 1 M sodium hydroxide prior to the treatment. Absence of enzyme in the secondary phase of clotting was confirmed by measuring the enzyme activity in the washings of the reactor bed and simulated milk ultrafiltrate (supernatant liquid of the salted out milk).

4.5 Results and Discussion

The physicochemical properties of the synthesized crosslinked resinous material salicylic acid-resorcinol-formaldehyde (SRF) are discussed in Section 2.4.1.

4.5.1 Activation of support

The results obtained in the immobilization of pepsin, trypsin and chymotrypsin on various polymeric supports using different hydroxyl activation processes are given in Table 4.1. It was observed that SRF shows maximum coupling and retention of pepsin activity on p-tolyl sulphonyl chloride as well as p-benzoquinone activation (Table 4.1). Cynuric chloride activation gave better retention of trypsin activity for the protein coupled on the

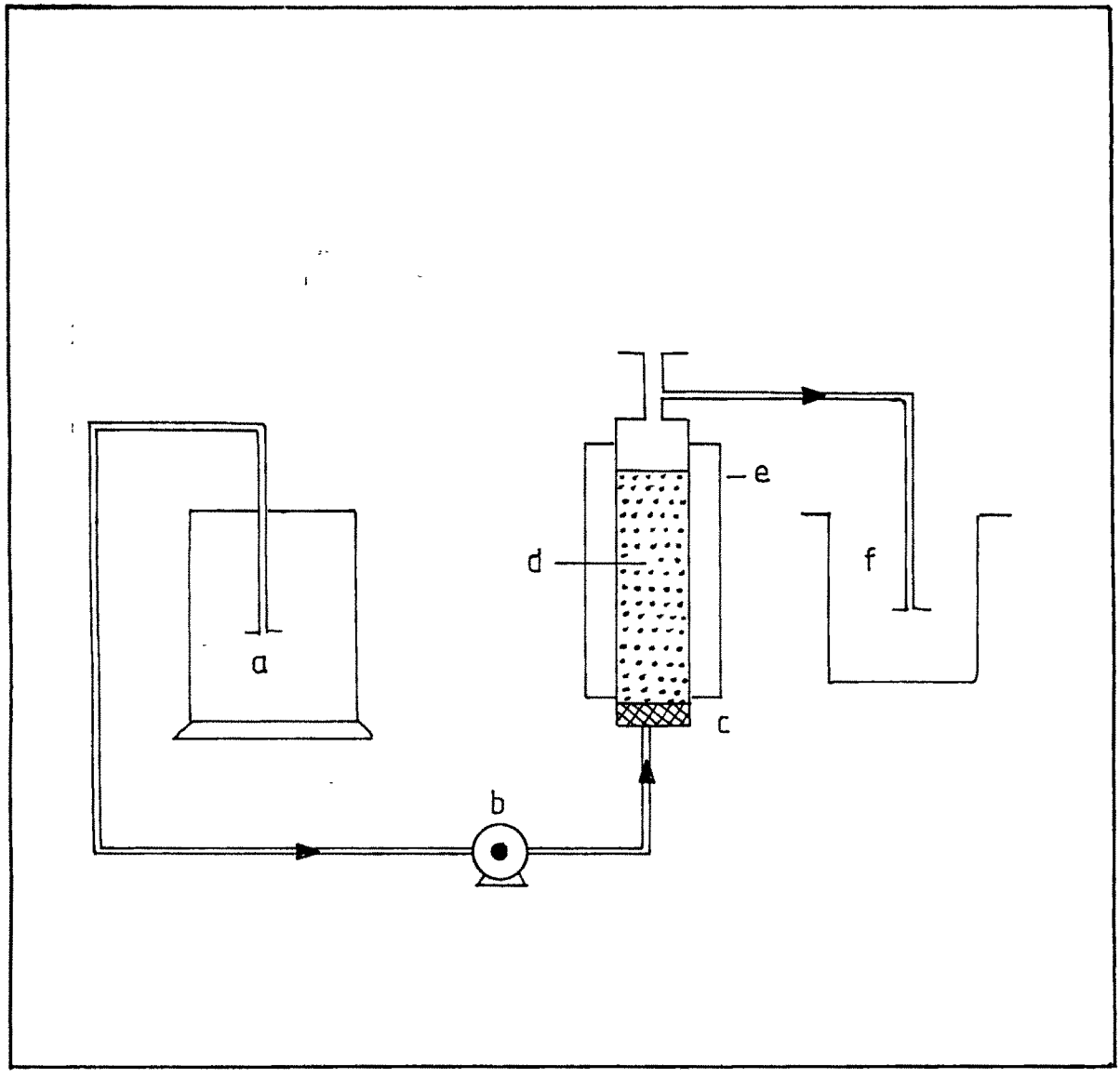

Fig 4.1 Schematic diagram of fluidized bed reactor
(a) Milk reservoir, (b) Peristaltic pump, (c) Porous glass disc, (d) Suspended IME, (e) Temperature controller, (f) Treated milk.

resinous support (SRF). Whereas chymotrypsin showed maximum coupling and retention of enzyme activity through p-benzoquinone activation route (Table 4.2). Other two supports poly(vinyl alcohol) and amberlite IRA-400 showed highest retention of pepsin activity only on p-benzoquinone activation. However, all these supports gave negligible retention of trypsin and chymotrypsin activity for all activation processes. The observed variation for different enzymes and same supports can be attributed to the difference in the nature of the enzyme support and activator/support relationship. Hence further studies were carried out by activating all the supports with p-benzoquinone for pepsin immobilization. Cynuric chloride and p-benzoquinone activation was used for SRF support for trypsin and chymotrypsin respectively. The observed superior activity on p-benzoquinone activation may be due to the quinone moities generated during activation processes which may have higher affinity towards the enzymes under study[75,76].

4.5.2 Optimization of coupling conditions

Immobilization of trypsin and chymotrypsin on activated support SRF was carried out at pH 2-10. The enzyme activity was observed to be markedly influenced by the local microenvironment (Fig 4.2). Trypsin and chymotrypsin-SRF systems showed the maximum coupling and retention of their activity at pH 7-8 and 4-7 respectively. The Chymotrypsin-SRF system was observed to be less critical of pH of coupling medium.

Immobilization of pepsin on activated supports [SRF, Amberlite-IRA-400, Poly(vinyl alcohol)] was carried out in the coupling media of pH 1-8. From the results given in Fig 4.3, it was observed that coupling of the protein is pH independent but ~~it its~~ retention of activity is maximum at pH 1-2 for all the systems under study.

The optimum time required for the immobilization of pepsin on the supports was determined by measuring the amount of protein coupled on the support at different time intervals ranging from 30 min to 16 hrs. From the results illustrated in Fig 4.4, it is evident that the rate of immobilization is fast and almost quantitative coupling takes place within one hour time. Retention of activity shown by all the three supports is considerably different. The SRF-pepsin system shows highest retention of pepsin activity (100%) on activation through p-tolyl sulphonyl chloride. SRF activated with PBQ shows 80% retention of pepsin activity and Amberlite IRA-400 and poly(vinyl alcohol) show 70% and 45% pepsin activity on immobilization.

Table 4.1Effect of various activation methods on immobilization of pepsin

Methods	SRF		Poly (vinyl alcohol)		Amberlite IRA-400	
	Protein coupled (mg g^{-1})	Retention of activity (%)	Protein coupled (mg g^{-1})	Retention of activity (%)	Protein coupled (mg g^{-1})	Retention of activity (%)
p-Benzoquinone	8.50	80	9.40	45	8.8	70.4
Cynuric chloride	9.00	3.3	8.40	7.9	8.5	3.5
p-tolyl sulphonyl chloride	8.50	100	9.70	34.5	3.6	53

Time 18h at 277° K, amount of supports used: 200 mg, enzyme used (2.0 mg).

Table 4.2 Effect of various activation methods on immobilization of Trypsin and Chymotrypsin.

Methods	Salicylic acid-Resorcinol-Formaldehyde (SRF)			
	Trypsin		Chymotrypsin.	
	Protein coupled ($mg\ g^{-1}$)	Retention of activity (%)	Protein coupled ($mg\ g^{-1}$)	Retention of activity (%)
p-Benzoquinone	70.4	17.6	20	1.1
Cynuric chloride	50.4	18.5	00	00
p-tolyl sulphonyl chloride	68.0	4.2	63.8	1.5

Trypsin: Time 18h at 277° K, pH 7, amount of supports used: 200 mg, enzyme used 16 mg.

Chymotrypsin. Time 18h at 277° K, pH 7, amount of supports used: 200 mg, enzyme used 16 mg.

