DESIGN, SYNTHESIS AND EVALUATION OF POLYMERS FOR AFFINITY BASED ENZYME SEPARATIONS

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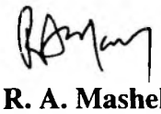
CERTIFICATE

Certified that the work incorporated in the thesis entitled "**Design, Synthesis and Evaluation of Polymers for Affinity Based Enzyme Separations**" submitted by Mr.A.A.Vaidya, was carried out under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.



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ABSTRACT

A wide range of techniques have been developed for the separation and recovery of different biomolecules. Amongst these, the affinity based techniques viz. affinity chromatography, affinity ultrafiltration and affinity two-phase aqueous extraction are attractive as they provide high specificity and selectivity during separation as compared to the conventional methods. Although selective, these techniques are beset with major shortcomings. To name a few, fouling of the membranes in affinity ultrafiltration, low capacity and flow rate limitations in affinity chromatography and contamination of the polymeric phase with final product in two-phase extraction. The affinity precipitation technique overcomes many of the problems associated with membrane filtration and affinity chromatography. It offers ease of scale up, concentration and purification, which could be achieved in a single step. It is amenable to continuous operation and the affinity ligand can be recycled. A large number of enzymes separated using this technique.

With continued investigations, many shortcomings of affinity precipitation too were realized. It suffers from limited stability of many natural affinity ligands, decrease in the affinity of ligand when incorporated in the polymer due to crowding effect, and steric hindrance posed by the high molecular weight polymers. The present investigation was undertaken to design and synthesize new affinity polymers that would overcome the crowding effect and enhance the ligand-enzyme binding in affinity thermoprecipitation process. To adapt and demonstrate this methodology for the recovery of a commercially valuable enzyme, lysozyme was selected as a candidate. A comparative analysis of recovery of lysozyme by acidic thermoprecipitating polymers versus affinity based synthetic/natural ligands has been done.

In the recent past, molecular imprinting technique has been employed in various bioseparations. In this, secondary valance interactions such as hydrogen bonding, ionic and hydrophobic interactions are exploited not only during synthesis of an imprinted polymer but also during rebinding studies. Since only weak interactions are involved in the rebinding of the desired molecule, the selectivity and capacity of such imprinted receptors is often low. In order to improve on this the enzyme-affinity ligand interactions are exploited during synthesis of molecularly imprinted polymers.

This work is presented in seven chapters and a brief outline of each chapter follows:

Chapter 1: Literature survey

Conventional separation techniques and separation based on affinity techniques have been reviewed. The principle, basic steps involved in each technique, pros and cons and finally applications of each technique with special reference to the polymers used therein have been discussed. A detailed discussion on the affinity precipitation follows which includes both homo-bifunctional and hetero-bifunctional mode. The smart polymers are an indispensable part of hetero-bifunctional precipitation. Therefore, the literature on the smart polymer is also briefly reviewed. In this a thorough discussion on affinity thermoprecipitation is given with appropriate examples that have been listed in the literature so far. The prerequisites for an ideal polymer to be used in affinity precipitation are summarized. Lastly, a section on molecular imprinting technique is included.

Chapter 2: Objectives and Scope of the work

The objectives in undertaking the present investigation in view of the prior results obtained in the field of hetero-bifunctional affinity precipitation and molecular imprinting have been summarized. The scope of the work is mainly confined to the improvement and developing new polymers to overcome the limitations experienced so far.

Chapter 3: Enhancing ligand-protein binding in affinity thermoprecipitation: Elucidation of spacer effects

The copolymers of N-isopropylacrylamide (NIPAM) and N-acryloyl amino acids of varying chain length were synthesized. Para aminobenzamidine (PABA) was chemically linked to the pendant carboxyl groups of these polymers to obtain thermoprecipitating affinity polymers. The inhibition constant (K_i) of these polymers for trypsin decreased, i.e. the efficiency of PABA - trypsin binding increased with increase in the spacer chain length. It was demonstrated that the enhancement in trypsin binding by the polymers was due to the spacer as well as microenvironmental effects. Moreover, the recovery of trypsin from aqueous solution and from chymotrypsin mixture was found to increase with spacer chain length. Finally, it was demonstrated that the inhibition constants of these affinity polymers were not adversely affected by the crowding effect.

Chapter 4: New synthetic ligands for lysozyme thermoprecipitation

New synthetic ligands containing acetamido group and a spacer were synthesized. These were conjugated with an acrylic monomer and copolymerized with NIPAM to yield the affinity thermoprecipitating polymers. These polymers inhibited lysozyme far more efficiently than those containing N-acetylglucosamine (NAG). Spectrophotometric analysis revealed that the mode of binding of pendant acetamido groups in the monomer

with lysozyme was identical to that between natural ligand i.e. NAG by lysozyme. The amount and activity of lysozyme recovered from aqueous solution as well as lysozyme-ovalbumin mixture increased with the length and the hydrophilicity of the spacer. In sixteen cycles, the thermoprecipitating polymer comprising synthetic ligand exhibits increased stability as compared to the polymer containing NAG. Thus, this investigation led new synthetic ligands for the recovery of lysozyme. The approach is generic and could be extended to other enzymes as well.

Chapter 5: Acidic thermoprecipitating polymers for lysozyme recovery

A comparative study on the recovery of lysozyme using acidic thermoprecipitating polymer against affinity based polymer has been made. Various synthetic acidic monomers were copolymerized with NIPAM to yield thermoprecipitating polymers exhibiting lower critical solution temperatures (LCST) in ambient range. These polymers bind to lysozyme by electrostatic interactions with basic amino acids on the surface whereas NAG binds at active site of enzyme. This resulted in enhanced efficiency of binding by acidic polymers than those containing NAG and synthetic N-acetamido ligands reported in previous chapter. The total number of binding sites (q^{app}_{max}) was higher below LCST than above LCST. The dissociation constant (K_d) was marginally higher above LCST. The polymer comprising acrylic acid exhibited higher recovery of specific activity of lysozyme than the polymer containing acryloyl-N-acetylglucosamine (Ac. NAG). More than 90 % of lysozyme activity could be recovered from egg white using this polymer. The approach, therefore, could provide a method for selective recovery of lysozyme from egg white.

Chapter 6: Enhanced selectivity of the receptor by affinity-imprinting technique

Molecular imprinting technique has been used for the synthesis of bio-receptors. In this work trypsin imprinted receptor was synthesized by employing affinity based molecular imprinting technique. In this trypsin and N-acryloyl paraaminobenzamidine complex was polymerized with comonomer acrylamide and crosslinker methylenebisacrylamide. Template trypsin was eluted out from the polymer. Various control experiments were performed to demonstrate the synergistic affinity and imprinting effect. It was shown that imprinting efficacy is governed by the percentage of crosslinker used. At an optimized percentage of crosslinker (50 %) trypsin imprinted receptor exhibited exclusive recognition of trypsin from a mixture of trypsin and chymotrypsin. Moreover, the uptake capacity of trypsin imprinted gel was hundred folds higher than in the case of conventional molecularly imprinted silica gels employing mainly hydrogen bonding interactions.

Chapter 7: Conclusions and suggestions for future work

In this chapter all the results of present investigation have been summarized. Based on the conclusions arrived at, suggestions for future work to validate the consequences resulting from this work and to enhance the ligand – enzyme binding have been made.

Chapter 1

Literature Survey

1.0.0 Introduction

Separation of various enzymes and proteins from their natural sources is one of the important areas in biotechnology that combines multidisciplinary knowledge and expertise. Bio-separations enable recovery of desired biomolecules in pure form, which further find diverse applications in food, pharmaceutics and chemical industries. So far, various techniques for separation of proteins/enzymes from their natural sources have been developed that range from conventional salting out of proteins to modern membrane ultrafiltration units. Amongst these, the affinity based separation techniques which rely on selective recognition between biomolecules (ligate) and their respective ligands are most suitable. The basic principle used in affinity separation is to form a complex between desired enzyme and its affinity ligand. Ligands are generally selected from active site inhibitors, dyes, substrates and coenzymes that have very high affinity for the given enzyme. The enzyme-ligand complex is then processed. Based on the process used, affinity separations are further classified as affinity chromatography, affinity ultrafiltration, affinity precipitation etc. Affinity precipitation in particular is an attractive technique as it offers simplicity of operation, scale up and reuse. In this, the enzyme-ligand complex is precipitated out from the solution by various means. The complex is then dissociated and the enzyme is obtained in pure form.

Affinity precipitation has been broadly classified into homobifunctional and heterobifunctional precipitation based on the differences in mechanism of precipitation. In homobifunctional precipitation, two bifunctional ligands are joined together through a spacer arm. This macroligand forms complex with the different binding sites on two different biomolecules. Eventually, this complex grows in size forming large three-

dimensional networks. At an optimum ratio of ligand to biomolecule and at the proper length of spacer, the complex precipitates out from solution. In heterobifunctional affinity precipitation, the macroligand consists of two parts. The first part is the ligand that binds to biomolecule and forms the complex. The second part of this system is stimuli sensitive. Upon change in the environment such as pH, temperature, light etc., this stimuli sensitive part precipitates causing the precipitation of the entire macroligand-enzyme complex. The stimuli sensitive part is usually made up of a smart polymer, which is sensitive to the changes in the external environment.

The use of smart polymers in affinity separation has a number of advantages. One can tailor the smart polymer according to the specific application. Unlike macroligands used in homobifunctional precipitation, these synthetic polymers are very stable and are resistant to denaturation due to pH or temperature shocks. However, there is still a large scope to improve the properties and performances of macroligands based on smart polymers. In the present work we have directed our efforts to design a new family of thermosensitive polymers that can be used in a variety of heterobifunctional affinity precipitations. These polymers provide a number of advantages such as precipitation at ambient temperature, higher accessibility of the attached ligands for biomolecules and also improved binding of enzyme to the macroligand. We have also developed some new synthetic ligands that bind far more efficiently than their natural counterparts. In addition to this we have developed a new technique that combines merits of molecular imprinting and affinity separation.

The present work has been undertaken after a systematic review of the literature available. As mentioned above, our work is focused on designing novel smart polymers

and polyligands for affinity separation. Therefore, although there is vast literature available on the bio-separations, we have briefly overviewed different non-affinity based conventional separation techniques. Further, we have confined our literature survey mainly to the various affinity based separation techniques and the polymers used therein.

The literature survey starts with a brief description of conventional protein separation techniques. This is followed by a description of the importance of affinity separations over the earlier techniques. A brief description is given on the affinity based recognition between biomolecules such as enzymes and their inhibitors. This is followed by detailed review of various affinity based enzyme purification techniques that have been developed so far. These are discussed based on the principles they use and the merits and demerits of each technique. Various examples cited in the literature pertinent to each of the affinity separation technique have been described. Then literature survey deals with a separate section on what are smart polymers and how they respond to different stimuli. As, smart polymers are frequently used in affinity precipitation technique it is followed by a detailed discussion on this technique, with reference to the reported work in the literature, which describes the present state of the art. There is a section on molecular imprinting technique with special reference to non-covalent imprinting approach used in bioseparations. Different examples related to protein/enzyme separation using this approach has also listed. Finally, the literature survey ends with concluding remarks which comment on the present state of the affinity precipitation art, which calls for the design of novel smart polymers and polyligands that can help to solve the existing problems in this technique.

1.1.0 Separation of proteins based on solubility differences

There are two types of interactions that generally help protein to solubilize in water. One is hydrogen bonding and ionic interactions between surface polar amino acids and surrounding aqueous medium. The other is hydrophobic interaction amongst bulky non-polar amino acids so as to remain away from water. Any protein precipitates if these interactions are weakened or disrupted. So far, four different approaches have been used to lower the solubility of the protein.

a) Change in pH

The isoelectric point (pI) of a protein is the pH at which there is no net charge on the protein and at which it is least soluble in the aqueous medium. The intra- or inter-molecular electrostatic repulsion is at minimum, which causes protein to coalesce and precipitate. Different proteins have different isoelectric points depending upon the content and composition of the amino acids. Lysozyme has pI of 11.0 because of high proportion of positively charged amino acids e.g. Arg., Asn., Lys. In order to achieve precipitation of the desired protein, the pH of the solution is adjusted to its pI. The advantage of this procedure is that precipitated protein remains in its native conformation and can be resolubilized by adjusting pH and salt concentration. The major disadvantages are non-selectivity during precipitation, as the pI of different proteins lie in close vicinity and the pH sensitive enzymes can not be purified by this method.

b) Change in ionic strength

The solubility of a protein progressively decreases with increase in ionic strength. The salting out of a desired protein can be achieved by increasing the ionic strength of the solution so as to precipitate the protein. The salt concentration required to salting out the

desired protein depends upon the total number of ionic/polar amino acids. At high salt concentration the ionic interactions between salt and polar amino acids predominate over hydrogen bonding interactions with water (water of hydration), which precipitates the protein. This process of salting out is complex and complete physio-chemical details of the process are still unknown. Ammonium sulfate [$(NH_4)_2SO_4$] is frequently used for salting-out as it provides high ionic strength without denaturation of protein and its solubility in water is also high. This technique of protein precipitation is generally used in initial stages of protein separation in bulk, as it is easy to scale-up and often yields are high. The major disadvantage is the product obtained by this method is crude which requires further processing by other techniques such as gel filtration, dialysis or solvent fractionation.

c) Solvent fractionation

Protein solubility is affected by dielectric constant of the medium. At a constant pH and ionic strength, water miscible organic solvents decrease dielectric constant of the aqueous medium. With decrease in dielectric constant, the interactions between oppositely charged amino acids are enhanced which correspondingly reduces their hydrogen bonding with water. This results in increase in the apparent hydrophobicity of the protein with respect to surrounding water. At a particular concentration of organic solvent the protein precipitates out from solution. This process is economical, easy to scale up and not energy intensive. The major drawback of this process is the high risk of enzyme deactivation and nonselectivity during precipitation. Normally, all precipitation processes mentioned above are not employed individually but are used in combination with each other or with chromatographic techniques as illustrated by following examples.

In one of the earlier reports on protein precipitation, El Nockrashy et al (1977) isolated two meal proteins using countercurrent solvent extraction and isoelectric precipitation as follows. 0.02 N sodium hydroxide (NaOH) with a meal-to-solvent ratio of 1:25 was used during solvent extraction and subsequently a two step isoelectric precipitation was performed at pH 6.0 and 3.6 to yield two different fractions of meal proteins with 93 % and 98.6 % recovery. Taha et al (1981) isolated a meal protein from defatted sunflower seed meal by first carrying out counter current solvent extraction using 0.04 M NaOH and 0.1 % sodium sulfite (Na_2SO_3) with solvent to meal ratio of 20:1. The obtained crude protein was precipitated by isoelectric precipitation at pH 4.0. This resulted in 88 % recovery of meal protein.

For separation and purification of amylase secreted by *Streptomyces aureofaciens* 77 grown in liquid inorganic salts-starch medium, different separation strategies were successively employed as described in the following. Initially, $(NH_4)_2SO_4$ fractionation at a concentration of 50-70 %, was used to precipitate amylase from cell free extract. The salt was removed by dialysis and then crude enzyme was applied to DEAE-cellulose column to increase the purity of the enzyme by 60 fold. Further purification was carried out by gel permeation chromatography using Sephadex-G 200 column. This resulted in an increase in purification by 74 fold. The purified amylase can be used in the manufacture of various detergents. The above mentioned reports clearly illustrate that different conventional methods have to be employed in combination with one another to obtain enzymes having desired purity [Ibrahim et al (1990)].