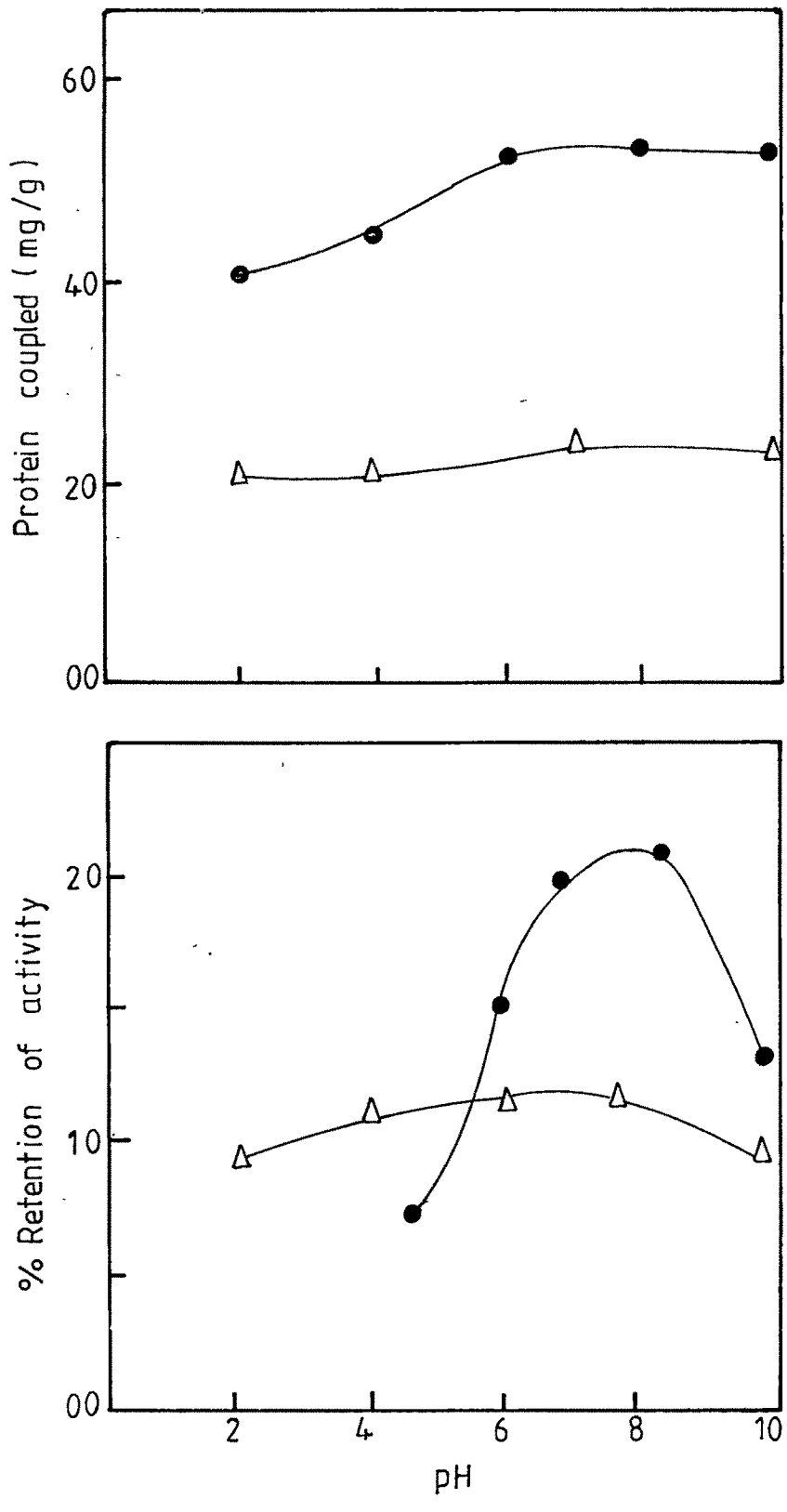

Fig 4.2 Effect of pH of coupling medium on immobilization of trypsin and chymotrypsin
(●) trypsin, (Δ) chymotrypsin.
Support SRF Temperature 277°K, Time 18 h, Enzyme concentration 16 mg trypsin, 16 mg chymotrypsin, Activation method of cynurichloride for trypsin, p-benzoquinone for chymotrypsin, Amount of support 0.2 g.

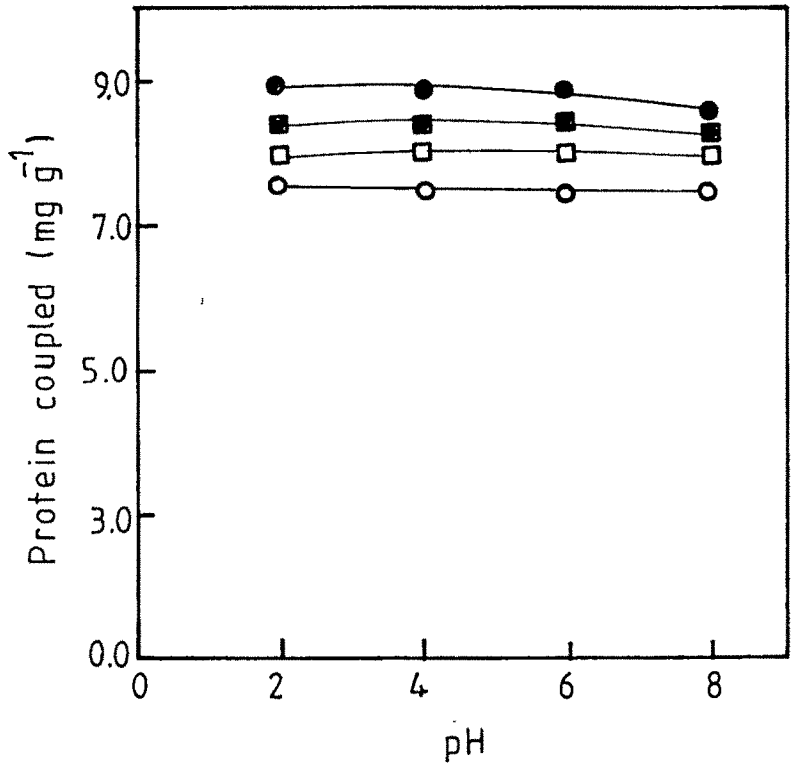

Fig 4.3.a Effect of pH of coupling medium on coupled protein
(●) Pepsin-SRF/PTS, (■) Pepsin-SRF/PBQ,
(□) Pepsin-IRA/PBQ, (○) Pepsin-PVA/PBQ.
Temperature 277°K, Enzyme concentration 2 mg, Time 18 h,
Supports 0.2 gm.

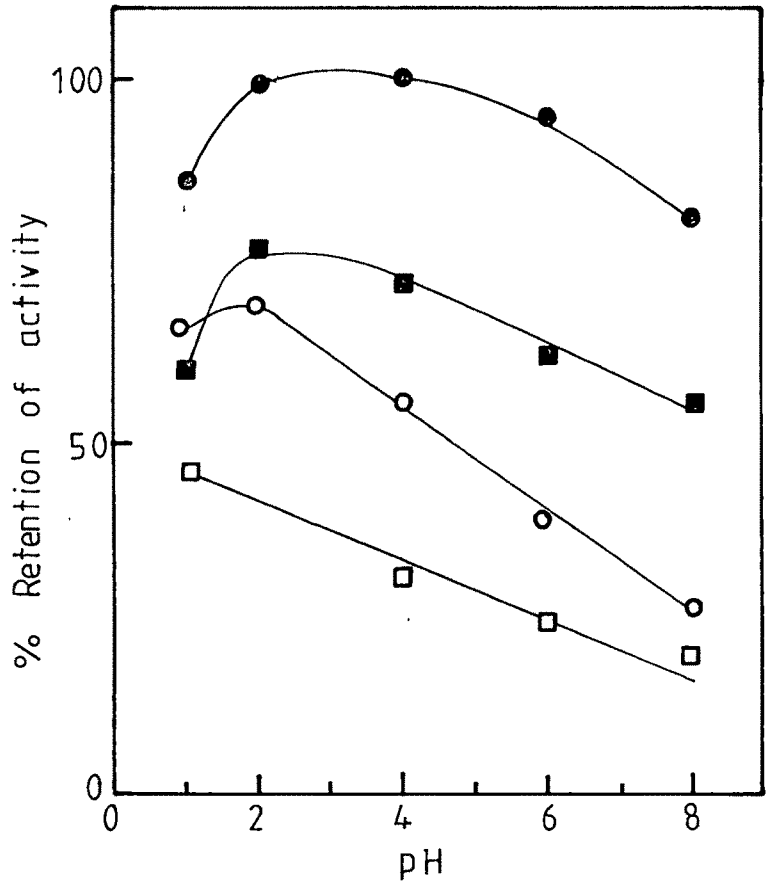

Fig 4.3.b Effect of pH of coupling medium on % retention of activity
(●) Pepsin-SRF/PTS, (■) Pepsin-SRF/PBQ,
(□) Pepsin-IRA/PBQ, (○) Pepsin-PVA/PBQ.
Temperature 277°K, Enzyme concentration 2 mg, Time 18 h,
Supports 0.2 gm.

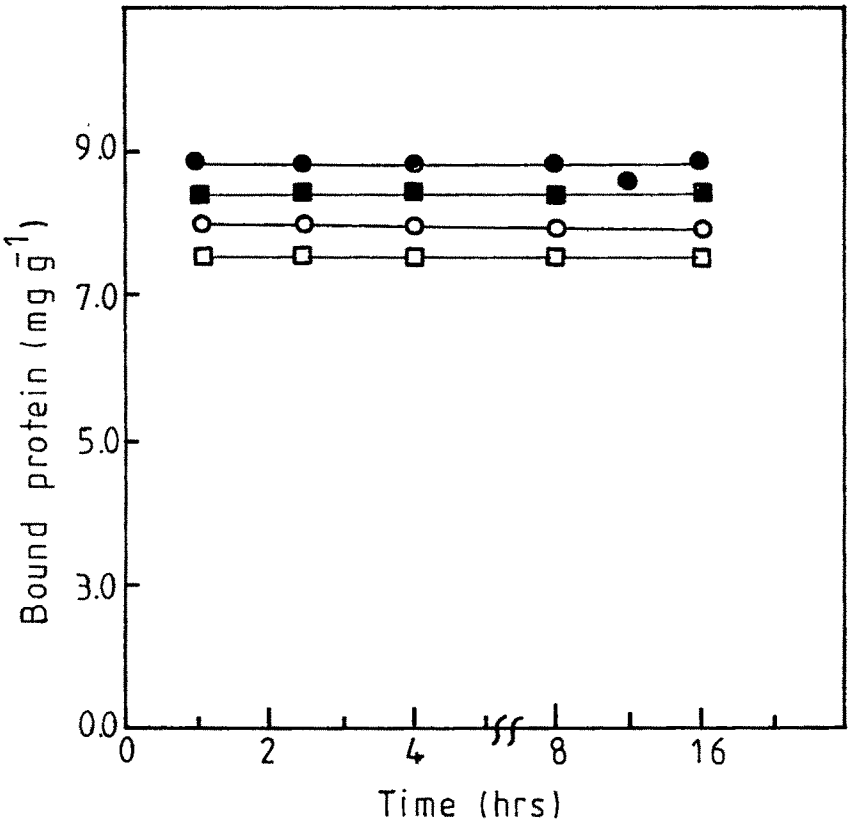

Fig 4.4.a Effect of time on protein coupled (●) Pepsin-SRF/PTS, (■) Pepsin-SRF/PBQ, (□) Pepsin-IRA/PBQ, (○) Pepsin-PVA/PBQ. (Conditions as described in Fig 4.3)