Chang et al (1991) used organic solvent i.e. pure dimethyl sulfoxide (DMSO) instead of water as the reaction medium for protein separation. Bovine pancreatic trypsin

was purified by first dissolving it in DMSO (1-50 mg/ml) and then adsorbing it onto carboxymethyl-cellulose column in DMSO. Trypsin was desorbed from the column using inorganic salts such as sodium chloride (NaCl). Further, in the same investigation, a mixture of bovine pancreatic chymotrypsinogen and chicken egg ovalbumin was fractionally precipitated in DMSO using varying concentrations of ethyl acetate, the solvent which does not dissolve proteins.

Recently, Mineyama and Saito (1998) purified pyroglutamyl peptidase enzyme from *Enterococcus faecalis* ATCC 19433 by employing different techniques after cell lysis with N-acetylmuramidase. The proteins were salted out, then subjected to gel filtration and finally to anion-exchange chromatography. This gave 46-fold purification of enzyme.

d) Thermoprecipitation

At temperatures greater than 40 ^{0}C, H-bonding and ionic interactions between different polar side chains of amino acids begin to break down and most proteins denature exposing their interior hydrophobic core out-side, which causes the protein to precipitate. Although, this method is fast and requires no extra addition of chemicals, it is used seldomly as most of the proteins particularly enzymes are thermolabile and may get deactivated during isolation at high temperature.

Although, all above techniques are simple to perform, fast and easy to scale-up, they require the prior knowledge of certain physical properties of the desired biomolecule. For isoelectric precipitation, the knowledge of pI and for salting out the concentration of salt required to selectively precipitate the desired proteins are essential. Also, in case of solvent fractionation and thermoprecipitation the risk of denaturation of

protein is very high. Most of the times the product obtained by salting-out or isoelectric precipitation is a mixture of different proteins. Thus all these conventional techniques lack the selectivity and specificity for the recovery of target protein/enzyme. As, the demand for the high purity enzyme products is increasing day by day; it enforces biotechnologist to search for new methods for selective enzyme recovery [Lehninger 1975)].

1.1.1 Ion-exchange chromatography

The net-charge on the protein is affected by composition of amino acid, pH and ionic strength of the solution. Different proteins have different amino acid composition and thereby exhibit different net charges at physiological pH. The proteins can be separated from each-other depending upon their net charges. This is the basis used for separation of various proteins by ion-exchange chromatography. In this technique, the desired protein is allowed to adsorb reversibly on ion-exchangers under the conditions that give stable and tight binding. The elution of the protein is carried out by change in pH, ionic strength or by using chaotropic agent in eluting buffer. The ion-exchangers generally comprise insoluble, three-dimensional network or matrix, which is made up of dextran or cellulose or styrene. To this matrix the charged groups are covalently linked. Depending upon the net charge on the matrix the ion-exchangers are further divided into

a) Cationic-exchanger

Negatively charged e.g. sulphonic ($-SO_3^{-2}$), carboxyl ($-COO^-$) groups are covalently linked to matrix. This exchanger binds to the proteins containing positive net charge.

b) Anionic-exchanger

Positively charged e.g. quaternary amino ($-N^+R_3$) groups are covalently linked to exchanger. This binds to the negatively charged protein.

Following are the routine steps used in conventional ion-exchange chromatography –

1. The columns are packed with ion-exchange resin.
2. The resin beads are equilibrated with loading buffer.
3. A mixture of proteins containing target protein is loaded on the column.
4. The unbound proteins are washed away from the column.
5. Finally, the desired protein is eluted by either gradient or step-wise elution.

This technique provides certain advantages over conventional precipitation technique such as improved purity of the product, sequential separation of different proteins is possible and the eluted protein is in active form. But, it has few draw backs such as poor resolution, diffusional spreading of bands under low flow rates reduces final yield of the product, it is time consuming and tedious method. [Freifelder (1982)]. Following are the pertinent examples, from the literature, which describe the applications of this technique.

There exists a problem of contamination of mucoproteins present in the mammalian stomach tissue during extraction of milk-coagulating enzymes (rennet solution). This was overcome by using ion-exchange resin. At pH 5.5, only milk coagulating enzymes were able to bind to anionic exchanger because at this pH the contaminating mucoproteins were uncharged and eluted out of the column, which resulted in the separation of rennet solution [Clarke (1976)].

The separation of highly unstable enzymes such as porcine and bovine carbonic anhydrases using ion exchange column was achieved by Bergenhem et al (1985). They used DEAE-cellulose ion exchanger for separations, which resulted in affording gram quantities of the product in 2-4 hours with high degree of enzyme homogeneity.

Shinano et al (1993) have examined in detail the adsorption capacity of lysozyme on strongly acidic cation-exchange resin with respect to the concentration of ionic groups. Acidic groups i.e. sulfopropyl groups (SP) were introduced in the microporous fibres by hydrolysis of epoxide group into diol which was reacted with propylsultone. The SP group density of the resulting hollow fiber ranged from 0.21 to 0.84 mol/kg of dry fiber. Lysozyme adsorption was examined during permeation of the solution (pH 6) through the pores across a hollow fiber. It was found that there was negligible diffusional resistance of lysozyme to the SP group. Also, the binding capacity of lysozyme on the fiber was constant in the SP group range studied. For comparison, the adsorption characteristic of cupric chloride solution during permeation was also examined. It was found that the binding capacity of Cu^{+2} to the fiber increased linearly with increasing SP group density, because cupric ions are smaller in size than lysozyme and thereby invade the depths of the grafted polymer branches.

Recently, Longa-Kowalik and Rogalski (1998) have demonstrated the purification of three isoforms of endo-1,4- β -glucanases from the *Phlebia radiata* using ion exchange column chromatography on DEAE-Sepharose and CM-Sepharose with 50 to 200-fold purification.

A slightly modified technique, which combines ion-exchange chromatography with partitioning, is used in resolving a mixture of weakly polar biomolecules such as

sugars. It is known as reversed-phase chromatography. In this technique, a mixture of sugars dissolved in a polar/non-polar mixed solvent system is applied to an ion-exchanger column. The polar solvent binds to the column matrix forming a polar stationary phase and sugars are differentially partitioned on this polar stationary phase and a mobile non-polar phase. Ghosh et al (1993) resolved the anthraniloyl derivatives of sugars by reverse-phase chromatography. These derivatives exhibit differences in their hydrophobicity, which render them to partition differentially on the column depending upon the hydrophobic content of individual sugar.

1.1.2 Molecular-sieve / Gel chromatography

The overall three-dimensional shape and size of a protein is governed by composition and sequence of amino acid in a peptide, secondary/tertiary structure and molecular weight. The separation of different proteins based on shape and size dissimilarity is the basis of gel chromatography. It comprised of a three-dimensional crosslinked network of acrylamide or dextran or agarose with different pore sizes used as a stationary phase. An aqueous solution of a mixture of protein is passed through the column. Protein with size larger than pores moves through spaces between the individual stationary phase particles and hence not retarded by the column. On the other hand, proteins with smaller size than the pores diffuse in and out of matrix particles and thereby are slowed down moving through column. Therefore, the largest protein will elute first or if the shapes of different proteins are relatively constant, then high molecular weight protein will come out of column first. At very low ionic strength other factors such as net charge on protein affects separation. The steps involved in gel chromatography are similar to ion-exchange chromatography.

The fractionation of proteins using this method is independent of temperature and pH. This technique is applied to separate enzymes, which are sensitive to the changes in their external environment such as pH and temperature. It is a useful tool in determination of molecular weight of enzymes. The disadvantages in this technique are non-specific adsorption of proteins on gel, zone-spreading under low flow rates and at high ionic strength the gel behave partly as ion-exchanger. Sephadex G-100 was successfully used for the purification of acid protease from *Humicola lutea* 120-8. This recovered 72 % (on protein basis) acid protease [Nikolova et al (1980)].

Davis and Radke (1982) modified the conventional gel chromatography to determine activity of the enzyme and heterogeneity in the molecular weights of the active fraction simultaneously. This was achieved by halting flow rates while the enzyme is still present on the column, it provides information on the relative distribution of enzyme activity. A very low level of enzyme activity could be detected by continuous monitoring the absorption profile with respect to the stable baseline. This provides an information on the presence of a small amount of active enzyme with differences in their molecular weights. This method was applied to various systems such as separation of aldolase from rabbit muscle, pyruvate dehydrogenase and α-ketoglutarate dehydrogenase from bovine heart, and hexokinase and glucose 6-phosphate dehydrogenase from yeast.

The gel chromatography may not be directly applied for the purification of protein from crude source. Because the sample needs a thorough pretreatment in order to remove small particulates from the solution which otherwise will clog the column. This can be exemplified as follows. The final stage purification of cell wall antigen from *Coxiella burnetii* was done by gel chromatography. Prior to chromatography, in order to improve

the purity of the crude antigen it was extracted by following treatments - heat, ultrasonication, phenol extraction and treatment with SDS or sodium deoxycholate [Sting and Westphal (1993)].

1.1.3 Hydrophobic interaction chromatography

Proteins are folded into a tertiary structure so as to keep the interior hydrophobic domain away from the aqueous solution. The hydrophobic amino acids interact with each other to provide minimum contact with surrounding water. In strong polar solvents, such hydrophobic regions come close to each other and interact strongly with hydrophobic matrices. The binding of a desired protein from a mixture depends upon the hydrophobicity of that protein, the type of solvent used for binding and elution and nature of support. The routine steps involved in this chromatography are as follows –

1. A column is packed with hydrophobic stationary phase e.g. phenyl-agarose or octyl-agarose.
2. A mixture of proteins is loaded on the column under high ionic strength.
3. The desired protein is eluted from the column by reducing polarity of mobile phase e.g. lowering ionic strength, addition of ethanol or ethylene glycol, addition of detergent or chaotropic agents or urea. These eluting agents disrupt the water structure surrounding the protein and thereby decrease the hydrophobic interactions with the matrix.

The major advantage of this technique is that a mixture of proteins with high salt (1 M $(NH_4)_2SO_4$) solution can be directly applied on the column for purification of desired protein. This is because the binding step is carried out under high ionic strength. Since the cumulative hydrophobic interactions between protein and hydrophobic surface of the matrix are comparatively stronger, usually harsh elution conditions are employed

to desorb the protein from the matrix. This may denature the desired protein or result in non-quantitative recovery of the product. This technique is used very rarely for enzyme separation as demonstrated in following example.

Recently, Zhu et al (1999) successfully applied this technique for purification of enzyme bromelain from crude stem of bromelainn. The phenyl groups were grafted on cellulose resin to create a hydrophobic stationary phase. The optimum adsorption-desorption conditions were as follows. On a 10 mL capacity hydrophobic resin 100 mL of crude bromelainn was applied. The column was run using a pH 5.0 mobile phase containing 1.5 mol/lit. $(NH_4)_2SO_4$. The desired protein was eluted at pH 8.0. By this method enriched bromelain with 4.4 fold enhancement in activity was obtained with total enzyme activity recovery of more than 120 %. The additional advantages offered by this support are - it can be used in the pH range of 1 to 13. As the support is rigid it can be used under low-pressure conditions. It was found that in fifty repeated cycles this support did not loose its efficiency.

1.1.4 Covalent chromatography

The principle of this chromatography technique is the formation of covalent bond (mainly disulfide bonds) between sulfur containing amino acids present on the surface of the desired protein with the stationary phase. The unadsorbed proteins are washed and the desired protein then eluted by breaking the covalent bond formed with the support. The most common stationary phase is activated thiol-sepharose containing 2,2"-dipyridyl disulfide which under mild conditions forms mixed disulfides with the thiol groups of proteins. The elution of the protein can be brought about by the addition of L-Cysteine in the mobile phase. This technique is most useful in the separation of proteins having a

large number of free thiol groups as exemplified by separation of papain and urease by Gemeiner et al (1977) on cellulose tolylisothiocyanate as a support matrix. Further, they showed that urease had a higher proportion of sulfur containing amino acids than papain. Subsequently, Kenichi et al (1980) et al used SH-SS exchange method for the purification of potato phosphorylase and rabbit muscle phosphorylase respectively.

Kitson (1982) successfully resolved identical enzymes from two different organelles. The cytoplasmic disulfiram-modified aldehyde dehydrogenase binds covalently to the SH groups of reduced thiopropyl-Sepharose 6B under the conditions in which, mitochondrial aldehyde dehydrogenase does not. The former enzyme was eluted from column by dithiothreitol solution.

Recently, the covalent chromatography was elegantly used to investigate the functional properties of distinct domains of a viral thiol containing protein e.g. M1. This protein is particularly resistant towards trypsinization in aqueous solution, as it is highly hydrophobic. Fedorova et al (1998) succeeded in trypsinization of M1 protein of influenza virus by using following procedure. Initially, a thio-disulfide exchange reaction was used to immobilize this protein on thiopropyl Sepharose-6B support. This attached protein was relatively easily digested by trypsin so as to elute noncysteine peptides of M1. However, cysteine-containing peptides remained attached on the support. Finally, thio-disulfide bond was reduced to detach cysteine-containing peptides from the support.

1.1.5 Two-phase aqueous partitioning

Proteins preferentially partition in either of the phases if they are dissolved in an aqueous solution (water > 80 %) containing two immiscible polymers or a polymer with a high ionic strength salt. This partitioning of a protein depends upon the molecular

weight of the protein, concentration and molecular weight of the polymer, temperature, pH and ionic strength of the solution [Albertsson (1986); Johansson (1985)].

Albertsson (1958) proposed this technique in late 1950s. He discovered that two polymer phases (e.g. PEG and dextran) which are mutually incompatible when threshold concentration of the polymer was exceeded, could be used to purify proteins and low molecular weight substances. He concluded that this was due to differential partitioning of the proteins in either of the phases. The main advantages of this technique are – 1) It is a simple method for the separation of both extracellular and intracellular protein. 2) It can be used for the recovery of desired protein in presence of cell wall and cell debris. Moreover, the technique can be operated semi continuously/continuously and scale-up of the process is easy.

Later Hustedt et al (1979) separated and purified pullulanase and 1,4-α-glucan phosphorylase from *Klebsiella pneumoniae* using PEG-dextran system. The enzyme selectively partitioned in PEG phase to give 70 % yield at 80 % purity. The disadvantage of dextran is its high cost, which limits its use in large-scale isolation of enzymes. Therefore, Tjerneld et al (1986) and Szlag and Giuliano (1988) replaced dextran with biocompatible and cheap polymers such as hydroxy propylstarch and maltodextrin respectively. Also, hydroxy propylstarch is easy to synthesize.

A detailed study was conducted by Dissing and Mattiasson (1994) on the influence of pH and inorganic ions on partition behavior of BSA, lactate dehydrogenase and myoglobin in aqueous two-phase system formed by poly (ethyleneimine) and (hydroxyethyl) cellulose. It was found that the anions, which interact strongly with

polyelectrolyte, enhance the partitioning of two phases by not only affecting the phase volume ratios but also the partition coefficients of the system.

The main disadvantage of using this separation technique is the need for the removal of the polymer phase from final product. This problem was overcome by Venancio et al (1995) by using the affinity ligand attached to one of the polymer phase. They used a cheap raw material guar gum derivative, (sold under the trade name Solvitose Gum Ofa) with PEG to generate an aqueous two-phase system. As a common drawback with other carbohydrate based polymers, Solvitose gum also faced with its low degree of purification. The 25 % improvement in the purification was achieved by using affinity ligand in the same system. Moreover, the cost of this system was one sixth of the cost of Reppal PES-PEG system.