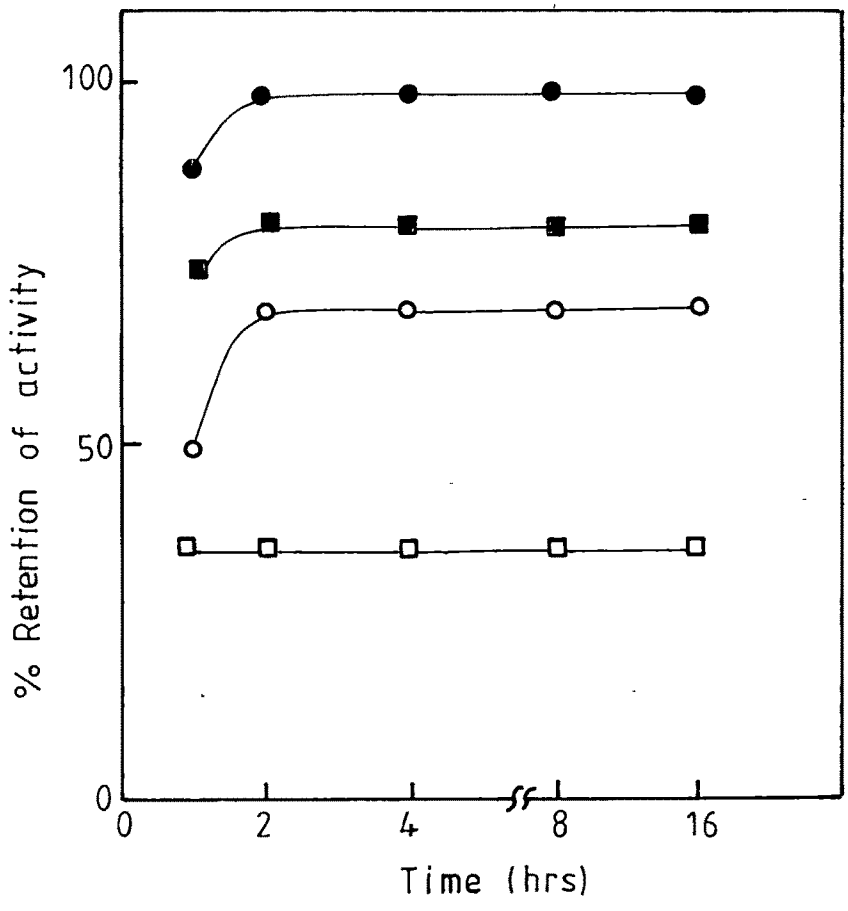

Fig 4.4.b Effect of time on % retention of activity (●) Pepsin-SRF/PTS, (■) Pepsin-SRF/PBQ, (□) Pepsin-IRA/PBQ, (○) Pepsin-PVA/PBQ. (Conditions as described in Fig 4.3)

The results obtained in the study of optimization of immobilization time for trypsin and chymotrypsin on resinous material (SRF) are given in Fig 4.5. It was observed that the rate of immobilization was fast and ~60% protein was coupled within 1-2 hour reaction time for Trypsin-SRF system whereas chymotrypsin-SRF showed ~45% coupled protein.

Effect of enzyme concentration on the extent of immobilization was studied by using 4 ml of 0.25-4.0 mg ml^{-1} pepsin. It was observed that all the supports show increased protein coupled but decreased % retention of protein activity with increased enzyme concentration (Fig 4.6). The decrease in the activity can be attributed to the coupling of the enzyme to the support through active sites at higher concentration and to the denaturation of the free and partially coupled enzyme under the action of the activator. Similar results were reported for urease[77].

4.5.3 Immobilization in the presence of substrate BTEE and Hemoglobin

Immobilization of pepsin and chymotrypsin was carried out under optimized conditions in the presence of hemoglobin (2%) and BTEE (0.16 M) substrates respectively. This was done to block the active enzyme sites during coupling of enzyme to support, so that the retention of enzyme activity can be improved. However, from the results given in Table 4.3, it was observed that with the increased concentration of enzyme percentage retention of enzyme activity decreased in presence of substrate. This may be due to substrate diffusional problems arising during immobilization process.

From optimization experiments, we have observed that trypsin and chymotrypsin showed poor retention of enzyme activity with all the activating reagents and for all the supports. Hence further characterization and applications were carried out only for immobilized pepsin.

4.5.4 Comparison of free and immobilized enzymes

(a) pH dependence of activity

The pH activity profile of the free and immobilized pepsin given in Fig 4.7, indicates that the free enzyme activity decreases with increasing pH of the substrate whereas pepsin immobilized on SRF through p-tolyl sulphonyl chloride and p-benzoquinone activation showed improved pH stability than that of free enzyme, with a very little change in its activity over the studied pH range.

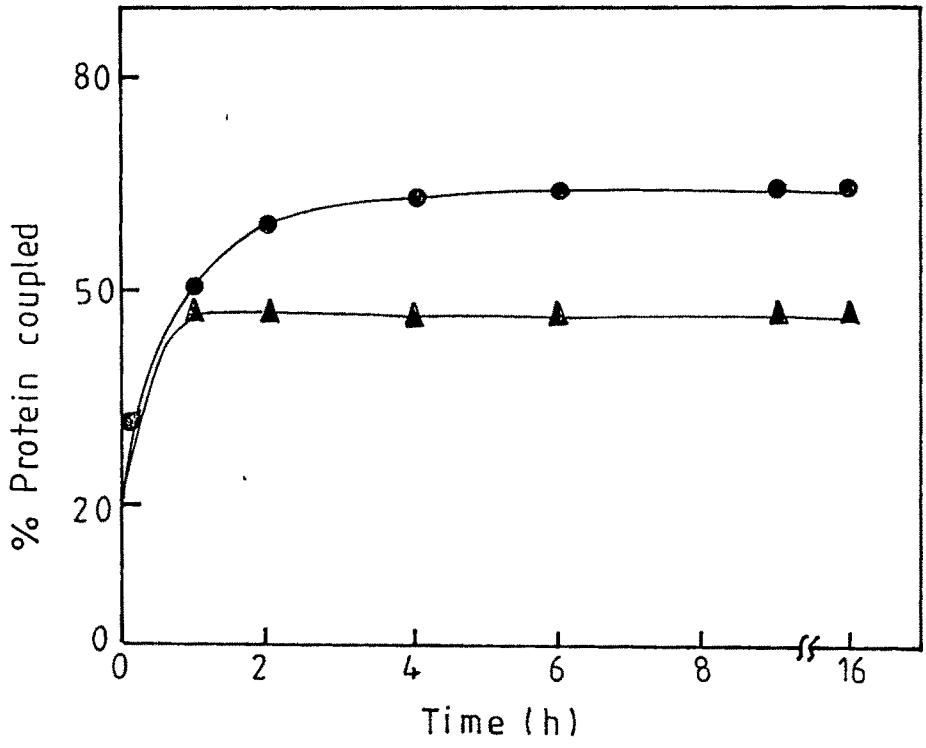

Fig 4.5 Effect of coupling time on immobilization of trypsin and chymotrypsin
(●) Trypsin-SRF, (Δ) Chymotrypsin-SRF
(Conditions as described in Fig 4.2)

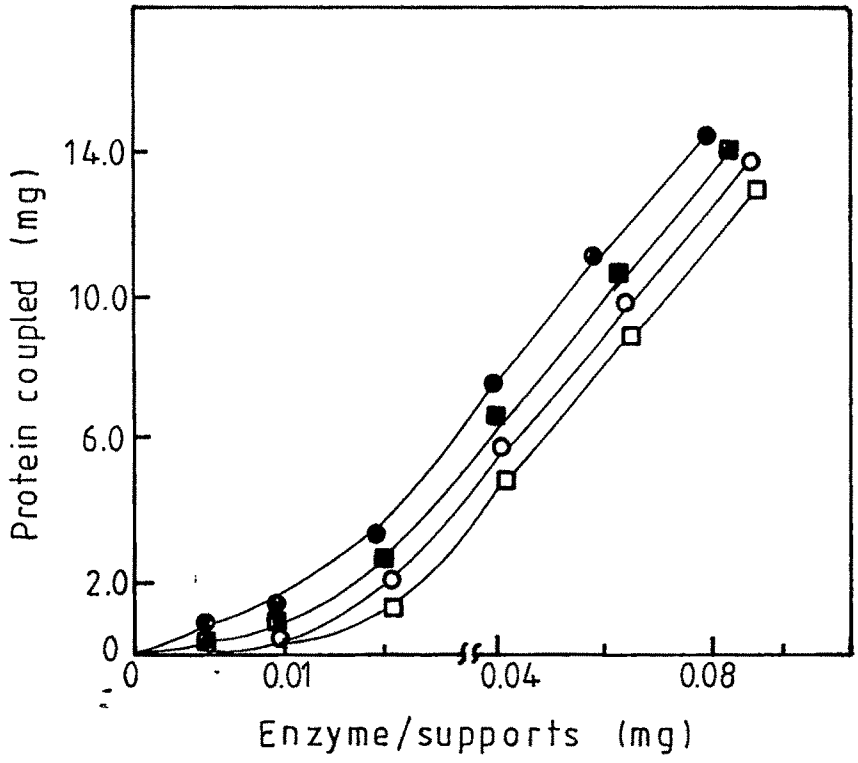

Fig 4.6(a) Effect of enzyme concentration on Protein coupled
(●) Pepsin-SRF/PTS, (■) Pepsin-SRF/PBQ,
(□) Pepsin-IRA/PBQ, (○) Pepsin-PVA/PBQ.
(Conditions as described in Fig 4.3)