As mentioned earlier, the concentration of polymer and salt both influence the purification of a given enzyme. Recently, Kulkarni et al (1999) demonstrated once again the importance of optimizing these parameters in semi-continuous extraction of xylanase from recombinant *E. coli* (pATBX 1.8). It was found that the optimum concentration of PEG and phosphate was 6 % and 12 % respectively to attain a purification ratio above 1 in top phase. In a 12 hours semi-continuous operation the maximum recovery of xylanase obtained in terms of specific activity was two folds. Thus, it reiterates the optimization of process parameters to get the maximum recovery of the product.

1.1.6 Membrane Separation

This technique is based on the differences in the molecular sizes of the protein. Two proteins can be separated using a porous membrane of a definite molecular weight cut off, if they differ by ten orders of magnitude in their molecular masses. This method

is very attractive since it does not require a change of phase as in two-phase extraction or addition of chemicals or large energy demands. Moreover, it is useful in processing large volumes at a high rate [Ramirez-Vick and Garcia (1996-97)].

These membranes are made of nitrocellulose, glass or polycarbonates. Various derivatives of these membranes with either acidic or basic functionality are used to enhance the purification or concentration of a desired biomolecule. The major constraints on this technique are membrane fouling which irreversibly modifies the porosity and surface properties of the membrane. Usually, the filtration is carried out by applying pressure, if broth containing the desired protein has cell debris or contaminants larger than the pore size of the membrane, it causes clogging of the membrane which decreases the flow rates. Inspite of these hurdles it is used commonly in the purification of proteins on large scale. The selectivity of the process is controlled by structure and physico-chemical properties of the membrane as illustrated by following examples.

The microfiltration and ultrafiltration techniques can be used in tandem as described by Sheehan et al (1990). They used a two step process for the separation and purification of extracellular protease. Initially, a crossflow microfiltration was applied to remove bacterial cells followed by ultrafiltration, which resulted in 10-fold concentration of extracellular protease product. This process exhibited consistently high yield (> 90 %) of enzyme recovered from 100 liter fermentation broth. High protease yield in the cell separation step involved transport of the enzyme through the microfiltration membrane. This was achieved under conditions of low transmembrane pressure and high crossflow recirculation rate. To maintain these conditions, the permeate chambers of the hollow fiber cartridges used for the cell separation was kept under pressure and also a pump on

permeate outlet was used to maintain a constant permeate flow rate. This resulted in increased flux performance and stability of the membrane keeping transmembrane pressure low. This process gave 100 % recovery of the protease and it was found to be twice as cost effective as centrifugal filtration.

Recently, Pouliot et al (1999) demonstrated the use of charged ultrafiltration membrane for the separation of tryptic digested peptides obtained from whey protein. In this investigation various process parameters such as pH and salt concentration which affect the yield of protein separation were also studied. The membrane with a molecular weight cut off between 1000 and 5000 g/mol showed the best separation properties accompanied with high fluxes. Further, for the same membrane, the flux and transmission of nitrogen was increased when pH of the solution was raised from 5 to 9, at 0.5 M NaCl. This also decreases the tendency of the membrane to fouling. At higher concentration of NaCl both permeability and fouling of the membrane was increased concomitantly. It was observed that neutral and positively charged peptides were predominant in permeates.

The applied pressure in ultrafiltration causes enzyme deactivation as observed during concentration of xanthine oxidase which results into 31 % reduction in the activity but overall specific activity remain constant [Ozer et al (1999)].

The methods described so far for the separation of protein/enzyme are based on the physico-chemical properties of the biomolecule. It has been found that for many biomolecules especially for proteins, a large number of such properties e.g. molecular weight and pI, are comparable. Therefore, any of the above technique has to be complimented with others to obtain the protein products in desired purity. This major

drawback of conventional techniques has been demonstrated in many examples illustrated above. Eventhough, the separation/purification factors obtained using above techniques are significant for high volume-low value products, and thus are generally used for their recovery on industrial scales. This is good enough for food and other consumer products industry. However, in biotechnology and pharmaceutical industries the purity of the product is a crucial factor, which governs not only the cost but also final application of the desired product. Therefore, to improve the existing methods available for protein purification the researchers began to explore new methods, which could provide better selectivity and specificity during separation. The affinity interactions are just suitable in this regard. A brief discussion on bio-specific recognition in general is given in following paragraph before going into the review of affinity based techniques used for separations in biotechnology.

1.2.0 Bio-specific recognition

In natural biological systems, it is well known that there are numerous macromolecular pairs, which recognizes each other selectively and specifically to form very strong complexes. This includes enzymes and their inhibitors, substrates, cofactors or effectors; agonists and receptors; lectins and glycoproteins; antibodies and antigens etc [Sada (1990)]. This exclusive recognition amongst biomolecule and ligands or affinants occurs mainly through four types of non-covalent interactions. These are – van der waal's forces, electrostatic forces, hydrogen bonding interactions and hydrophobic interactions [Alberts et al (1983)]. These interactions are called as "affinity-interactions". In last three to four decades a lot of research is going on to exploit these interactions for developing new separation strategies.

The separation of proteins and enzymes with different degrees of purity are finding vast applications in the field of cosmetics, food, medicines, diagnostics, pharmaceutics and allied industries [Geisow (1992)]. Purification of proteins is at least five times more expensive than that of low-molecular weight substances and comprises up to 50-80 % of all the costs of production of proteins and enzymes [Narayanan (1994)].

As mentioned earlier, precipitation of proteins [using $(NH_4)_2SO_4$, polyoxy ethylene, and organic solvents] is a most sought after industrial method, as it requires simple equipment and provide high yields. But the low selectivity of the process results in lower degree of purification [Galaev and Mattiasson (1997)]. Therefore, to provide selectivity in separations affinity-interactions were adopted. So far, various affinity-based techniques have been developed for separation of biomolecules. The important techniques are affinity chromatography, immobilized metal affinity chromatography, membrane affinity filtration, affinity partitioning and affinity precipitation.

1.3.0 Affinity Chromatography

The exclusive affinity between the ligand (either synthetic or natural) and ligate (biomolecule) is exploited in this technique. The principle of this technique is that when a biomolecule comes in contact with the affinity ligand, a complex is formed between them. The strength of this complex depends upon different interactive forces as discussed previously in bio-specific recognition. The unadsorbed or weakly adsorbed biomolecules are eluted more easily from the column as compared to the adsorbed one. The affinity complex of biomolecule and ligand that is retained strongly on the column is then dissociated using either change in pH, ionic strength or addition of other more potent

affinity ligand. This gives a much more homogeneous purified product as compared to other non-affinity based separation techniques.

The affinity chromatography technique was initiated in 1910, a significant progress in the field started in the late 60's when Axen et al (1967) and Axen and Ernback (1971) developed a method of binding affinity ligands to a highly porous hydrophilic agarose matrix by activating the hydroxyl groups with cyanogen bromide. Further, extensive research by Cuatrecasas (1970) Cuatrecasas and Anfinsen (1971), Cuatrecasas and Wilchek (1968) and Cuatrecasas et al (1968) led to development of affinity chromatography to its present form that uses affinity ligands covalently bound to adsorbents as the stationary phase in a chromatography column.

The typical steps involved in affinity chromatography technique are –

1. The column is packed with affinity beads.
2. The packed column was equilibrated with a buffer solution.
3. A mixture of crude protein is loaded on the column.
4. The impurities are washed out with a buffer solution.
5. The desired protein, which is retained on the column, is eluted out by change in pH or ionic strength or by adding a specific eluant [Katoh et al (1978)].

So far various support materials have been used as the stationary phases in affinity chromatography such as agarose, sepharose, polyacrylamide etc. The affinity ligands used are either group specific (binds to a class of substances such as thiol groups, RNA molecule etc.) or substance specific (binds to a particular molecule such as inhibitor-enzyme, antigen-antibody, DNA-repressor protein etc.).

In an early investigation Lee et al (1977) developed a two-step affinity column procedure to purify 20 different enzymes and their genetic variants from mouse and drosophila tissues. The biospecific elution method comprising different coenzymes or inhibitors, or by sequential passage of the tissue extract through several cofactor-related affinity columns was employed. Initially the low affinity enzyme was separated from unadsorbed proteins. Then, saturation-readsorption procedure was used to further separate the high affinity enzymes from the low affinity ones. In this procedure the column was over loaded with tissue extract and then the eluted low affinity enzyme was readsorbed on a second affinity column, which eliminates competitive high affinity enzymes.

The affinity column was used as a support matrix in the high-performance liquid chromatography to separate lactate dehydrogenase from liver alcohol dehydrogenase in less than 10 minutes. The bioaffinity support was synthesized by coupling N_6-(6-aminohexyl)-adenosinemonophosphate (AMP) to glycosil followed by sodium borohydride ($NaBH_4$) reduction to yield an AMP-silica. It was used as a support in the high-performance liquid chromatography mode. Lactate dehydrogenase was eluted using nicotinamideadeninedinucleotide (NAD^+) + pyruvate, whereas NAD^+ + pyrazole was used for the elution of alcohol dehydrogenase. A mixture of bovine serum albumin and human serum albumin was also rapidly separated on an anti-human serum albumin-silica column using this integrated approach [Ohlson, et al (1978)].

The use of group specific ligands such as procion Yellow H-E3G and procion Red H-E7B dyes is a very common method for purification of dehydrogenases. In an expanded bed with perfluoropolymer support these dyes were immobilized and the

resulting affinity column was used to purify malate dehydrogenase (MDH) and glucose 6-phosphate dehydrogenase (G6PDH) from homogenized yeast cells. It was possible to selectively elute MDH using nicotinamideadeninedinucleotide protonated (NADH) as an eluting agent. The yield was dependent on concentration of NADH used. The overall recoveries for both enzymes were greater than 94 %. It shows that the expanded bed approach for purification of MDH is a safe method. [McCreath et al (1995)].

Penzol et al (1998) demonstrated the effect of hydrophilic spacer (dextran) on the binding of protein A to the immunoglobulin (IgG) in affinity chromatography column. A low-density layer of long, flexible, hydrophilic and inert dextran molecules was formed on the internal part of agarose fibers. Protein A was immobilized on dextran. It was found that due to incorporation of dextran as a spacer the protein A (ligand) exhibited minimal steric hindrance to the incoming IgG molecules. Further it was shown that this new affinity support adsorbed two molecules of IgG per molecule of protein A.

In the modification of this technique instead of affinity ligand a metal ion is used to bind to surface histidine residues of an enzyme. The enzyme gets separated depending upon number of histidine residues and its strength of interaction with metal ion. This technique is known as "immobilized metal affinity chromatography" (IMAC). Two closely related enzymes viz. pectin lyase and pectin esterase were separated using sepharose-iminodiacetic acid-Cu (II) as IMAC support. Pectin lyase did not bind to the chromatography matrix at pH 8.0, while pectin esterase was retained and eluted only when the pH of the buffer was brought down to 3.0. It suggests that IMAC could discriminate between two forms of pectin degrading enzymes. It also demonstrates that

pectin lyase is devoid of accessible histidine residues, while pectin esterase could have one or more of them [Navarro et al (1994)].

Berna et al (1996) have compared the bioaffinity chromatography with that of pseudo-affinity chromatography. They demonstrated one-step purification of recombinant cyclodextrin glycosyltransferase (rCGTase) by using IMAC; which is a pseudo affinity chromatography. This enzyme exhibit negative affinity on Zn (II) column and positive affinity on Cu (II) column. The purification factor and activity recovery of rCGTase obtained by pseudo affinity chromatography was compared with the biospecific affinity chromatography, using β-cyclodextrin (β-CD) as the immobilized ligand. The same purification factor was obtained in both cases but with 79 % activity recovery with the β-CD ligand against 89 % activity recovery for IMAC. No further treatment was necessary to remove the eluting agent in case of pseudobiospecific affinity chromatography because the elution conditions used did not inhibit the enzyme. As opposed to this biospecific approach which required 10 mg/mL of β-CD or 150 mM of β-D-glucose as eluting agent which inhibit the enzyme activity. This suggests that at least one histidine residue on the surface of the enzyme was accessible for binding to Cu (II).

Thus, the technique provides many advantages such as one step purification of desired enzyme brought about by high selectivity in ligand-enzyme interaction. Overall it is a mild process used for separation of labile enzymes and it provides high protein and activity recoveries from a complex mixture of proteins. However, it has following drawbacks –

a) During binding of ligate to ligand in the absence of spacer the resistance to mass transfers (diffusion limitations and steric hindrance) is the common problem associated in

this chromatography technique, which retards binding rate and thereby reduces the available capacity of the column.

b) If this method is used early in the separation step without removing viscous and particulate material from the fermentation broth, it causes plugging of column, which results in high-pressure drops and reduced flow rates.

c) There is a high tendency of bead deformation after a prolonged use of affinity matrix at high flow rates, which enhances the channeling of proteins limiting the scaling-up of column [Senstad and Mattiasson (1989a and b)].

1.3.1 Affinity Membrane filtration (Affinity Ultrafiltration)

In this technique, the biomolecule to be purified is allowed to bind to an immobilized ligand, attached either to a water-insoluble crosslinked polymer membrane or to a high molecular weight (M.W.) water-soluble polymeric carrier. Thus, in the former case the biomolecule will be retained on the membrane. Whereas, in the latter case the biomolecule will bind to an affinity ligand attached to a high M.W. water-soluble polymer, which subsequently will be retained by the membrane. When affinity ligand is attached to a membrane the use of spacer is indispensable, as it will provide more accessibility for ligand to the enzyme. On the other hand the use of water soluble polymeric ligand forms precipitates after binding with enzyme which might cause drop in efficiency during elution step [Mattiasson and Ramstorp, (1984); Ling and Mattiasson (1989)].

The steps involved in this process are as follows.

1. Mixing of a protein solution with affinity ligands immobilized onto a high M.W. carrier such as dextran or starch.

2. Separation of high M.W. carrier-protein complex from the mixture by micro or ultrafiltration.

3. Dissociation of the bound protein from affinity ligand.

4. Concentration of the dissociated protein [Ling and Mattiasson (1987)].

Luong et al (1988) made first successful attempt to continuously purify trypsin from pig heart using affinity ultrafiltration. The affinity ligand m-aminobenzamidine was conjugated to a water-soluble high-molecular weight (M.W.>100,000) poly (acrylamide) to yield a macroligand. This macroligand was allowed to form complex with trypsin, which was retained using an ultrafiltration membrane. The complex was dissociated using arginine or benzamidine. The yield of recovered trypsin was 77 % with only 3 % contamination by other proteins. The major advantage of this process was that the affinity polymer could be easily reconditioned. It also possessed longer operative life. Similarly, Sigmundsson and Filippusson (1996) developed an affinity polymer for selective separation of trypsin using ultrafiltration. The affinity macroligand was synthesized by copolymerization of acrylamide and N-acryloyl-m-aminobenzamidine.

The silica nanoparticles were used as a carrier for purification of alcohol dehydrogenase and lactate dehydrogenases. But, due to the small dimension of the carrier, the binding capacity was improved considerably. It was found to be *at par* with high-performance liquid chromatography. The only limiting factor of the process was reduced filtration flow rate [Ling and Mattiasson (1989)].