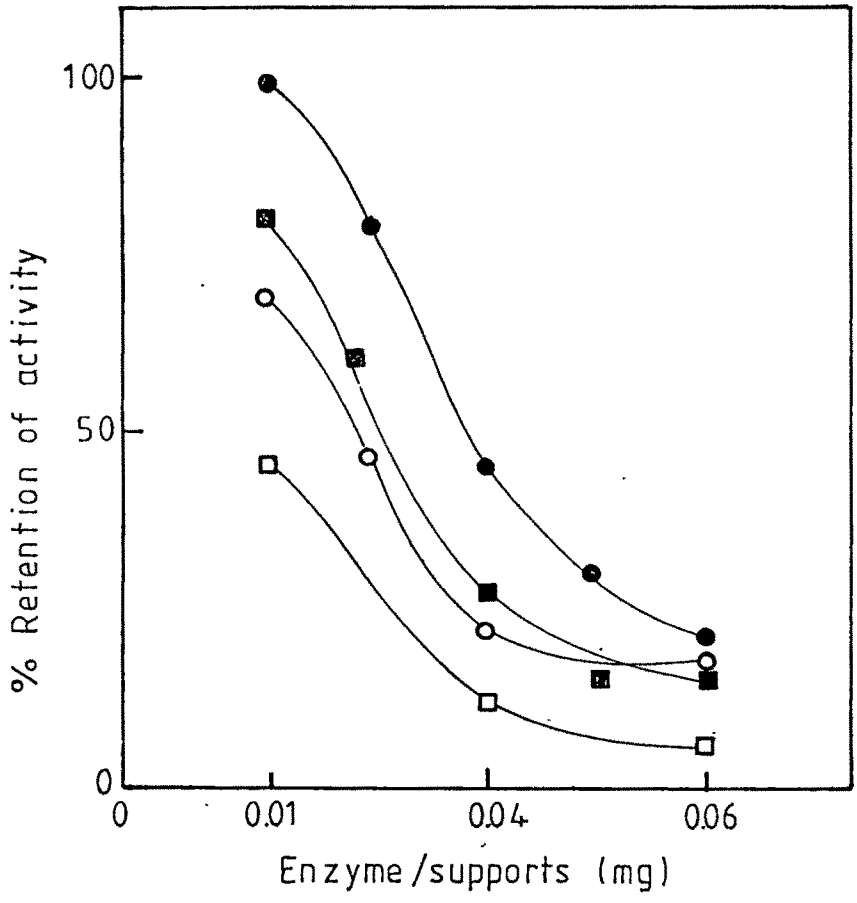

Fig 4.6(b) Effect of enzyme concentration on % retention of activity
(●) Pepsin-SRF/PTS, (■) Pepsin-SRF/PBQ,
(□) Pepsin-IRA/PBQ, (○) Pepsin-PVA/PBQ.
(Conditions as described in Fig 4.3)

Table 4.3 Immobilization in the presence of substrate

Conditions of Coupling		
ENZYME USED	Pepsin	Chymotrypsin
CROSSLINKING AGENT	p-tolyl sulphonyl chloride	p-benzoquinone
SUPPORT	SRF	SRF
IMMOBILIZATION TIME	18 h	18 h
pH OF THE MEDIUM	pH 2	pH 8
SUBSTRATE USED	Hemoglobin (2 %)	BTEE (0.16 M)

Enzyme Concentration (mg)	Retention of Activity(%)			
	Pepsin		Chymotrypsin	
	With substrate	Without substrate	With substrate	Without substrate
2	100	100	11.50	10
4	82	69	1.18	1.08
20	80	14	-	-

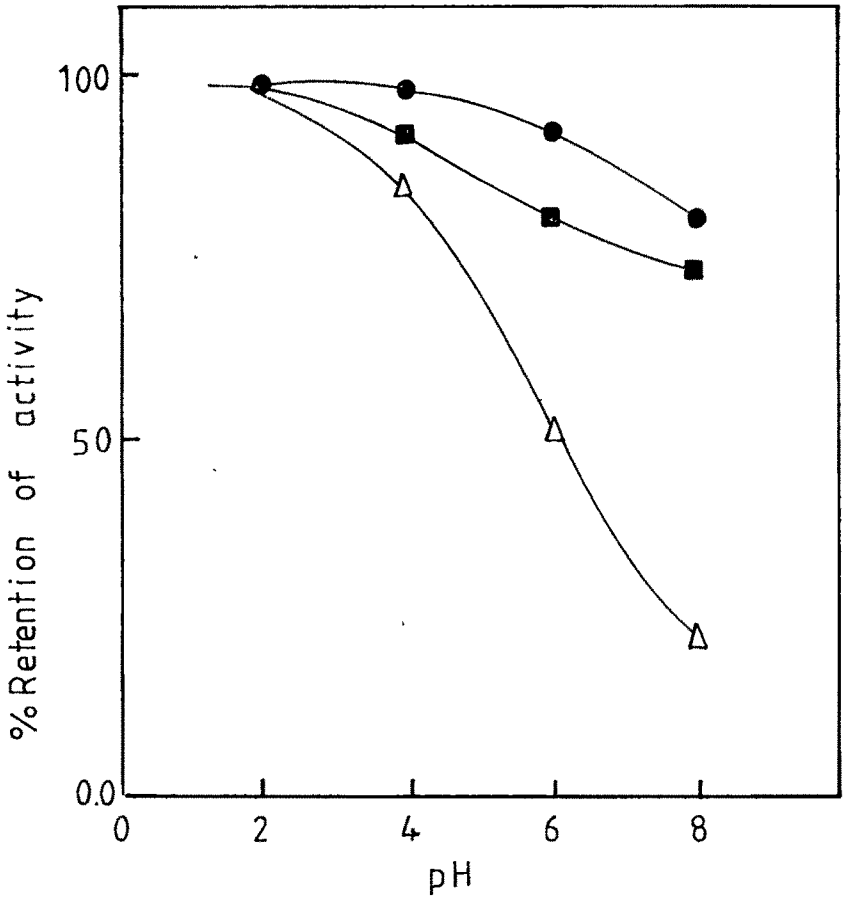

Fig 4.7 pH profile for free and immobilized enzyme
(●) Pepsin-SRF/PTS, (■) Pepsin-SRF/PBQ, (Δ) Pepsin.

(b) Thermal stability

Thermal stability plays an important role in designing the enzymatic applications. Hence, effect of temperature on the activity of free and immobilized pepsin was studied at 30°C to 70° C. From the results presented in Fig 4.8, it was observed that pepsin-SRF system shows the better retention of activity at the higher temperatures when compared with the free enzyme. This may be due to the higher resistance of covalently bound system against heat and denaturing than that of free form. From the results the activation energy of the free and immobilized systems was calculated by using the Arrehenius equation and plotting log V_{max} vs 1/T as discussed earlier in Section 2.4.6. The pepsin-SRF-system requires 1.37 and 1.85 Kcal/mole activation energy respectively for p-tolyl sulphonyl chloride and p-benzoquinone activation whereas free enzyme requires 2.7 Kcal/mole activation energy.

The decrease in energy of activation for immobilized system also confirms pore diffusion control of the process rather than kinetic control. This type of decreased activation energy in case of immobilized amino acylase by diazobinding method was observed by Maskova *et al*[78].

To study the thermal deactivation of the immobilized systems, they were further heated at 45°C for a longer time durations. The results are given in Fig 4.9. It was observed that free enzyme loses more than ~50% of its activity after 90 min incubation whereas immobilized enzyme retained 75% its activity after 3 hours incubation time. From the results, the thermodeactivation constant (K_d) was calculated as per the procedure reported by Hayashi and Ikada[79] and discussed earlier in Section 2.4.6. K_d was observed to be 1.18 and 6.49 min^{-1} respectively for free and immobilized pepsin.

(c) Michealis-Menten Kinetics (K_m and V_{max})

The enzymatic kinetics was studied by carrying out the reactions using different concentrations of substrate hemoglobin. The concentration of hemoglobin was varied from 1% to 6%. The Michaelis constant (K_m) and the reaction velocity (V_{max}) were calculated from Lineweaver-Burk plots of 1/v vs 1/s as discussed earlier and the results obtained are given in Table 4.4. Very similar K_m values for free and immobilized enzyme show that the enzyme has not undergone major structural changes during immobilization[80].

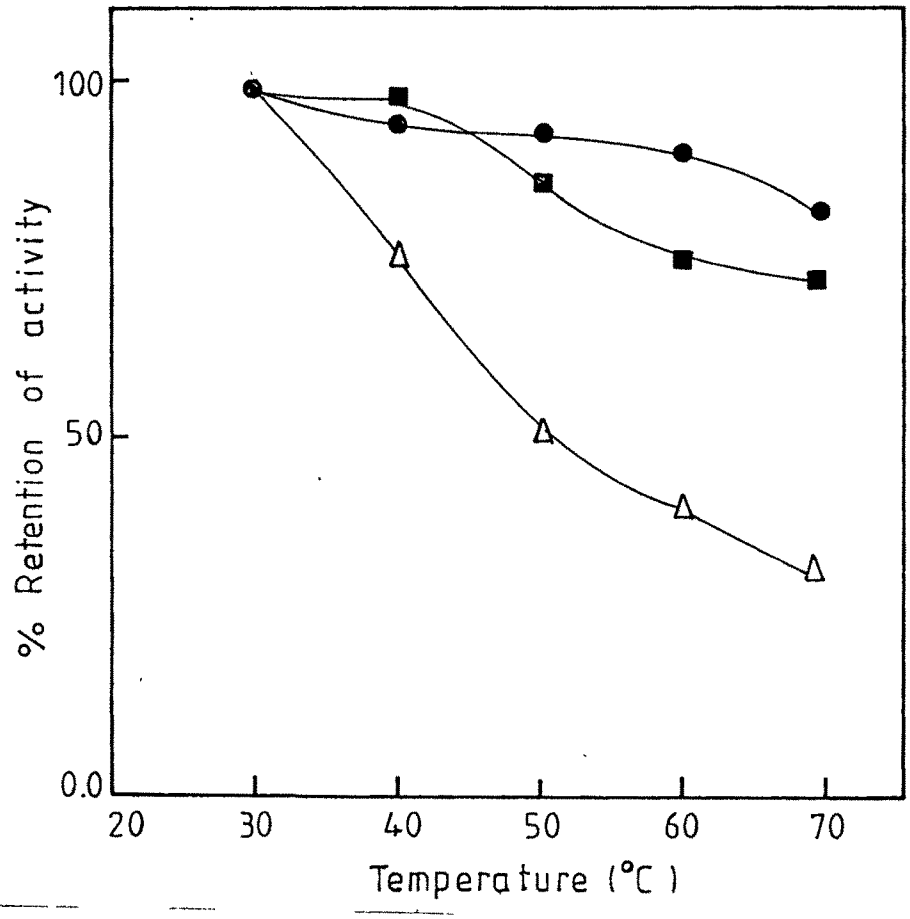

Fig 4.8 Effect of temperature on the activity of free and immobilized enzyme (●) Pepsin-SRF/PTS, (■) Pepsin-SRF/PBQ, (Δ) Pepsin.