In an earlier attempt to use anthraquinone dyes, cibacron blue F3GA was used as an affinity ligand in ultrafiltration. The dye was attached to water-soluble poly (vinyl alcohol) [PVA] to facilitate a homogeneous binding to the protein. The polymer-dye

provides sufficiently large size to prevent the target protein from entering the UF membrane. These early results demonstrate that PVA is a better carrier choice for UF of various proteins [Fisher et al (1989)]. Further, Male et al (1990) purified urokinase to 86 % recovery using p-aminobenzamidine as an affinity ligand in an ultrafiltration mode.

Recently, Onal and Telefoncu (1996) separated α-Galactosidase (EC 3.2.1.22) from watermelon with approximately 157-fold purification. They used dextran based water-soluble resin comprising α-D-galactosamine as an affinity ligand. Thus, from all the above examples it is clear that the affinity ultrafiltration provides certain advantages over affinity chromatography. These are as follows. The water soluble high molecular weight polymer is used to conjugate ligand and the complex formation of enzyme with ligand occurs in homogeneous solution. Thus the process overcomes the diffusion limitations prevalent in affinity columns. Moreover, the slow binding of ligand to enzyme and bead deformation observed in packed columns is also eliminated in this technique. Still, this technique has its own drawbacks to name a few –

a) The use of high M.W. water-soluble carriers to which affinity ligands are attached increases the cost of the overall process.

b) Particulate material in the initial protein mixture and rarely formed precipitate after ligand-enzyme binding may cause plugging of the membrane, which will reduce the capacity of the membrane to separate the desired protein.

c) This filtration process has to be carried out in batch mode.

d) Usually a second filtration step is necessary to concentrate the dissociated protein from the affinity ligand [Mattiasson et al (1987)].

1.3.2 Affinity Partitioning

As discussed already, in the conventional two phase aqueous extraction technique, a suitable affinity ligand is attached to a phase forming polymer in order to provide the selectivity during partitioning of desired protein. Thus during partitioning the two polymers get concentrated in opposite phases and the affinity interactions between enzyme and ligand helps in dragging the desired enzyme predominantly into one phase. This resulted in approximately ten thousand times enhancement in the partitioning of an enzyme. The purification of a desired protein in this magnitude could only be achieved by multiple extraction of the desired protein from the phase containing affinity ligand. The multiple extraction is done using three to four washing phases containing a second affinity ligand either to counter extract the target protein or to remove contaminants effectively, depending upon the relative affinity of desired protein for the second ligand. This extraction process is thoroughly reviewed in the separation technology and is most widely used in the field of down stream processing.

Normally, two phases comprise two immiscible polymers mixed together, the PEG / salt system is generally not suitable for affinity partitioning since high ionic strength of the system severely affects the ligand-protein interactions. A number of factors have a pronounced effect on protein partitioning behavior in an affinity two-phase partitioning system. Ligand, polymer and salt concentrations, M.W. of polymers, pH and number of ligands per polymer molecule are the most important ones.

The affinity based two-phase aqueous extraction involves following steps –

1. The affinity ligand is conjugated to a phase forming polymer.

2. Two-phase aqueous system is generated either dissolving the above polymer with another immiscible polymer or with a salt in the mixture of proteins.

3. The two phases are separated and the target protein complexed with the affinity ligand is dissociated using same eluents as used in conventional affinity chroamatography.

4. The dissociated protein is concentrated and further purified [Chen (1990)].

In early reports, Flanagan et al (1976) used for the first time an affinity partitioning method to purify acetylcholine receptor-rich membrane fragments from the electric organ of *Torpedo californica*. Poly (ethylene oxide)-phase forming polymer was modified by an affinity ligand viz. α, ω-[p-(trimethylammonium)phenylamine]. A two phase partitioning system was generated using dextran T 500, unsubstituted poly (ethylene oxide) and α,ω-[p-(trimethylammonium)phenylamino] poly (ethylene oxide) in sodium phosphate buffer (pH 7.4) containing NaCl. A high degree of purity of the product was obtained in the affinity phase.

Later, various triazine dyes were used as a group specific affinity ligand for selective partitioning of various kinases, dehydrogenases, and aminotransferases. Kopperschlaeger et al (1983) made first effort in this direction by conjugating various triazine dyes to polyethylene glycol (PEG) and mixing it with dextran so as to create a new two-phase extraction system. In continuation with their earlier work with triazine dyes Kopperschlaeger and Birkenmeier (1986) studied the effect of Remazol Yellow GGL dye on the separation of phospho fructokinase and glucose 6-phosphosphate dehydrogenase from baker's yeast. For the first time they showed that the partition coefficient of these proteins was dependent upon the concentration of reactive dye used in the PEG and dextran two-phase system.

In the parallel work, Johansson and Andersson (1984) partitioned several glycolytic enzymes from bakers' yeast in two-phase system comprising dextran and PEG. It was commonly observed that the glycolytic enzymes exhibited high affinity for the lower dextran-rich phase. This affinity was reversed by using procion type triazine dyes bound to polyethylene glycol. This resulted in enrichment of enzymes in the upper PEG phase. It was observed that by this method 10-500 times enrichment in top phase could be achieved as compared to the bulk of the proteins, which remain in the dextran phase. Usually, it was observed that enzymes precipitated at high concentrations of polyethylene glycol (12.5 % wt./wt.). But, Johansson and Joelsson (1986) observed that when polyethylene glycol-bound triazine dyes (Procion yellow HE-3G and Procion olive MX-3G) were used in the extraction of glucose 6-phosphate dehydrogenase and 3-phosphoglycerate kinase from baker's yeast, no precipitation of enzymes occurred even at high concentrations of PEG. This specific prevention of precipitation of desired enzymes was useful in their purification from crude extracts. A 3.4-fold purification of glucose 6-phosphate dehydrogenase was achieved with good recovery (93 %). Further purification was possible by combining the recovered (enzyme-containing) supernatant liquid with a dextran solution, which generates a normal two-phase system. The lower phase, containing dextran extracts the unwanted bulk proteins leaving the target enzyme in the upper phase.

In the extension of this work, the same group studied the effect of ligand and polymer concentration, type and concentration of salt and concentration of dye-dextran on the partitioning of procion yellow HE-3G bound to dextran (M.W. 70,000) in an aqueous two phase system comprising dextran and poly (ethylene glycol). At moderate

dye:dextran molar ratios (5-8), the partitioning was found to be strongly salt dependent. Dye-dextran conjugate can be directed to either the upper or the lower phase with partition coefficients from 0.02 to 28 by changing the concentration of salts. The dye linked to dextran in the two-phase system affects the partitioning of dye-binding enzymes e.g. lactate dehydrogenase, glucose-6-phosphate dehydrogenase and 3-phosphoglycerate kinase towards the dye-containing phase. Also, it was observed that the affinity of the dye for the enzyme was increased by increasing ligand:dextran ratio [Johansson and Joelsson (1987)] .

The high value products such as restriction endonucleases viz. EcoRI, EcoRV, and BamH I were extracted using dextran-polyethylene glycol (PEG) system. The PEG was derivatized by conjugating either triazine dyes or herring DNA. This affinity phase was used to partially purify EcoRI (52-fold) and EcoRV (37-fold) using a combination of affinity partitioning and ion-exchange chromatography [Vlatakis and Bouriotis (1991)].

In the modification of the conventional affinity partitioning, immobilized metal ion affinity partitioning (IMAP) was integrated with it. This integrated system was used to extract and purify a costly enzyme D-2-hydroxyisocaproate dehydrogenase from *Lactobacillus casei*. Both polyethylene glycol/dextran as well as polyethylene glycol/salt two phase systems were used successfully. It was found that the partitioning of the enzyme was influenced strongly by the inclusion of iminodiacetic acid as a chelating ligand coupled to polyethylene glycol, which was loaded with Cu^{+2} in the phase system. With this technique, enhancement in the enzyme recovery of up to 1000-fold was observed. It was further concluded that approximately 6.4 histidine residues were involved in the enzyme-metal chelate complex formation. The only draw back of this

system was that it could not be used directly for the extraction of enzyme from cell homogenate or cell debris. To overcome this, a combination of pre-purification step by fractional precipitation with polyethylene glycol which, is followed by immobilized metal affinity partitioning resulted in a purification factor of 11 with a yield of 90 % [Schustolla et al (1992)].

Similarly, Franco et al (1997) used this integrated approach by combining affinity partitioning comprising thermoprecipitating polymers with immobilized metal affinity partitioning. This system was developed for purification of recombinant lactate dehydrogenase carrying an affinity tag of 6 histidine residues (His_6-LDH) from a crude *E. coli* extract. It will be discussed in detail in affinity thermoprecipitation section.

Later, Han and Lee (1997) demonstrated the effect of surface characteristics and net charge on protein during their separation by affinity two phase extraction. In PEG/dextran aqueous two-phase system, the partition coefficient of bovine serum albumin (BSA) with negative surface charged in the neutral pH region, decreased by the addition of phosphate salt. However, the partition coefficient of lysozyme, which is positively charged at neutral pH, increased as phosphate salt was added. The neutral protease produced from *Bacillus subtilis* exhibited enhancement in partition coefficient in PEG/salt aqueous two-phase system with increasing phosphate salt concentration. When PEG-palmitate was substituted for PEG, *Bacillus subtilis* neutral protease moved to the upper PEG-palmitate phase and was purified to 10-fold compared to the crude enzyme. Palmitic acid in PEG-palmitate showed not only high affinity to *Bacillus subtilis* neutral protease but also enhanced the proteolytic activity (by a factor of 2.5). In the PEG - palmitate phase, the neutral protease exhibits stronger hydrophobic interactions with

palmitate leading to increased proteolytic activity. The results of this study demonstrate that aqueous two-phase systems are suitable for purifying proteins and enzymes by bulk liquid partitioning, and can also give useful information on the surface properties of proteins.

The effect of molecular weight of PEG on the separation of the desired enzyme was shown by Silva et al (1997). P-aminophenyl 1-thio-β-D-galactopyranoside (APGP) was attached to activated PEG. The partitioning of β-galactosidase enzyme from *Kluyveromyces lactis* exhibit highest purification factors (83 % recovery) when 6 % PEG of M.W. of 4000 was used with 12 % dextran T 505,000 to generate a two phase aqueous system.

Recently, Sun et al (1999) demonstrated the use of reversed phase micelles in two phase extraction of lysozyme from its synthetic mixture with BSA as well as from egg white. The effect of surfactant on the partitioning of lysozyme was also shown. In this investigation, Cibacron Blue F3G-A (CB) as an affinity ligand was immobilized to reversed micelles composed of soybean lecithin. The enhanced solubilization of lysozyme by incorporation of nonionic surfactant Tween 85, resulted in the formation of mixed micelles which increased the W0 (water/surfactant molar ratio) and micellar size. By addition of Tween 85 the dissociation constant was decreased, which consequently increased the lysozyme binding to the immobilized CB. It was observed that by addition of Tween 85 (20 g/lit.) to 50 g/lit. lecithin/hexane micellar phase containing 0.1 mmol/lit. of CB, the extraction capacity for lysozyme was increased by 42 %. Moreover, the CB-lecithin micelles with or without Tween 85 showed significant size exclusion for BSA due to its high molecular weight. Thus, lysozyme and BSA were separated from artificial

mixture. The affinity-based reversed micellar phase containing Tween 85 was recycled three times for lysozyme purification from crude egg-white which increased the lysozyme purity by 16 to 18 fold, reaching 60-70 % at the end of third cycle.

The affinity partitioning process occurs in homogeneous solution thereby it alleviate the problems of steric hindrance, slow binding of protein to ligand, plugging of columns/membranes as well as it can be used at very early stages of protein recovery in down stream processing i.e. directly from fermentation broth containing cells. Moreover, the higher loading of the affinity ligand on the polymer did not exhibit irreversible binding with enzyme due to multiple site attachment of the ligand unlike observed in affinity beads. The extraction can be carried out at room temperature and the stabilizing effect of PEG keeps the enzyme in active form. Also, this process is easy to scale-up. Albeit, similar to all other techniques, it has few drawbacks.

a) The phase forming polymer component, contaminate with the final product.

b) Cost of phase forming component is high.

c) Usually a second purification step is required to separate desired protein from affinity ligand attached to phase forming polymer.

d) Time required to separate two phases should be critically optimized as it may take few minutes to several hours [Chen (1990)].

In summary affinity chromatography provides high selectivity and low productivity whereas, conventional precipitation technique results in high productivity but low selectivity. Therefore both these techniques were combined together in a manner as to exploit the advantages of each of these techniques i.e. high selectivity and high productivity [Mattiasson and Kaul (1993)]. This new hybrid technique is called as

"Affinity Precipitation". In following paragraphs a detailed discussion on affinity precipitation is given.

1.4.0 What is Affinity Precipitation?

In affinity precipitation, an affinity ligand (either mono or multidentate) is coupled to a water-soluble smart polymer or to a spacer to form a macroligand. This macroligand when mixed with protein solution forms a complex with the target protein. Phase separation occurs with or without changing the environment, which makes the polymer backbone insoluble. At this point one of the two methods is followed. In the first the target protein is eluted from the insoluble macroligand-protein complex which is precipitated. In the second, the precipitate is dissolved, the protein is dissociated from the macroligand and the dissociated macroligand is precipitated again which can be reused for next cycle of protein purification. The protein remaining in supernatant is in purified form [Mattiasson et al (1998); Galaev et al (1996)]. This process is schematically illustrated in Figure 1.1.

The affinity precipitation technique is classified depending on the route followed to induce precipitation of the complex formed between affinity macroligand and the biomolecule. The nature and type of polymer chosen for the synthesis of an affinity macroligand indirectly affects the route by which this precipitation could be achieved. Depending on this the classification is done as follows.

A] Homo-bifunctional or primary effect or Bis-ligand affinity precipitation and

B] Hetero-bifunctional or secondary effect affinity precipitation.

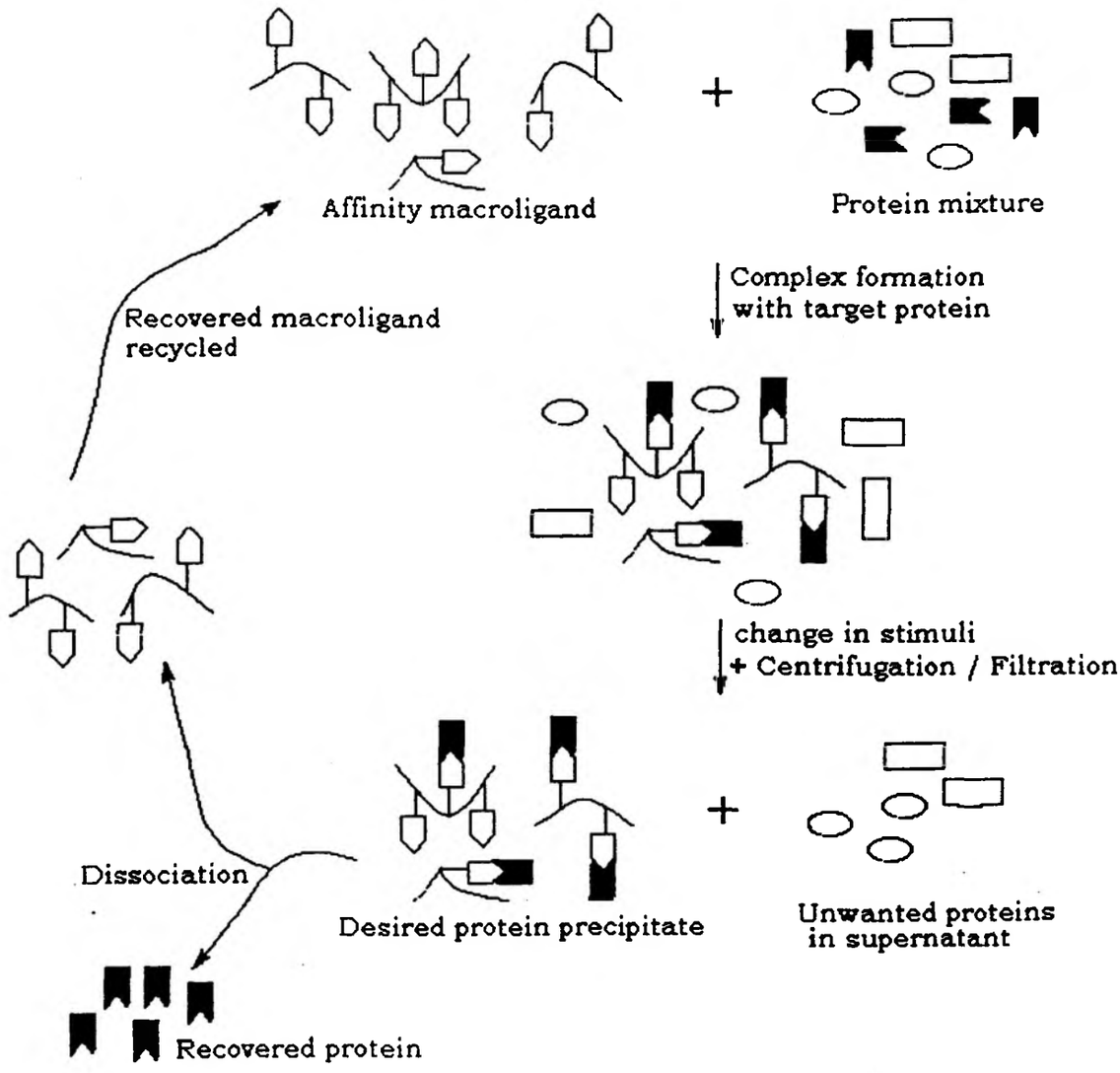


Figure 1.1: A schematic representation of affinity precipitation.

1.5.0 Homo-bifunctional or primary effect or Bis-ligand affinity precipitation

It involves the synthesis of a bivalent ligand (bis-ligand) in which two identical ligands are linked via a spacer arm as shown in Figure 1.2. The bis-ligand thus synthesized can bind to two proteins at two different binding sites provided the inter-linking spacer is long enough to cover the binding sites of two different oligomeric proteins. The oligomeric protein binds to more than one bis-ligand and gives rise to lattice formation. After growing to a particular size, the giant three-dimensional protein aggregate precipitates from the solution. Dissociation of the complex and then centrifugation or filtration helps recover the desired protein. The advantage of this technique is that it is a single step purification process and does not require chromatographic columns or high-speed centrifugation [Irwin and Tipton (1995; 1996)]. In Table 1.1 the use of different bis-ligands in affinity precipitation is given.

Irwin and Tipton formulated the conditions under which the bis-ligand approach works successfully. These are –

1. The protein/enzyme to be purified should be oligomeric i.e. contain more than one coenzyme binding site.

2. The bis-ligand should have a strong affinity for the enzyme ($K_a = 10^5$ to 10^9 M).

3. The spacer connecting the two ligands has to be long enough to bridge the distance between two ligand-binding sites on two different enzyme molecules.

4. The ratio of bis-ligand to enzyme subunit should be optimum. If this is low, the concentration of coenzyme ligand is less than the available subunit sites on the enzyme, which will not allow forming the lattice structure, as there are not enough crosslinks

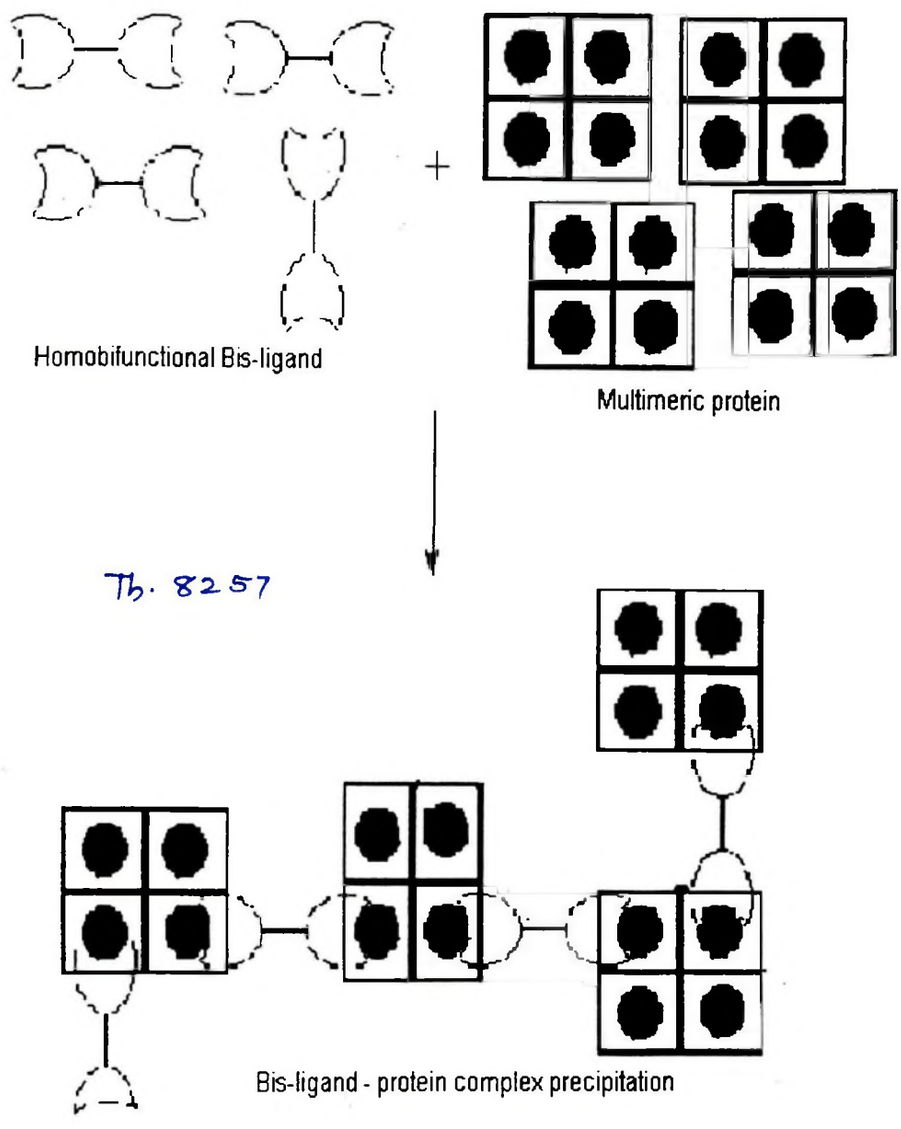


Figure 1.2: Schematic representation of homo-bifunctional bis-ligand.

Table 1.1 Use of various bis-ligands in affinity precipitation of proteins.

No.	Protein recovered	Bis-ligand*	Reference
1	Phosphofructokinase	Bis-ATP	Beattie et al (1987)
2	Rabbitactate dehydrogenase	Bis-Cibacron blue F3G-A	Hayet and Vijayalakashmi (1986); Lowe and Pearson (1983)
3	Bovine serum albumin	---"---"---	---"---"---
4	Rabbit lactate dehydrogenase	Methoxylated p-sulfonated isomer of Procion blue H-B	Pearson et al (1986); Pearson et al (1989)
5	Recombinant Glutamate dehydrogenase	EGTA $(Zn)_2$	Lilius et al (1991)
6	Human hemoglobin, sperm whale myoglobin	$Cu(II)_2$EGTA,$Cu(II)_2$ polyethylene glycol-(iminodiacetic acid)	Van Dam et al (1989)
7	Plasminogen	Procion Red HE-3B	Bertrand et al (1985).
8	Beef heart lactate dehydrogenase	Cibacron blue conjugated to polyethylene imine - polyacrylic acid	Dissing and Mattiasson (1996)

* The use of bis-NAD^+ as a homobifunctional affinity ligand in precipitation of various proteins is given in Table 1.2

available during complex formation. If this ratio is too high, each enzyme binding site may be occupied by one end of a bis-ligand molecule but it will not give intermolecular crosslinking which is essential for precipitin reaction.

To illustrate the effect of bis-ligand concentration on affinity precipitation of tetrameric protein it is schematically shown in Figure 1.3. For tetrameric protein such as lactate dehydrogenase (LDH) the optimum ratio of bis-ligand/enzyme was found to be 1.25. This ratio is identical to immunoprecipitation reaction in which two antigens (Ag) can bind to one antibody (Ab) [Feinstein and Rowe (1965)].

Following are the different homo-bifunctional affinity ligands used so far.

1.5.1 Bis-derivatives

The most thoroughly studied representative of this class is bis-NAD^+ derivative. The credit goes to Larsson and Mosbach (1979) for carrying out the pioneering work in the field of affinity precipitation and in particular for the first time synthesizing the homo-bifunctional affinity ligand i.e. bis- NAD^+. The N_2, N'_2- adipodihydrazido-bis-(N^6-carbonylmethyl)- NAD^+, known as bis-NAD^+, was synthesized by conjugating two ligands via a spacer of adipic acid dihydrazide as shown in Fig.1.4.

Flygare et al (1983) demonstrated the use of bis-NAD^+ and lactate dehydrogenase in precipitation–diffusion experiment (OUCHTERLONY double diffusion) which was performed in agarose gel containing pyruvate. The authors stated that this technique would have a potential clinical application in the detection of abnormal levels of enzymes and other serum proteins without raising antibodies against them. Also, it was shown that bis-NAD^+ could be used for affinity precipitation of ox heart LDH with greater than 90 %

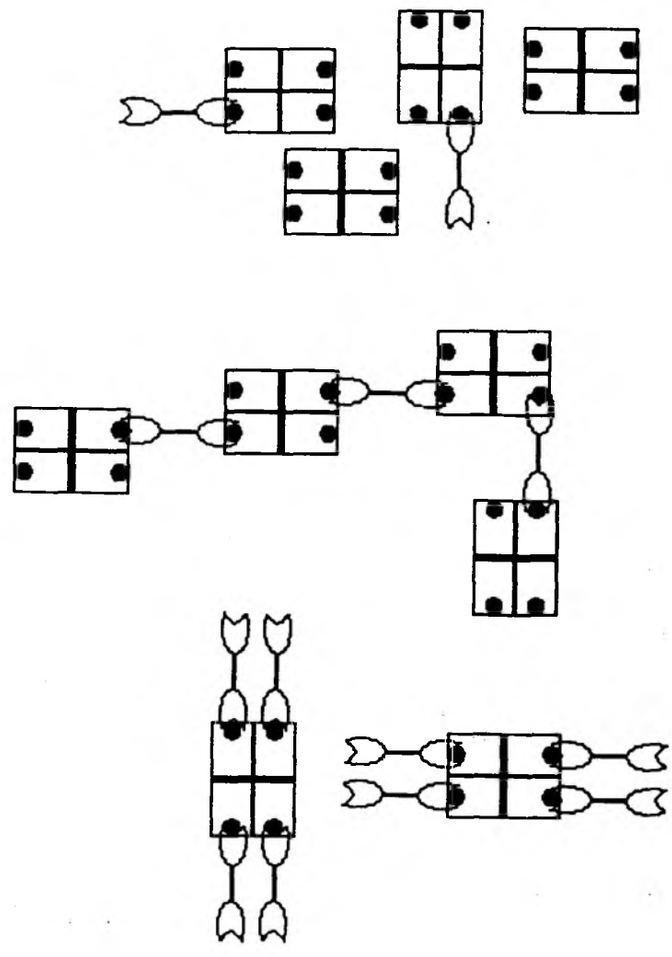
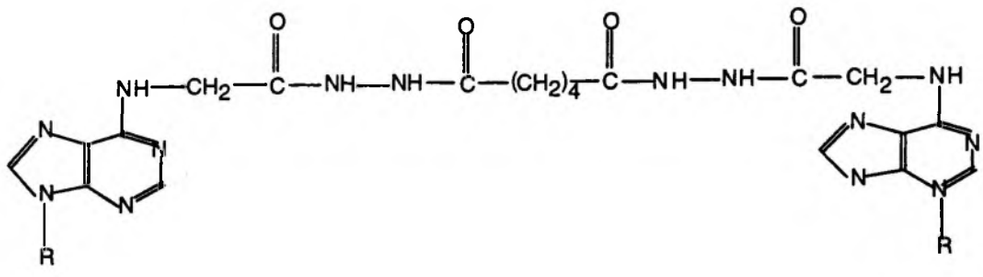


Figure 1.3 Effect of bis-ligand concentration on affinity precipitation of tetrameric protein

a) If the ratio of bis-ligand to tetrameric protein is low, affinity precipitation will not occur.

b) At an optimum ratio, maximum lattice formation and precipitation will occur.

c) When the bis-ligand is in excess than tetrameric protein, a decrease in precipitation will occur because each binding site on protein is occupied by different bis-ligand molecule.



Where, R= Nicotinamide mononucleotide Phosphoribose

Figure 1.4: Structure of Bis-NAD+ with spacer length of 1.7 nm.

yield, when it was mixed with a closely related enzyme i.e. yeast alcohol dehydrogenase, which did not precipitate.

Bis-NAD^+ shown in Figure 1.4 has a spacer length of 1.7 nm. This optimum spacer length allows easy access of bis-ligand to the active site of two LDH molecules simultaneously. Other forms of bis-NAD^+ with shorter (0.7 nm) and longer (3.2 nm) spacer arms were synthesized and these analogues were used to set up a novel immobilized two-enzyme system. It consists of coimmobilization of LDH and horse liver alcohol dehydrogenase (ADH) by glutaraldehyde coupling. Prior to immobilization both enzymes were saturated with NAD^+, as it is well known that one NAD^+ moiety occupied an active site of each enzyme. This ensures that active sites were positioned against each other, even after removal of the co-enzyme analogue. The advantage of this co-immobilization is the facilitated diffusion of the product of first enzyme i.e. NADH to the active site of nearby other enzyme [Mansson et al (1983)].

As, shown in Table 1.2 the bis -NAD^+ ligand have been used to separate a variety of dehydrogenase enzymes. It was observed that many enzymes, particularly dehydrogenases and kinases require adenine nucleotide coenzyme for their reaction. Hence, NAD^+ and ATP were used for affinity binding with the enzymes belonging to the above mentioned classes. Moreover, these ligands do not show selectivity for a particular enzyme in either dehydrogenase class or kinase class; therefore these ligands are called 'general ligands'.

Buchanan et al (1989) made use of bis-NAD^+ in abortive complex formation to resolve LDH iso-enzymes from mammalian cells. The mammalian LDH iso- enzymes

Table 1.2 Use of Bis- NAD^+ in homo-bifunctional affinity precipitation of various dehydrogenases.