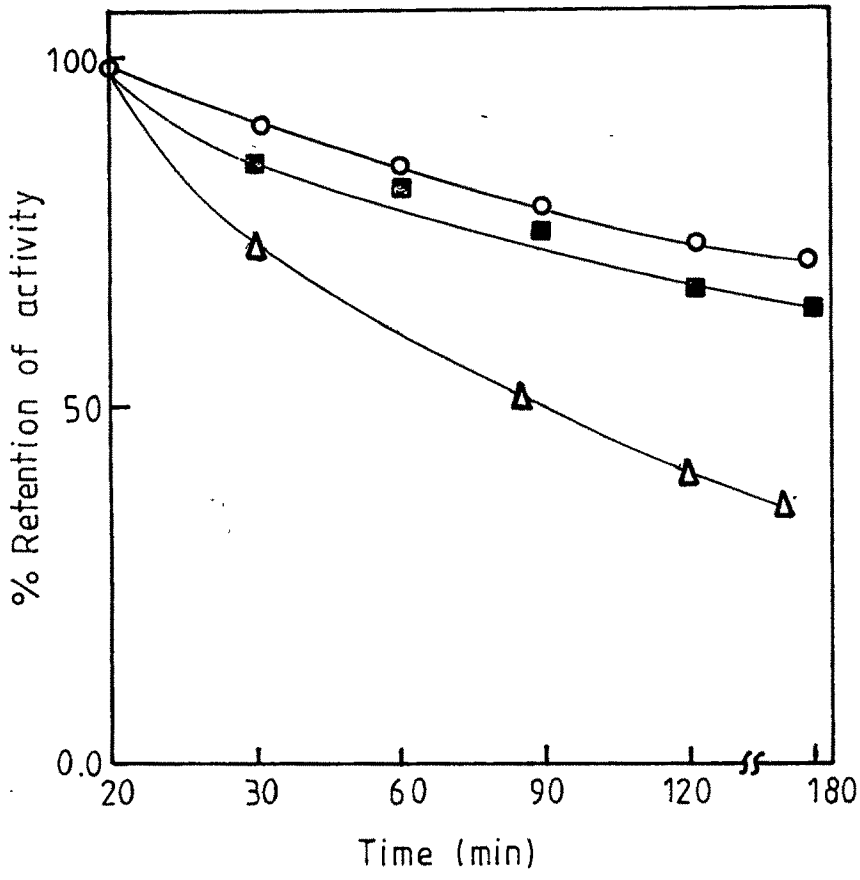

Fig 4.9 Kinetics of thermal deactivation at 45°C for free and immobilized enzyme through hemoglobin hydrolysis at pH 4
(○) Pepsin-SRF/PTS, (■) Pepsin-SRF/PBQ, (Δ) Pepsin.

Table 4.4 Kinetic parameters for free and immobilized Pepsin.

Sample	Michaeli's constant (K_m) [µg]	Maximum Velocity (V_{max}) [µg mL^{-1} min^{-1}]
Pepsin-SRF/PTS	0.76	2.28
Pepsin-SRF/PBQ	0.75	2.38
Pepsin-PVA/PBQ	2.01	1.30
Pepsin	0.76	1.36

Hemoglobin hydrolysis at pH 4, Temperature 37° C for 30 min.

(d) Storage stability

All the immobilized pepsin systems showed 50% or above retention of enzyme activity even after 3 months moist storage at low temperature as shown in Fig 4.10. Retention of activity was determined with respect to the activity of enzyme just after immobilization using hemoglobin substrate.

(e) Stability towards repeated use

Stability of immobilized pepsin is very important in application as it is subjected to repeated hydrolysis reaction as reported earlier. Figure 4.11 shows the effect of repeated use on the residual activity of immobilized pepsin on hemoglobin hydrolysis. The immobilized pepsin activity was observed to be retained 50% or above even after 5 repeated uses of the enzyme.

4.5.5 Clotting of milk by using fluidized bed reactor

Conventional methods of milk coagulation for the production of cheese are batch processes and involve expensive non reusable enzymes. This difficulty can be overcome by using immobilized proteolytic enzymes.

Coagulation of milk can be divided into two consecutive phases. In the primary phase (enzymatic stage) proteolysis takes place which is followed by aggregation of micelles producing clotting in the secondary phase (non enzymatic stage). In proteolysis hydrolysis of k-casein to para k-casein and to polypeptide takes place. Non enzymatic reaction is highly temperature dependent, during which micelles containing αs_1, αs_2 ß-caseins and k-caseins are formed.

The two basic proteins present in the milk are **casein** proteins and **whey** proteins. The hydrolysis of casein proteins leads to the coagulation of milk as mentioned above whereas whey proteins are discarded.

In general, most of the proteolytic enzymes can clot milk under suitable conditions. Of the many proteolytic enzymes investigated, pepsin displayed the best properties regarding activity, stability and eventual curd forming capacity[17]. Some of the factors such as pH, temperature, enzyme concentration, salt balance and protein content influence milk coagulation. This can be regulated accordingly by using the continuous and mechanised process of immobilized pepsin. From our studies it is observed that pepsin-SRF system shows higher coupling and stability. Hence it is used for the study

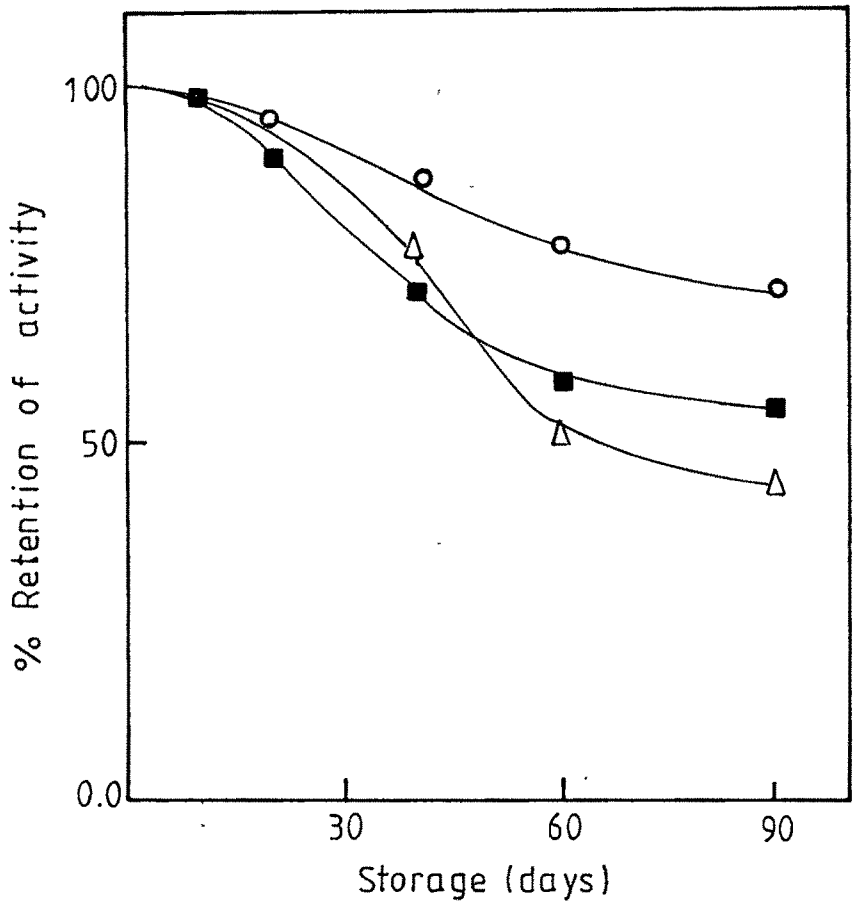

Fig 4.10 Storage stability for free and immobilized pepsin
(○) Pepsin-SRF/PTS, (■) Pepsin-SRF/PBQ, (Δ) Pepsin.

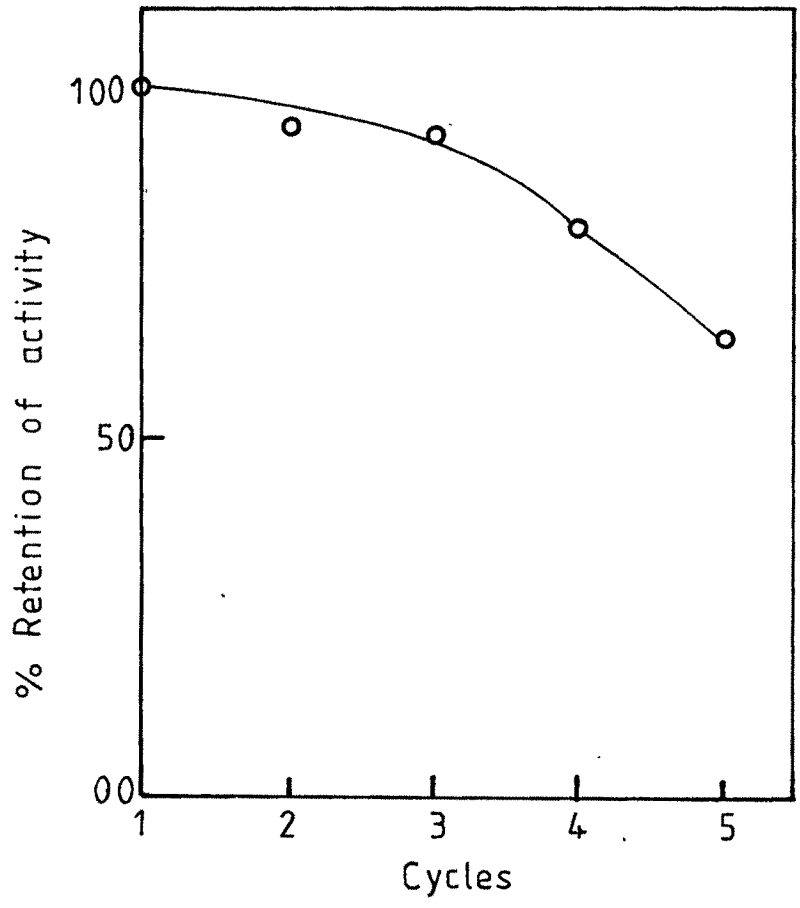

Fig 4.11 Immobilized enzyme for repeated use

of coagulation of milk using fluidized bed reactor. A low pressure drop and resistance to plugging can be achieved through the use of fluidized bed reactor.