No.	Protein recovered	Reference
1	Lactate dehydrogenase	Larsson and Mosbach (1979); Flygare et al (1983); Larsson et al (1984); Beattie et al (1985); Buchanan et al (1989)
2	Glutamate dehydrogenase	Flygare et al (1983); Larsson et al (1984); Beattie et al (1985); Graham et al (1985)
3	Yeast alcohol dehydrogenase	Flygare et al (1983); Beattie et al (1985)
4	Isocitrate dehydrogenase	Beattie et al (1985)

found in somatic cells have been designated as H (heart) and M (muscle) as they are found predominantly in these organs. The homo-tetrameric enzyme H_4 and M_4 have different kinetic, immuno-chemical, physical, and electrophoretic properties. The H_4 forms an abortive complex with NAD^+ and pyruvate, whereas, M_4 iso-enzyme did not. Bis- NAD^+ gave rise to affinity precipitation of H_4 in the presence of oxamate, which is a competitive inhibitor with respect to pyruvate but M_4 iso-enzyme did not precipitate under these conditions.

1.5.2 Locking-on Effect

As, discussed already, since NAD^+,$NADP^+$ and ATP are general ligands these ligands have a drawback that after complex formation and precipitation of the complex, the desired protein was eluted by either using competitive ligand or salt or coenzyme, which leads to elution of more than one enzyme simultaneously. This seriously impaired the selectivity of the process. This problem was circumvented by the efforts of O'Carra (1978) who used the 'locking-on effect' concept, which involved increasing the strength of enzyme binding to the general affinity ligand by adding analogues of substrates, specific for the enzyme of interest e.g. LDH binding strength to bis-NAD^+ increased by adding oxalate, a structural analogue of lactate to the irrigating buffer. The omission of oxalate from eluting buffer weakened the binding, allowing LDH to readily elute from the column.

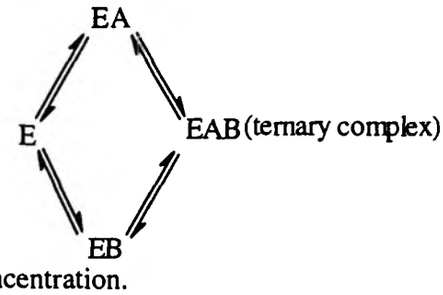
The locking-on effect would apply if enzyme (E) has an ordered binding mechanism, in which the leading ligand (coenzyme) (A) binds to the enzyme before the second ligand or substrate (B) binds. In this case an unproductive, competitive analogue of the second substrate will displace the coenzyme binding equilibrium to ternary

complex formation, it effectively increased the strength of binding of enzyme to the coenzyme.

$$E \rightleftharpoons EA \rightleftharpoons EAB \text{ (ternary complex)}$$

When (B) is at saturating concentration.

In the case of random sequential binding of an enzyme (E), the locking-on effect could be observed only under the condition when the equilibrium of the reaction under the conditions used is such that the binding of the second substrate (B) favors the binding of the coenzyme (A).



when (B) is at saturating concentration.

In the context of recovery of an enzyme by affinity precipitation the locking-on effect has two fold implications.

a) It allows selective removal of a particular dehydrogenase from a crude extract e.g. the addition of bis-NAD^+ and glutarate selectively precipitate glutamate dehydrogenase (GDH) from bovine liver extract eventhough, other NAD^+- dependent dehydrogenases are present [Graham et al (1985)].

b) As the dissociation constant of binary complex of bis-NAD^+ and LDH is weak, of the order of $3 * 10^{-4}$ M. The addition in low concentration of bis-NAD^+ alone to a crude extract will not allow affinity precipitation of LDH. This could be enhanced by the addition of pyruvate or oxalate e.g. The addition of pyrazole enhances the NAD^+ binding to horse liver alcohol dehydrogenase by 1600 times [Theorell and Yonetani (1963)].

It was found that if the second substrate (B) or its analogue is not present at saturating level than NADH displaces bis-NAD^+-dehydrogenase complex readily as NADH binds an order of magnitude higher to many dehydrogenases than NAD^+ [Dalziel (1975)]

Another homo-bifunctional affinity ligand in the class of bis-ligand is bis-ATP i.e. N_2, N_2'-(adipodihydrazido)-bis-(N^6-carbonylmethyl)-ATP. This ligand is applied to precipitate phosphofructokinase from bovine heart. The mechanism of precipitation in this case is not simple locking-on effect as discussed above because ATP acts both as a substrate and an allosteric inhibitor of this enzyme. The allosteric inhibition of ATP is potentiated by citrate. Hence in the presence of citrate bis-ATP was used to precipitate enzyme [Beattie et al (1987)]. This bis-ligand is not used frequently, as it is highly unstable due to phosphate-catalyzed cleavage of the dihydrazido-groups in the spacer arm.

1.5.3 Different dyes used in homobifunctional affinity precipitation

A wide range of dyes have been used for large scale purification of protein since

a) Many synthetic procedures are available for conjugating dyes to adsorbent matrices and these dyes interact efficiently with many proteins

b) They are chemically stable, cheap and available in bulk.

The most commonly used are the triazine dyes. One of the earliest attempts to use triazine-dye for affinity precipitation started with precipitation of plasminogen from plasma using Procion Red HE-3B [Bertrand et al (1985)]. Subsequently, Pearson (1987) used triazine dye to precipitate rabbit muscle LDH and bovine liver GDH respectively.

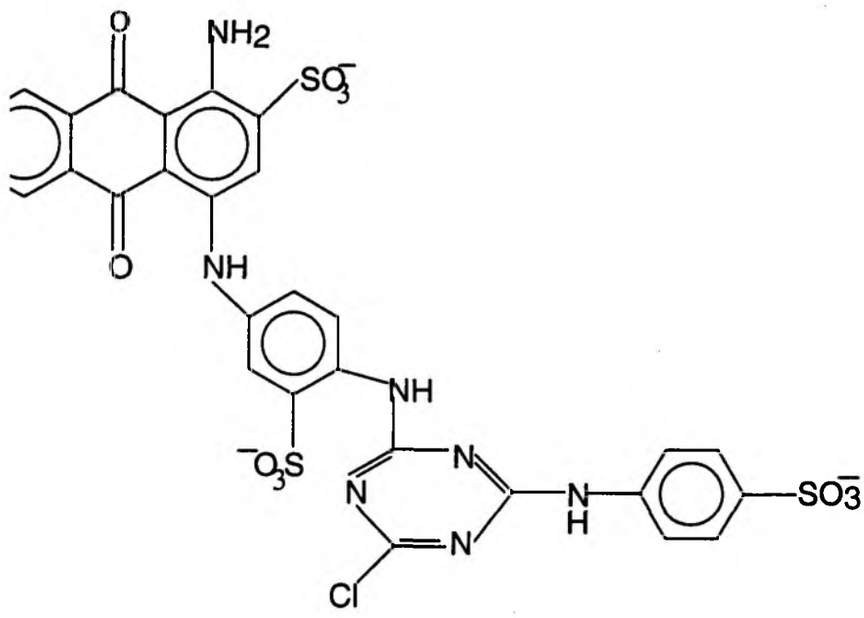
However, Bertrand et al (1985) showed that the interactions between these dye molecules and protein are non-specific and attributed to electrostatic interactions between negatively charged functional groups of dye molecules with the positively charged protein surfaces forming insoluble aggregates.

Therefore, attempts were made to use cibacron blue F3G-A, which exhibit affinity for a number of NAD^+-dependent dehydrogenases. The structure of cibacron blue F3G-A is shown in Figure 1.5.

Dissing and Mattiasson (1996) carried out the initial work using cibacron blue as an affinity dye. They conjugated this dye with polyethylene imine and polyelectrolyte complexes (PECs) were formed with polyacrylic acid sodium salt. It was concluded that precipitation behavior of PECs were governed by stoichiometry between oppositely charged polymers, net charge density on resultant PECs and salt concentration. These PECs were used to precipitate beef heart LDH with a yield of 85 %.

Subsequently, a bis-derivative of cibacron blue was synthesized by conjugating two dye molecules through their sulphonate groups using carbodiimide coupling to yield the sulphonamide derivative of bis-ligand. This bis-dye ligand was used to precipitate LDH with 90 % yield [Hayet and Vijayalakshmi (1986)].

Morris et al (1993) have pointed out that above 5 μM concentration of this dye it shows self-association or "stack" which inhibits precipitation. The reason for this stack formation was provided from their earlier work on affinity precipitation of avidin using poly (acrylamide) – biotin as an affinity polymer system. The authors observed that the precipitate formed was negligible for biotin substitution frequency of 30 per 100,000 MW polymer. This low yield was explained as follows. At a high local concentration of



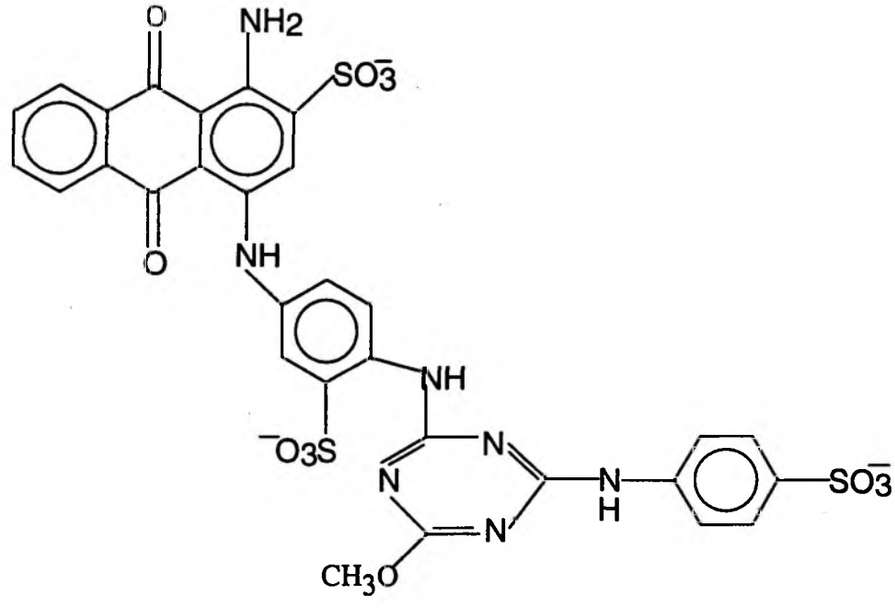


Figure 1.5 a) Cibacron Blue F3G-A i.e. Procion Blue H-B.

b) Methoxylated p-sulphonated isomer of Procion Blue H-B.

ligand and due to the polymer flexibility, a "wrapping" as opposed to "bridging" interaction was favored. Wrapping is defined as the binding of a second ligand on a polyligand molecule to the same protein molecule already bound to that polyligand, whereas bridging is defined as the binding of ligands on the same polyligand molecule to different protein molecules. Moreover it was observed that with further increase in the biotin substitution frequency on the polymer the precipitation of avidin was found to increase due to increased bridging interactions.

Another bis-derivative comprising this dye was synthesized by reacting it with 6-aminohexyl cibacron blue F3G-A to give a bifunctional dye derivative. It selectively precipitates rabbit muscle LDH in presence of transition metal ions such as Co^{+2} and Ni^{+2}.

Pearson (1986) formulated certain criteria's to distinguish true affinity precipitation as against non-specific electrostatic precipitation. These are –

a) Precipitation should depend upon dye / enzyme ratio.

b) Precipitation should be reversed upon addition of competitor with bifunctional ligand for the dye binding sites.

c) Only bifunctional dye derivative must promote precipitation and not mono-functional dye.

d) Alteration of pH, temperature or ionic strength of the medium affect precipitation in as much as it affects dye-enzyme binding site interaction.

e) Complete recovery of enzyme activity could be achieved upon resolubilization of the precipitate.

The mono-functional dye viz. p-methoxylated o-sulphonate isomer of procion Blue H-B (as shown in Fig. 1.5) selectively precipitate rabbit muscle LDH with 97 % recovery whereas, a closely related enzyme porcine heart LDH did not precipitate even in low ionic strength buffer. Moreover, the precipitation of rabbit muscle LDH was independent of pH, in the range 6.5-9.0. At neutral or slightly alkaline pH the LDH is neutral or negatively charged therefore the non-specific interactions between anionic dye and enzyme was minimum. The precipitate formation was rapid and was completed in 8-10 minutes. Also, the precipitated protein resolubilized by addition of 95 μM NADH in just 2 minutes. The precipitation of rabbit muscle LDH was found to be dependent on enzyme subunit:dye ratio, the optimum being 2:1. An explanation for this was given as follows. One enzyme binding site was occupied by anthroquinone ring to form a complex, while the terminal methoxylated triazine and p-aminobenzene sulphonate ring (please refer to the Figure 1.5) formed a cross-link with a similar binding site of a nearby enzyme molecule [Pearson et al (1986)].

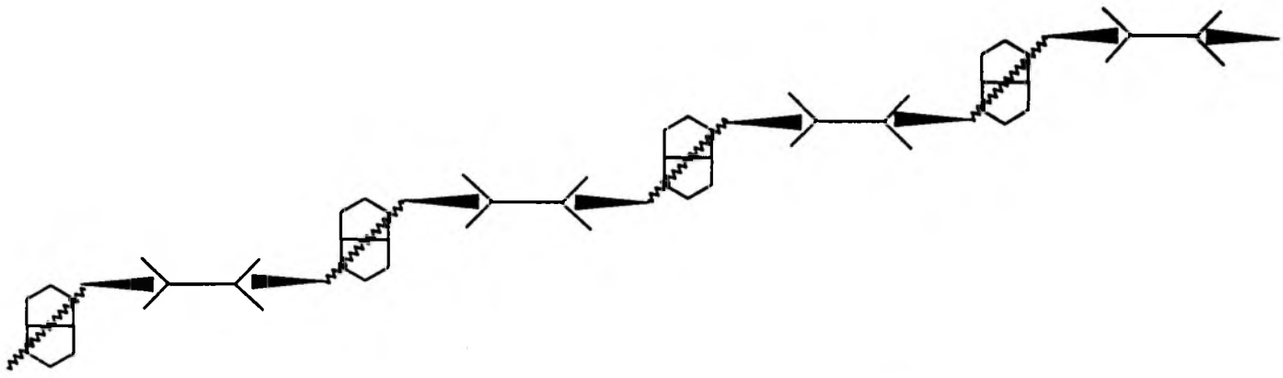
1.5.4 Use of Metal-ion in homo-bifunctional affinity Precipitation

This methodology exploits the affinity between metal ions which belong to transition groups and metal-coordinating residues on protein surfaces e.g. histidine. In one of the early reports, Van dam et al (1989) synthesized two bis-chelates viz. a short chain chelate consisting of two Cu^{+2} ions chelated by a molecule of ethylene glycol bis-(β-aminoethyl ether) - N',N''-tetra acetic acid (EGTA) and a long chain chelate, PEG-Cu (II), which comprised Cu^{+2} ions chelated by iminodiacetic acid, immobilized on each end of a molecule of PEG. Human haemoglobin (Hb) with 26 surface accessible histidine residues and sperm-whale myoglobin (Mb) with 6 surface accessible histidines were

separated using above mentioned chelates. Hb was 100 % precipitated at Cu^{+2} : surface–accessible histidine ratio of unity at pH 8.0 (highly pH dependent) using any of bis-chelates, whereas, Mb gave only 10 % precipitation under identical conditions.

A multimeric protein e.g. Con. A having more than one binding site for the metal ion was purified to 4.5 fold by simply adding Cu^{+2} solution at pH 6.0 in partially purified extract of jack-bean. At an optimum concentration of Cu^{+2} the complex grows in size to an extent where it precipitates out of the solution. This is the simplest application of the metal ion precipitation [Agarwal and Gupta (1994)].

An interesting modification of metal-ion affinity precipitation was exploited for the purification of recombinant enzyme. Lilius et al (1991) used recombinant galactose dehydrogenase as a model protein. A DNA fragment encoding five histidine residues was fused to the 3' terminal end of galactose dehydrogenase gene from *Pseudomonas fluorescens*, which was expressed in *Escheria coli.* As, this recombinant enzyme is a homo-dimer each chain carries a terminal poly-histidine tail. A bis-metal chelate EGTA $(Zn)_2$ was able to bind such two enzyme sub-units giving rise to formation of long linear polymer as shown in Figure1.6. At 10 mM concentration of metal chelate complex, this method yielded 90 % precipitation of the recombinant enzyme. The enzyme complex was dissolved by the addition of EDTA as a chelating agent to recover Zinc. The recombinant enzyme released in the filtrate was converted to its native form by the digestion of penta-histidine tail using carboxypeptidase A. This method is inexpensive and ligands used are stable. But, it would be a highly non-specific method if it has to be used for one step purification of non-recombinant proteins directly from broth. Later, Carlsson et al (1996)



b)

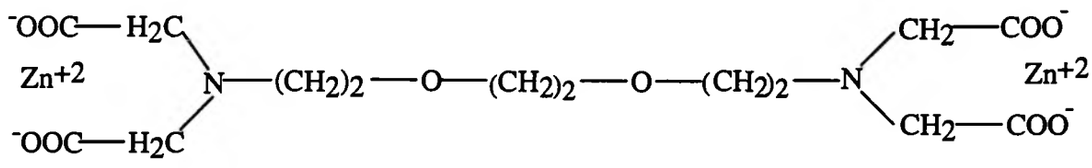
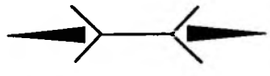
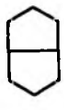
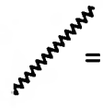


Figure 1.6 a) A linear lattice formation between recombinant GDH molecules, carrying one pentahistidine tail per subunit with bis-chelate i.e. EGTA(Zn^{+2}).

 = Bis-chelate EGTA $(Zn)_2$ complex;  = Zn^{+2} ion

 = one molecule of Galactose dehydrogenase (GDH);  = Pentahistidine tail.

b) Bis-chelate i.e. EGTA$(Zn^{+2})_2$ structure.

used this method to precipitate LDH, β-glucuronidase and galactose dehydrogenase using EGTA and Zn^{+2} as a precipitant

The other affinity precipitation technique viz. hetero-bifunctional affinity precipitation is discussed in details in subsequent paragraphs.

1.6.0 Hetero-bifunctional Affinity Precipitation or Secondary Effect Precipitation

This approach involves the synthesis of an affinity macroligand by conjugating an affinity ligand to a smart polymer, which exhibits reversible solubility characteristics in response to the external stimuli. Thus, one part of the macroligand exhibits affinity for the target protein while the other controls the solubility of the complex. A typical hetero-bifunctional affinity precipitation has been shown as a flow-chart in Figure 1.7.

The main steps include

1. Synthesis of a suitable hetero-bifunctional affinity macroligand (macroligand).
2. Addition of the macroligand to the crude protein mixture, which contains the desired protein/enzyme.
3. Precipitation of the macroligand-target protein complex by altering the solubility of the polymer. The choice of the stimulus for altering the solubility depends upon the polymer used. The precipitation could also be achieved by employing more than one stimulus.
4. Recovery of the macroligand-protein complex by centrifugation or filtration. In this step the target protein is separated from other contaminating proteins.
5. Dissociation of the target protein from affinity macroligand by using almost identical elution methods as used in affinity chromatography e.g. changes in pH, ionic strength or

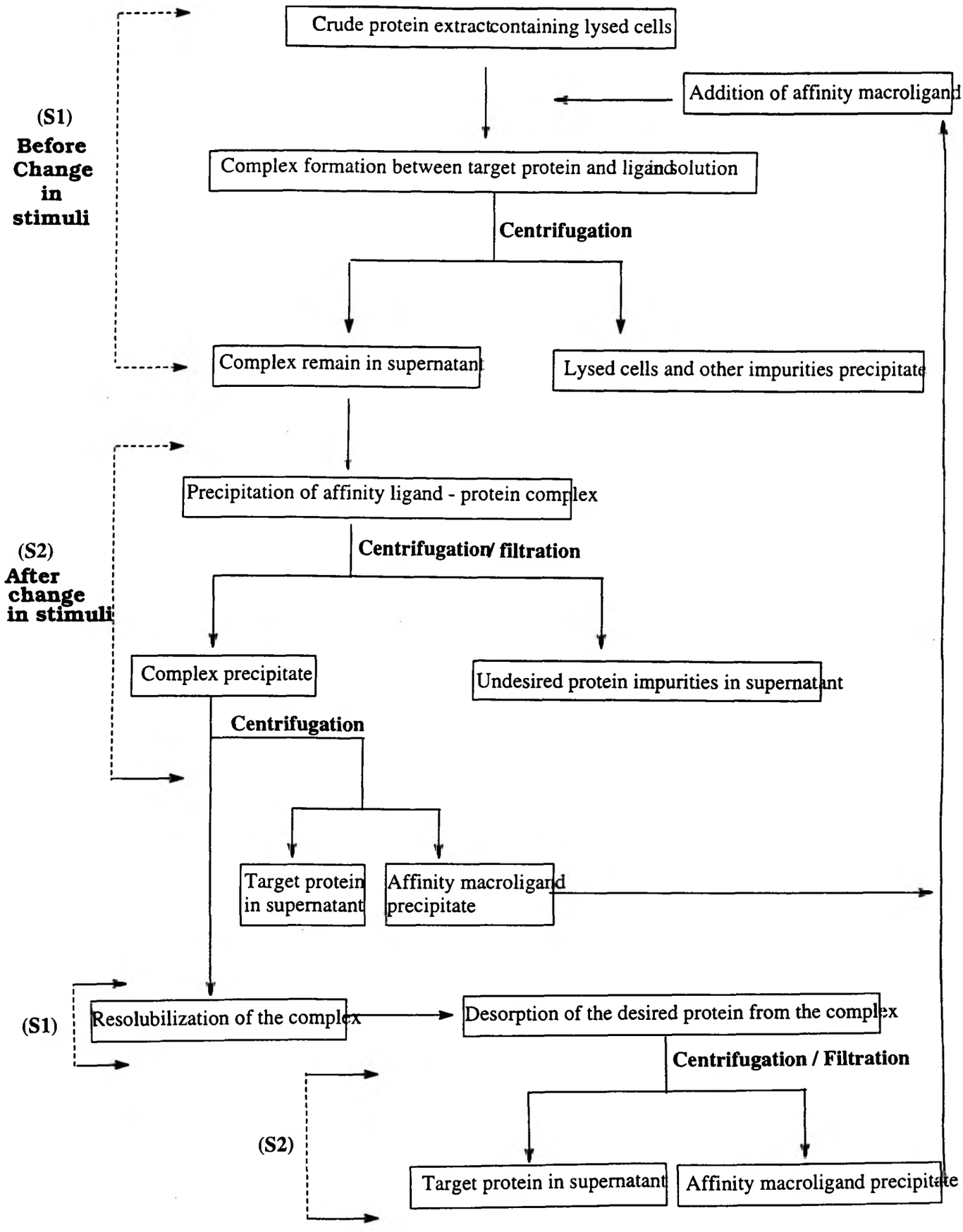


Figure 1.7 Flow - chart of hetero-bifunctional affinity precipitation.

using chaotropic agent or substrate analogue etc. The dissolution of the complex prior to dissociation sometime may increase the recovery of protein.

6. Elution of the desired protein by either of the two methods – i) Directly from insoluble complex. In that case the precipitated macroligand is ready to use for the next cycle. ii) Dissolution of the macroligand-protein complex then dissociation of the desired protein from the complex as well as precipitation of the unbound macroligand using identical stimuli. But if the dissociated protein and free macroligand are soluble, then a gel filtration or membrane filtration is used to recover the unbound macroligand [Gupta et al (1996)].

This technique has certain merits over the homo-bifunctional approach, which are summarized below.

a) It does not require optimization of the concentration of affinity ligand and protein to achieve the best precipitation yield of the desired protein. This is because the precipitation of macroligand-protein complex could be controlled by external stimuli only.

b) This approach can be used for purifying monomeric as well as multimeric proteins.

c) It is more robust and adaptable to scale-up for various ligand-protein systems.

d) The synthetic polymers can be tailored as per the specific application.

e) The technique uses homogeneous condition for enzyme binding. Thus it eliminates the problems associated with affinity chromatography and affinity ultrafiltration.

f) The overall process requires less equipment.

g) Synthetic polyligands can be recycled much more efficiently than homo-bifunctional ligands described earlier.

h) Precipitation of enzyme-smart polyligand complex can be achieved at low temperature and ambient pH and ionic strength so that, thermosesitive and highly unstable enzymes can be easily separated and recovered form the fermentation broth [Chen (1990)].

In view of its flexibility, hetero-bifunctional affinity precipitation is considered one of the most promising techniques in bioseparations. The smart polymer to which ligands are covalently linked to form stimuli sensitive polyligands constitutes an important component of the system. Therefore, before reviewing the prior work in this area, it will be appropriate to briefly discuss the smart polymers in general.

1.7.0 Smart polymers

These polymers undergo fast and reversible changes in the microstructure brought about by small changes in the property of the medium e.g. pH, temperature, ionic strength, presence of specific chemicals, light, magnetic field etc. These, microscopic changes in polymer structure manifest themselves at the macroscopic level as a precipitate formation in a solution or many-fold changes in the water content of hydrogels. These responses of smart polymers to environment are reversible and predictable [Gisser et al (1994)]. These properties of smart polymers were exploited for the development of new protein purification techniques in down stream processing which is called as affinity Precipitation [Mattiasson et al (1998)]. In Table 1.3 the use of smart polymers in affinity precipitation of different biomolecules are listed.

1.7.1 Classification of Smart Polymers

All smart polymers are generally divided into three categories depending on the stimuli used for their precipitation.

Table 1.3 - Use of smart polymers in affinity precipitation.

No.	Smart polymer*	Affinity ligand	Protein recovered	Change in stimuli	Reference
1	Alginate	Soybean trypsin inhibitor	Trypsin	Addition of calcium	Linne et al (1992)
2	---"---"---	_____	Endo-polygalacturonase	Addition of calcium at pH 3.8	Gupta et al (1993)
3	---"---"---	Guar gum	Peanut lectin	Addition of calcium	Tyagi et al (1996)
4	Chitosan	_____	Wheat germ agglutinin	PH	Senstad and Mattiasson (1989b)
5	---"---"---	Soybean trypsin inhibitor	Trypsin	PH	Senstad and Mattiasson (1989a)
6	---"---"---	_____	β-glucosidase	PH	Homma et al (1993)
7	---"---"---	_____	Lysozyme and rice, potato and tomato lectins	PH	Tyagi et al (1996)
8	---"---"---	_____	β- glucosidase	PH	Agarwal and Gupta (1996)
9	Dextran	Cibacron blue	Lactate dehydrogenase	Con. A addition	Mori et al (1994)
10	Blue dextran	Cibacron blue	Lactate dehydrogenase	Con. A addition	Senstad and Mattiasson (1989c)
11	Galactomannan	Protein A	Human IgG	Tetraborate addition	Bradshaw and Sturgeon (1990)
12	Hydroxypropyl methyl cellulose acetate Succinate	IgG	Protein A	pH	Taniguchi et al (1989)
13	---"---"---	_____	Cellobiohydrolase and endo-β-1,4-glucanase	pH	Homma et al (1992)
14	Poly(acrylamide-N-acryloyl m-amino benzoic acid)	m-aminobenzamidine	Trypsin	pH	Schneider et al (1981)

* Use of Eudragit polymer in affinity precipitation is given in Table 1.4.

* The polymers used in affinity thermoprecipitation are listed in Table 1.5.

a) Changing the pH

This can be achieved by changing pH which neutralizes charges and reduce repulsion between polymer segments e.g. copolymer of methyl methacrylate – methacrylic acid precipitate from aqueous solution at pH 5.0. This is due to protonation of carboxylic groups, which reduces the net charge on the polymer. Therefore hydrophobicity of the polymer increases as compared to the surrounding water which causes polymer to precipitate. But replacing methacrylic acid by dimethylaminethyl methacrylate precipitate the polymer from alkaline condition (pH ≈ 9.0) [Galaev et al (1996)].

Alternatively the net charges on the polymer can be neutralized by adding an efficient low M.W. counterion or a polymer molecule bearing opposite charges as to form polycomplex which are very sensitive to changes in pH or ionic strength [Dublin et al (1994)] e.g. The complex of poly (N-ethyl-4-vinyl pyridinium bromide) and poly (methacrylic acid) quantitatively precipitates and dissolves with a change of 0.3 pH units [Margolin et al (1984)].

b) Changing the hydrophobic-hydrophilic balance of the polymer

Uncharged water soluble polymers exhibit solubility due to hydrogen bonding with water. These polymers could be precipitated by increasing temperature or ionic strength which changes hydrophobic/hydrophilic balance of the polymer due to decreased tendency of the polymer to form hydrogen bonds with water e.g. poly (N-isopropylacrylamide) precipitates from aqueous solution at 32-34 ^{0}C. This polymer is said to have a lower critical solution temperature (LCST) of 32-34 ^{0}C. Similarly, poly (vinyl methyl ether) and poly (N-vinyl caprolactam) has LCST of 34 ^{0}C and 32 - 40 ^{0}C respectively [Galaev and Mattiasson (1993)].

c) Use of small ions

There are few polymers which interact reversibly and noncovalently with certain metal ions and inorganic acids so as to form an insoluble polymer network e.g. calcium-alginate [Charles et al (1974)] and boric acid- polyols [Wu and Wisecarver (1992)].

Smart polymers can also be classified as -

a) Synthetic polymers – These are usually made up of copolymers of vinyl derivatives comprising active functional groups such as hydroxyl, epoxy, carboxyl, amino etc. e.g. Acrolein-acrylic acid copolymer, poly (N-isopropylacrylamide – glycidylmethacrylate).

b) Natural polymers – These are derivatives of cellulose, chitin, alginic acid or milk casein.

c) Polymer complexes – It was found that a stoichiometric mixture of oppositely charged polymers form strong electrostatic bonds with each other and the resulting complex precipitates when it grows to a particular size. But, if one polymer is added in excess the precipitation/solubility properties of the resulting complex will depend upon the pH and ionic strength of the solution e.g. poly (N-ethyl-4-vinyl pyridinium bromide) and poly (methacrylic acid) quantitatively precipitate and dissolves with a change of 0.3 pH units [Fujii and Taniguchi (1991)].

The stimuli, which precipitate the macroligand–protein complex in hetero-bifunctional mode are discussed below.