Retardation of the clotting process at lower temperature of secondary phase is a well studied phenomenon and forms a basis for continuous coagulation of skimmed milk with immobilized enzyme[81]. Accordingly in the present chapter, we have examined the effect of temperature, and pH on the coagulation of skimmed milk treated with the pepsin immobilized on SRF. The immobilized pepsin was used in a water jacketed glass column (1 x 15 cm) reactor as discussed earlier (Fig 4.1). The primary enzymatic reaction was carried out at 15° C and the secondary stage of coagulation was carried out at 30-50° C.

(a) Effect of pH on milk coagulation

Ferrier *et al*[19] observed decay of immobilized enzyme activity at lower pH values for the pepsin immobilized on controlled pore glass. This may be due to the adsorption of milk casein on the glass resulting into a lower clotting activity at lower pH. In our study the treatment of milk at lower pH has resulted into greater clotting activity, may be due to the reduced adsorption of milk casein on the support. Moreover, the difference between the clotting time for the untreated and treated milk goes on decreasing at different temperatures as the pH increases (Fig 4.12). The activity measured at zero and predetermined time intervals indicates no leakage of the enzyme from the reactor. This was further confirmed by mixing the whey of the clotted milk with equal volume of fresh skimmed milk at the same pH.

(b) Effect of temperature on milk coagulation

The results obtained in the study of variation in temperature of secondary phase are given in Fig 4.13. Considerable decrease in clotting time was observed with increasing temperature of the treated milk. The clotting time shows pH dependence at lower temperatures but at higher temperature (50° C) it becomes pH independent. On the other hand the clotting time required for untreated milk was high even at high temperature and was pH dependent. The sharp decrease in clotting time for the treated milk can be attributed to the strong attractive forces between the micelles resulting into lowering of the clotting time. Similar results were obtained by Payens[82]. Green[83] suggested that higher potential of pepsin treated micelles at high temperatures results into lowering the clotting time.

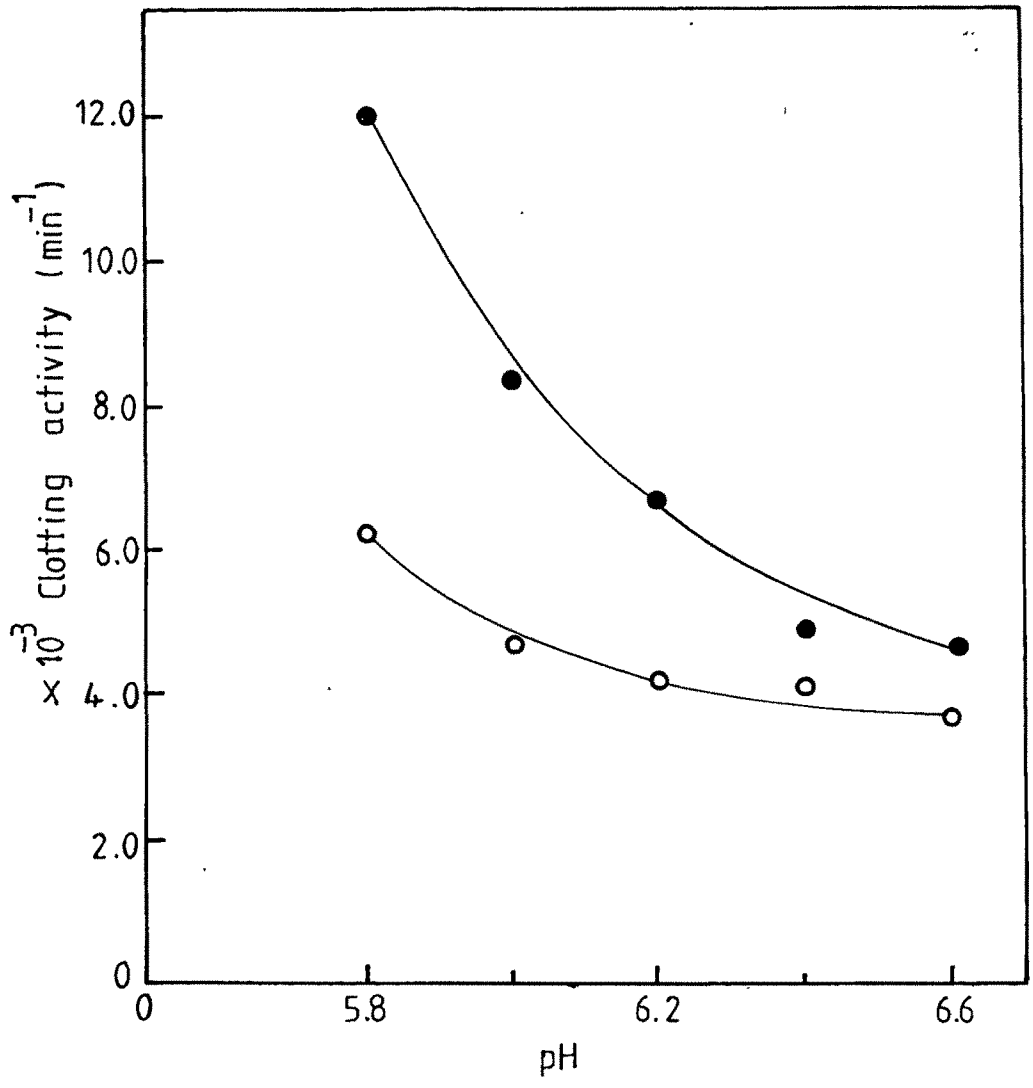

Fig 4.12.a Effect of pH on milk clotting at 30°C
(○) treated milk, (●) untreated

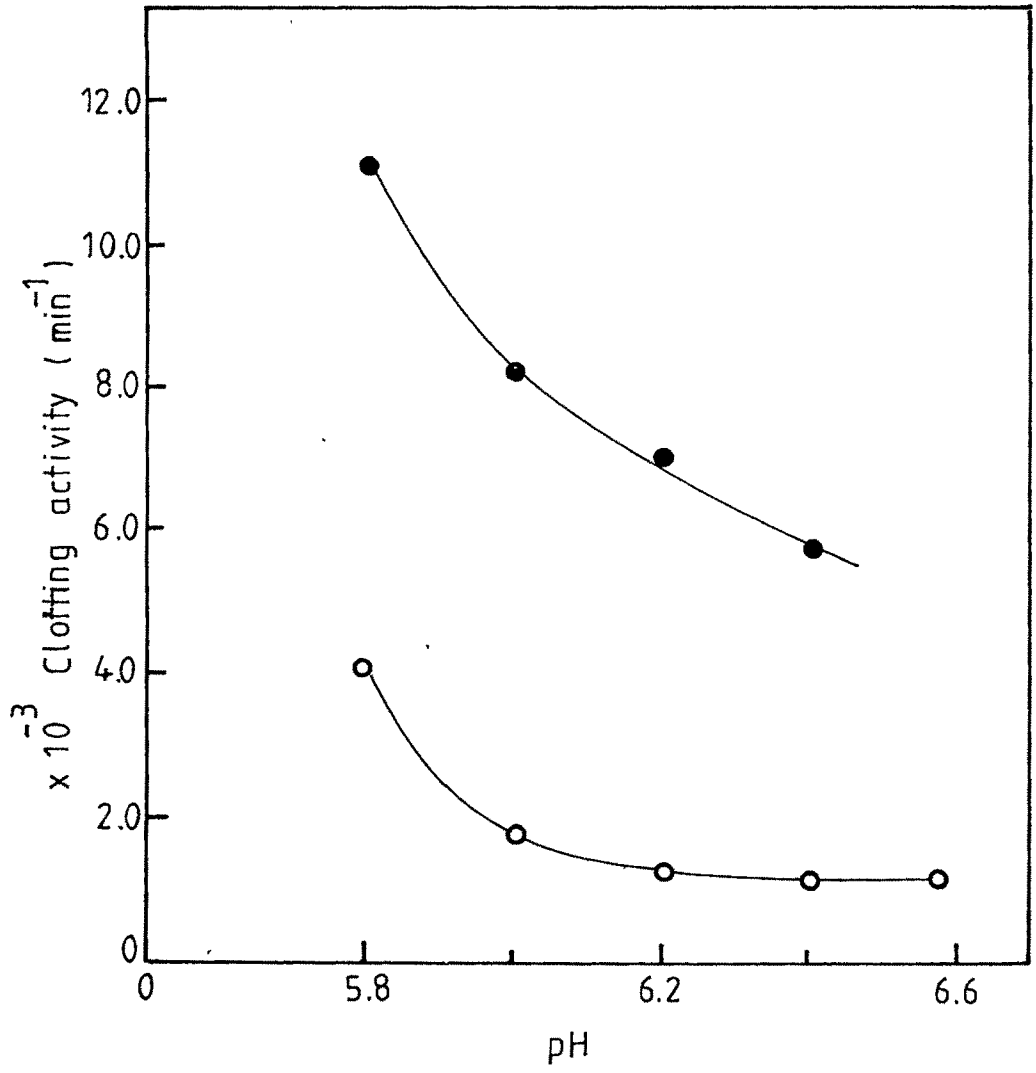

Fig 4.12.b Effect of pH on milk clotting at 40°C
(○) treated milk, (●) untreated

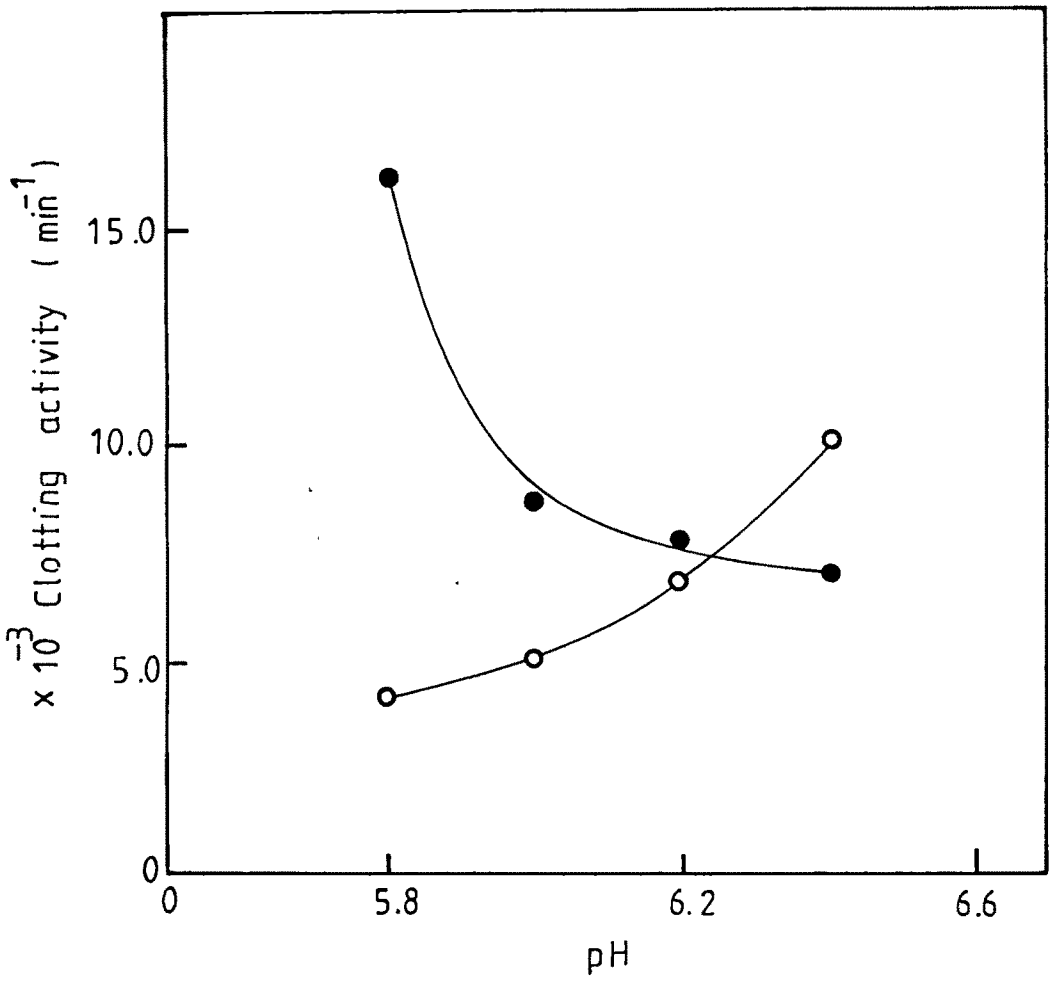

Fig 4.12.c **Effect of pH on milk clotting at 50°C (○) treated milk, (●) untreated**

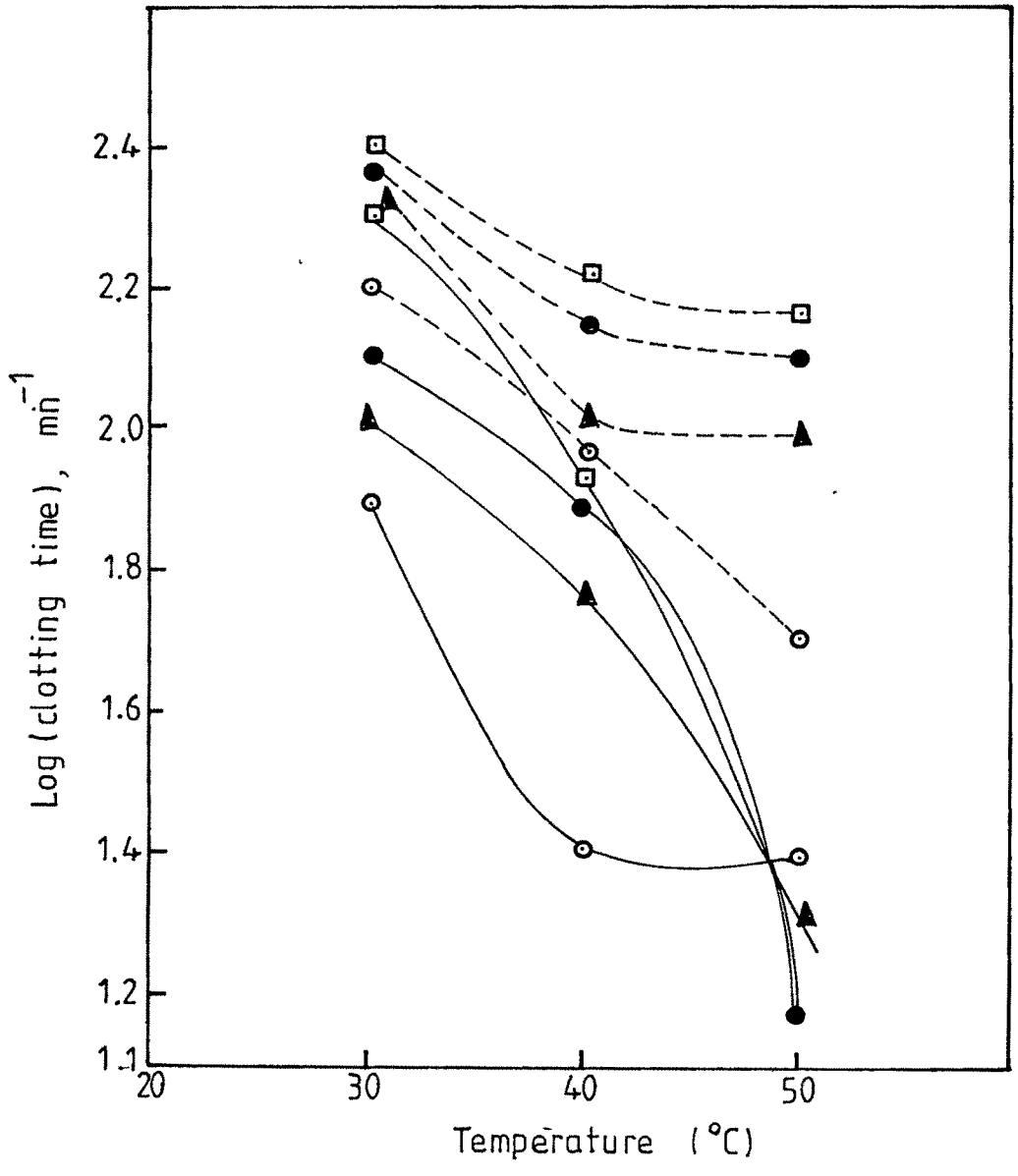

Fig 4.13 Effect of temperature on secondary phase of milk clotting
(—) treated milk, (----) untreated milk
(○) pH 5.80, (▲) 6.0, (●) 6.2, (□) 6.4
Flow rate 2 ml min^{-1}, space time 0.5 gm ml^{-1} min^{-1}

(c) Effect of dilution

Dilution effect was studied by mixing the milk fractions emerging from the reactor with equal volume of whey water and fresh skimmed milk. It was observed for both whey and water that clotting time increases almost identically as the volume ratio of treated milk: diluent increases. Whereas for the dilution with treated milk the time required for clotting was observed to be higher indicating that it is not only the dilution effect but also the increased population of untreated micelles interfering in the clotting process.

4.6 Conclusion

The feasibility of immobilized proteases (pepsin) for the continuous coagulation of milk has been greatly enhanced by using a fluidized bed reactor. However, overall it was observed that the SRF-support offers a high capacity polymeric material and gave a better performance during immobilization of pepsin. In the fluidized bed reactor studies, clotting of milk was observed to be less dependent on the pH of the milk. The deactivation of the reactor and the adsorption of casein were negligible. The immobilized pepsin showed better storage, thermal, and pH stability than the free pepsin.

4.7 REFERENCES

1. Bergmann, M., *Advances in Enzymol* ., 1942, **vol. 2**, 49.

2. Smith, E.L., *The Enzymes*, 1st Ed. 1951, **vol. 2**, 793.

3. Green, M.N. and Neurath, H., in *The proteins*, Academic Press, New York, 1954, **vol. 2**, 1057.

4. Davies, N.C. and Smith, E.L., *Methods of Biochem. Anal.* 1955, **2**, 215.

5. Laskowski, M., *Methods in Enzymol'*, Academic Press, New York, 1955, **vol. 2**, 8.

6. Northrop, J.H., Kunitz, M. and Herriott, R., *Crystalline Enzymes*, 2nd Ed. Columbia University Press, New York, 1948, 125.

7. Boyer, P.D., Lardy, H. and Myrback, K., *The Enzymes*, Academic Press, New York, 1960, **vol. 4**, 103.

8. Bergmayer, H.V., *Methods in Enzymology*, Academic Press, New York, 1965, **vol. 2**, 1046.

9. Mitz, M.A. and Schtueter, R.J., *J. Am. Chem. Soci.*, 1959, **81**, 4024.

10. Katchalski, E., *Polyamino acids Polypeptides and Proteins* (Ed. M.A. Stahmann), The University of Wisconsin Press, 1962, p. 283.

11. Manecke, G., *Pure App. Chem.,* 1962, **4**, 507.

12. Manecke, G. and Singer, S., *Mackromol. Chem.,* 1960, **39**, 13.

13. Mosbach, R., Kochschmidt, A.C. and Mosbach, K., In Immobilized Enzymes *Methods in Enzymology*, Academic Press, New York, 1976, **vol. 44**, 33.

14. Line, W.F., Kwong, A. and Weetall, H.H., *Biochem. Biophys. Acta.*, 1971, **242**, 194.

15. Vretblod, P. and Axain, R., *FEBS Letters*, 1971, **18**, 154.

16. Valaris, M. and Harper, H.J., *J. Food Sci.*, 1973, **38**, 477.

17. Cheryan, M., Vanwyk, P.J., Olson, N.F. and Richardson, T., *Biotechnol. Bioeng.*, 1975, **17**, 585.

18. Cheryan, M., Vanwyk, P.J., Olson, N.F. and Richardson, T., *J. Dairy Sci.*, 1974, **58**, 477.

19. Ferrier, L.K., Richardson, T., Olson, N.F. and Hicks, C.L., *J. Dairy Sci.*, 1971, **56**, 726.

20. Goldstein, L., *Biochem. Biophys. Acta.*, 1973, **327**, 137.

21. Beddows, C.G., Mirauer, R.A. and Guthrie, J.T., *Biotechnol. Bioeng.*, 1980, **22**, 311.

22. Hirano, S. and Miura, O., *Biotechnol. Bioeng.*, 1979, **21**, 711.

23. Abdel-Hay, F.I., Beddows, C.G. and Guthrie, J.T., *Makromol. Chem.*, 1981, **182**, 717.

24. Puvanakrishnan, R. and Boss, S.M., *Biotechnol. Bioeng.*, 1980, **22**, 919.

25. Puvanakrishnan, R. and Boss, S.M., *Indian J. Biochem. Biophys.*, 1984, **21**, 323.

26. Gorgani, M.N. and Karimian, K., *Biotechnol. Bioeng.*, 1984, **26**, 565.

27. Lastra, M.C. and Ortega, M., *AuR. Acad. Farm.*, 1985, **51**, 123.

28 Higeta Shoyu Co. Ltd., Japan, Kokai, Tokyo, 59, 91, 883, 84.

29. Dumitriu, S., Popa, M. and Dumitriu, C., *J. Macromol. Sci. Chem.*, 1987, **24**, 1135.

30. Ivanov, A.E. and Turkova, C.M., *Biocatalysis*, 1990, **3**, 235.

31. Gustovo, G. and Jacqueline, A., *J. Chem. Technol. Biotechnol.*, 1995, **63**, 247.

32. Mitz, M.A. and Schlueter, R.J., *J. Am. Chem. Soci.*, 1959, **81**, 4024.

33. Brandenberger, H., *Rev. Ferment. Ind. Aliment*, 1956, **11**, 237.

34. Surinov, B.P. and Manoilov, S.E., *Biochem.* (USSR) (English Trans.) 1966, **31**, 337.

35. Katchalski, E. and Bar-Eli, E., *Nature*, 1960, **188**, 856.

36. Takami, T. and Ando, E., *Seikagoku* (Jpn), 1970, **44**, 547.

37. Brummer, W., Hennrich, N., Klockow, M., Lang, H. and Orth, H.D., *Eur. J. Biol. Chem.*, 1972, **25**, 129.

38. Lee, E.C., Senyk, G.F. and Shipe, W.F., *J. Food Sci.*, 1974, **39**, 1124.

39. Robinson, P.J., Dunhil, P. and Lilly, M.D., *Biotechnol. Bioeng.*, 1973, **15**, 603.

40. Johanson, A.C. and Moshbach., *Biochem. Biophys. Acta.*, 1974, **370**, 339.

41. Stanley, W.L., Watlers, G.G., Chan, B.G. and Mercer, J.M., *Biotechnol. Bioeng.*, 1975, **17**, 315.

42. Coulet, P.R., Juthard, J.H. and Gantheron, *Biochem. Biophys. Acta.*, 1975, **391**, 272.

43. Dillon, J.G. and Wade, C.W.R., *Biotechnol. Bioeng.*, 1976, **18**, 133.

44. Abdel-Hay, F.I., Guthrie, J.T., Morrish, E.J. and Beddows, C.G., *Poly. Bull.*, 1979, **1**, 755.

45. Beddows, C.G., Mirauer, R.A., Guthrie, J.T., Abdel-Hay, F.I. and Morrish, C.E.J., *Polym. Bull.*, 1979, **1**, 749.

46. Beddows, C.G., Gil, M.H. and Guthrie, J.T., *Biotechnol. Bioeng.*, 1982, **24**, 1371.

47. Barker, P.S., Bottom, R.A. and Guthrie, J.T., *Polym. Photo Chem.*, 1982, **2**, 87.

48. Abdel-Hay, F.I., Beddows, C.G., Gil, M.H. and Guthrie, J.T., *J. Poly. Sci.*, 1983, **21**, 2463.

49. Cohenford, A.M., Santoro, P.E., Urbanowski, C.J. and Dain, J.A., *Biotechnol. Bioeng.*, 1986, **28**, 736.

50. Raghuram Reddy, C.P., Ram Reddy, C., Raghunath, K. and Joseph, K.T., *Biotechnol. Bioeng.*, 1986, **28**, 609.

51. Banateam Daniela, Shell, Bentia, H.D., Dascula, T. Simona., *Stud. Ceret. Biochim.* (Rom..,) 1986, **29**, 115.

52. Beddows, C.G., Gil, M.H. and Guthrie, J.T., *Biotechnol. Bioeng.*, 1985, **27**, 579.

53. Ulbrich, R., Schellenberger, A. and Dameran, N., *Biotechnol. Bioeng.*, 1986, **28**, 511.

54. Ortega, F. and Ortiga, M., *An. R. Acad. Farm.* (Spain), 1986, **52**, 525.

55. Masko, A.A., Marosova, A.A., Lyga, L.K., Galushko, N.A., *Prikl. Biolchim. Microbiol.* (Russ.), 1992, **28**, 211.

56. Okubo, M., Kamei, S., Tosaki, Y., Fukunaga, K. and Matsumoto, T., *Colloid. Poly. Sci.,* 1987, **265**, 957.

57. Beddows, C.G., Gil, M.H. and Guthrie, J.T., *J. App. Poly. Sci.*, 1988, **35**, 135.

58. Devi, S., Gurhrie, J.T. and Beddows, C.G., *Radiat. Phys. Chem.*, 1990, **36**, 707.

59. Dragamova, R., Tsolova, E. and Aleksier, B., *Khim. Ind.,* 1986, **58**, 272.

60. Fujimura, M., Takao, M. and Tosa, T., *Biotechnol. Bioeng.*, 1987, **29**, 747.

61. Serralheiro, M.L., Empis, J.M. and Cabral, J.M.S., *Biotechnol. Letters*, 1990, **12**, 167.

62. Alcantara, A.R., Simisterra, J.V., Torres, C., Guisan, J.M., Gil, M.H. and Williams, A., *Prog. Biotech.*, 1992, **8**, 443.

63. Cheng, E., Miranda, M.T.M. and Tomuaga, M., *Int. J. Peptide Protein Res.*, 1988, **31**, 116.

64. Heras, A., Martin, M.T., Acosta, N. and Debaillon V., *Prog. Biotechnol.*, 1992, **8**, 339.

65. Martin, M.T., Simisterra, J.V. and Heras, A., *J. Mol. Cat.*, 1993, **80**, 127.

66. Luthra, A.K., Andrew, W. and Pryee Robert, J., *J. Chem. Soci. Perkin Trans.*, 1987, **2**, 1575.

67. Jkamer, W., Robinshai, Shatzmiller and Tsaffrir, R., *J. Am. Chem. Sci.*, 1993, **115**, 8690.

68. Heras, A. and Acosta, N., *Biocatalysis*, 1994, **11**, 305.

69. Guoliang, T. and Shintaro, F., *Poly. J.*, 1995, **27**, 111.

70. Sunyan, Jin, X.H., Dong, X.Y., Yu, K. and Zhou, X.Z., *App. Biochem. Biotechnol.*, 1996, **56**, 331.

71. Bergmayer, H.V., *Methods in Enzymology*, Acad. Press, New York, 1965, **2**, 1046.

72. Anson, M.L., *J. Gen. Physiol.*, 1939, **22**, 77.

73. Erlanger, B.F., Kokowsky, N. and Cohen, W., *Arch. Biochem. Biophy.*, 1961, **95**, 271.

74. Hummel, C.W., *Can. J. Biochem.*, 1959, **37**, 1393.

75. Rejikumar, S. and Devi S., *J. Macromol. Sci. Pure App. Chem.*, 1995, **32**, 2035.

76. Ponnuchamy, N.P. and Gupta, M.N., *Biotechnol. Bioeng.*, 1989, **33**, 927.

77. Dumitriu, S., Popa, M., Artenic, V. and Dan, F., *Biotechnol. Bioeng.*, 1989, **34**, 283.

78. Maskova, H., Barth, T., Jivovsky, B. and Rychlik, Z., *Collection Czech. Chem. Commun.*, 1973, **38**, 943.

79. Hayashi, T. and Ikada, Y., *J. App. Poly. Sci.*, 1991, **42**, 85.

80. Dumitriu, S., Popa, M. and Dumitriu, C., *J. Macromol. Sci. Chem.*, 1987, **24**, 1135.

81. Ferrier, L.K., Richardson, T., Olson, N.F. and Hicks, C.L., *J. Dairy Sci.*, 1972, **55**, 726.

82. Payenes, T.A., *J. Dairy Sci.*, 1966, **49**, 1317.

83. Green, M.L., *Neth. Milk Dairy J.*, 1973, **27**, 278.