1.8.0 Change in pH

The first successful application of hetero-bifunctional affinity precipitation for enzyme recovery using change in pH was reported by Schneider et al (1981). In this study the copolymer of acrylamide, N-acryloyl p-aminobenzoic acid and N-acryloyl m-

aminobenzamidine was synthesized. The polymer was added to a crude extract of bovine pancreas. This polymer exhibits affinity for trypsin due to the presence of m-aminobenzamidine and pH sensitivity was demonstrated by protonation/deprotonation of benzoic acid. The macroligand was allowed to form complex with trypsin at neutral pH and the resulting complex was precipitated at pH 4.0. The precipitate was washed and dissolved at pH 8.0. Then simultaneously, the complex was dissociated and the free macroligand was re-precipitated at pH 2.0. On centrifugation, the free trypsin was released in the supernatant, which was lyophilized to yield the 90 % product. The precipitated copolymer was regenerated upto 93 %, which was reused for next cycle. The prime advantages of this process are -

a) The method is specific for recovery of trypsin and in a single step a high overall yield is achieved.

b) Other valuable products could be recovered from the filtrate after removal of the trypsin.

c) The same polymer can be reused for 7-8 times without much loss in percent recovery thereby the overall process is economical.

d) The quantity of polymer required in this process is very low 0.1-0.5 %, which thereby alleviates the risk of non-specific adsorption of other proteins.

e) The process is easy to scale-up and does not require special installations.

Chitosan (partly deacetylated chitin) is a poly-cationic polymer rich in N-acetylglucosamine units. It exhibits reversible solubility at pH 6.5. Above pH 6.5 it quantitatively precipitates, because at this pH its amino groups are uncharged. This natural polymer was used in two separate studies to purify trypsin and wheat-germ

agglutinin (WGA). The STI was covalently linked to chitosan and the resulting polymer was allowed to form complex with trypsin at pH 5.5. The chitosan-STI-trypsin complex was precipitated by increasing the pH of the solution to 8.5. The precipitate was collected by centrifugation and was washed with ten volumes of buffer. The precipitate was re-solubilized and trypsin was dissociated at pH 2.5. At this pH the chitosan polymer was soluble. Therefore, to separate the dissociated trypsin from the soluble chitosan polymer, the solution was poured on gel filtration column to get 93 % recovery of trypsin activity from eluent [Senstad and Mattiasson (1989a)].

It is well known that NAG acts as an affinity ligand for WGA. This fact was explored in the second system by using chitosan as a polymeric ligand for WGA. The same procedure as in the prior case was adopted here to recover WGA. Only difference is the use of floating technique (which involves applying small bubbles to lift the precipitated material on the surface where it can be easily separated from solution) instead of centrifugation for collecting the WGA-chitosan complex on large scale. This operation gives a final 70 % yield [Senstad and Mattiasson (1989b)].

The sequential precipitation of cellulase and β-glucosidase using two oppositely charged polymers e.g. chitosan and Eudragit S-100 was carried out as follows. Homma et al (1993) established that chitosan, a positively charged natural polymer exhibits selective affinity for cellulase. This fact was used to remove cellulase activity from crude extract of *Trichoderma longibrachiatum,* which also contain β-glucosidase. The β-glucosidase was recovered from the supernatant using Eudragit S-100, which is a negatively charged synthetic polymer that exhibits selective affinity for the β-glucosidase. This process yields 99 % recovery of β-glucosidase in terms of activity units. Thus two different

polymers were used sequentially to precipitate two enzymes from the same source [Agarwal and Gupta (1996)].

An inherent disadvantage of using chitosan derivatives as pH sensitive polymers in affinity precipitation is its separation from final product. Usually, a time consuming gel filtration step is involved to separate the desired product from chitosan. This is because chitosan is soluble at low pH and lower pH is generally used to dissociate the target protein from the complex. This disadvantage can be overcome by using a different polymer, which is soluble at higher pH and become insoluble if pH is reduced as described in the following example.

The polymer that fulfils such solubility requirements is hydroxypropylmethyl cellulose acetate succinate. It is soluble above pH 5.0 and insoluble below pH 4.5, which was used to recover protein A from *Staphylococcal aureus.* This polymer was coupled to human immunoglobulin (IgG), which acts as an affinity ligand for protein A. Three consecutive steps were involved- complex formation at pH 7.2 at 2 ^{0}C for 1 hour, precipitation of the complex at pH 4.2 and elution of the protein A at pH 2.5 which gives a product with 53 % overall yield. The low recovery was attributed to non-specific adsorption of impurities present in the crude extract. This macroligand can be reused up to four times without affecting final yield and purity of the product. Thus, in this case lowering pH to 2.5 suffice both elution of protein A and precipitation of the polymer [Taniguchi et al (1989)]. The same polymer exhibits affinity for cellobiohydrolase and endo-β-1,4-glucanase, which are common components of any cellulase mixture. Therefore, it was used to affinity precipitate both of these enzymes except β-glucosidase which is also one of the components of cellulase mixture. After dissociating

cellobiohydrolase and endo-β-1,4-glucanase by 1 M acetate buffer (pH 3.5), the resulting supernatant was used to obtain cellobiose from cellulose powder with 50 % yield. This was a result of combined action of both the enzymes on cellulose [Homma et al (1992)].

Another well-studied pH sensitive polymer is Eudragit.

1.8.1 Eudragit – A Versatile Stimuli Sensitive Polymer

Eudragit is a copolymer of methyl methacrylate-methacrylic acid marketed under this trade name by rohm and Haas. It is used as an enteric-coating polymer, which exhibits complete phase separation at around pH 4.5. It was observed that depending upon the coupling efficiency of affinity ligands to this polymer, the pH required for phase separation shifts towards physiological pH i.e. 6.0 owing to the decreased number of free carboxyl groups. The quantitative precipitation of Eudragit by change in pH was found to be influenced by the presence of ammonium sulfate (salt) or PEG 8000 [Gupta et al (1996)].

Gupta et al (1996) listed the advantages of Eudragit polymer.

a) The precipitate formed is compact and easily separated from the supernatant. Moreover, water content of the precipitate is high enough which ensures the stabilization of the bound protein. In slightly alkaline buffer this precipitate dissolved readily.

b) In each step of precipitation-solubilization cycle, the polymer recovery is > 95 %. This process can be repeated many times.

c) It is cheap and easily available polymer.

d) Many enzyme exhibits affinity towards native Eudragit polymer e.g. xylanase [Gupta et al (1994) and D-lactate dehydrogenase [Guoqiang et al (1993)]. Therefore, Eudragit can be used directly to precipitate these enzymes without using any specific affinity

ligands. Thus, the cost and time for coupling the affinity ligands could be avoided. The use of Eudragit in some important applications in affinity precipitation is given in Table 1.4.

In the early reports Eudragit bound STI was used to selectively precipitate trypsin. In this study it was found that with increase in the polymer concentration above 1 %, the activity recovery was significantly low (28 %). This low activity recovery was attributed to the fact that at high polymer dosage the settling of the polymer was difficult and a small proportion of suspending STI bound polymer inhibited trypsin activity, which was reflected in lower recovery. Even then, 68 % of protein and 96 % of the precipitated activity could be recovered from crude extract of bovine pancreas using benzamidine as an eluting agent [Kumar and Gupta (1994)].

Larsson et al (1996a) carried out a detailed study on the precipitate formation of Eudragit S-100 conjugated with p-aminophenyl-α-D-glucopyranoside in the presence of Con. A using dynamic light scattering studies. It was found that the affinity macroligand-Con. A complex was formed in the solution prior to the formation of precipitate and the rheological measurements demonstrated that the inter- as well as intramolecular hydrophobic interactions were responsible for subsequent build-up of the precipitate. It was also shown that Con. A binds to polymer both via specific interactions with affinity ligand i.e. p-aminophenyl-α-D-glucopyranoside as well as non-specific hydrophobic interactions with Eudragit. The initial precipitation was achieved by only specific interactions and by selecting optimum conditions during precipitation the non-specific binding of the Con A can be reduced.

Table 1.4 Applications of Eudragit polymer in affinity precipitation of proteins.

No.	Affinity ligand	Protein recovered	Applied stimuli	Reference
1	p-aminophenyl-α-D-glucopyranoside	Con. A	PH	Linne Larsson and Mattiasson (1994)
2	Cibacron blue	D-lactate dehydrogenase	PH	Shu et al (1994)
3	---"---"---	L-lactate dehydrogenase	Acetonitrile (10 % v/v) + 0.15 M Ca^{+2}	Guoqiang et al (1994)
4	---"---"---	Pyruvate kinase	40 ^{0}C + 50 mM Ca^{+2}	Guoqiang et al (1994)
5	---"---"---	Alcohol dehydrogenase	50 mM Ca^{+2} at 40 ^{0}C in presence of Zn^{+2}	Guoqiang (1995a)
6	IgG	Protein A	Aqueous two phase extraction integrated with precipitation at pH 4.5	Kamihira et al (1992)
7	Soybean trypsin inhibitor	Trypsin	PH	Kumar and Gupta (1994)
8	_____	Xylanase	PH	Gupta et al (1994)
9	_____	D-lactate dehydrogenase	PH	Guoqiang et al (1993)

Subsequently, in the continuation of the same work using dynamic light scattering they concluded that the build-up rate of initial complex formation depends upon optimum ratio of affinity ligand to Con. A which was 40 ligands/Con. A. The rate of binding of Con. A was reduced above this ratio due to the diffusion limitations. The Con. A was desorbed from the precipitate using maltodextrin, which has a dextrose equivalent number of 16.5-19.5. It was found to be the most powerful desorbing agent for the Con. A from the Con. A - Eudragit polymer [Larsson et al (1996b)].

A microbial lipase as an antigen was conjugated to Eudragit S –100 (pH transition at 4.8). The resulting macroligand (0.1 % w/v) solution was added into diluted (1:3) hybridoma culture, which on affinity precipitation yields 50-55 % (by ELISA) monoclonal antibody [Taipa et al (1998)].

Eudragit S-100 can also be precipitated by various alternative modes. These are discussed below.

1.8.2 Alternative modes of Eudragit precipitation

Eudragit polymer is not only sensitive to pH but various additives influence the pH of precipitation e.g. the polymer precipitates at a relatively higher pH in the presence of $(NH_4)_2SO_4$, guanidine hydrochloride or PEG 8000, whereas NaCl did not have a significant effect. Further, it was demonstrated that $(NH_4)_2SO_4$ showed relatively stronger effect on Eudragit bound to cibacron blue. It was found that addition of Ca^{+2} precipitates Eudragit at neutral pH. This is a result of the complex formation of Ca^{+2} with charged carboxyl groups of Eudragit, which subsequently cross-linked into network thereby precipitating the polymer. In Eudragit-cibacron blue this precipitation was further

enhanced by strong interactions of Ca^{+2} with sulfonic acid groups. The reduction of intra- and inter-molecular repulsion in the polymer, also is a contributing factor.

Guoqiang et al (1994, 1995 a and b) have explored alternative stimuli for precipitation of Eudragit. It has been found that Ca^{+2} can precipitate Eudragit S-100 either when the temperature is raised or by incorporation of organic solvents. The polymer precipitates quantitatively at a relatively low concentration of acetonitrile (10 % v/v) + 0.15 M Ca^{+2} without changing pH. This precipitation route considerably decreases the degree of hydration of the precipitate, which resulted in compact pellet formation on centrifugation. It was observed that water-miscible organic solvents decreases the dielectric constant of the medium which enhances the electrostatic interactions between Ca^{+2} and free carboxyl groups of the polymer. In addition to it Eudragit S-100 precipitation by acetonitrile-Ca^{+2} reduces the non-specific interactions of proteins with the polymer. Using acetonitrile-Ca^{+2} precipitation mode Eudragit-STI resulted in 80 % recovery of trypsin. The change of pH on the other hand resulted in only 50 % recovery. Similarly, Eudragit-cibacron blue precipitates at 40 ^{0}C in the presence of 50 mM Ca^{+2} and resolubilized at room temperature in the absence of Ca^{+2} [Guoqiang et al (1994)].

In summary, diverse stimuli have been used for the successful precipitation of Eudragit polymer leading to development of different precipitation strategies for the purification of various enzymes. Thus LDH was thermoprecipitated at 40 ^{0}C from porcine muscle extract using 50 mM Ca^{+2}. It was observed that at low concentration of muscle extract both LDH and pyruvate kinase (which also exhibits affinity for cibacron blue) precipitates by Eudragit-cibacron blue polymer. On the contrary, at higher loading of muscle extract (65 % of total volume) LDH was preferentially and selectively

precipitated whereas pyruvate kinase did not precipitate at all. Thus, from high muscle extract loading more than 69 % LDH was recovered. Further, the sequential elution of these enzymes was carried out as follows. At lower KCl concentration (0.1 M) in the eluent, about 85 % pyruvate kinase was recovered and by increasing the KCl concentration to 0.5 M, 90 % LDH can be recovered. Thermoprecipitation of Eudragit was successfully used for the precipitation of alcohol dehydrogenase (ADH) from *Saccharomyces cerevisiae.* It was known that Zn^{+2} enhances the affinity of ADH for cibacron blue. The yeast cell homogenate was mixed with Eudragit-cibacron blue in the presence of Zn^{+2}. The enzyme-macroligand complex was precipitated by addition of 50 mM Ca^{+2} at 40 ^{0}C. The enzyme was desorbed by adding 200 mM iminodiacetic acid in the precipitate. It gives 66 % yield [Guoqiang et al (1995a)].

1.8.3 Change in Temperature (Affinity thermo-precipitation)

Polymers that undergo temperature dependent phase transition or exhibit lower critical solution temperature (LCST) are employed in affinity thermo-precipitation. Thermoprecipitation is very attractive technique since it requires only the input of energy i.e. heat but no matter i.e. acid, alkali or salt have to be added in the system. Thus waste and sterility problems are minimized. [Eggert et al (1998)]. Also, the bio-specific recognition process amongst enzyme and inhibitor, which involves electrostatic and ionic interactions, is dependent on pH and ionic strength of the solution. Therefore, dissociation of the desired enzyme prior to precipitation of the complex by change in temperature is negligible unlike in the case of pH dependent affinity precipitation. The polymers used in affinity thermo-precipitation are exclusively synthetic. Galaev and Mattiasson (1993) reviewed a large number of such polymers.

The affinity thermo-precipitation technique has developed in last two decades but now it is fast emerging a method of choice for separation of various proteins as seen from Table 1.5. Gupta and Mattiasson (1994) enlisted various criteria for the use of thermoprecipitating polymers in affinity thermoprecipitation. These are enumerated below.

a) The temperature range of phase transition should not be extreme. In general the polymers exhibiting LCST close to physiological temperature i.e. 37 ^{0}C is suitable. Also, the phase transition should be abrupt i.e. maximum 2-3 ^{0}C. Otherwise a temperature shock of ca. 5-10 ^{0}C could lead to enzyme deactivation except in case of thermostable enzymes [Ghose and Mattiasson (1993)].

b) The attachment of the affinity ligand should not drastically alter the properties of the thermosensitive polymer. This criterion is very important during designing new affinity macroligands.

c) The affinity of the affinity ligand towards target protein or enzyme should not alter upon attachment to the polymer.

Galaev and Mattiasson (1993b) demonstrated that the coupling of affinity ligands to thermoprecipitable polymers resulted in a 100-1000 fold reduction in the affinity of low M.W. ligands such as cibacron blue F3GA, Cu-iminodiacetic acid and p-aminobenzamidine for their respective target proteins. This decreased binding of ligands conjugated to the polymer is caused by steric hindrance of the polymer chain or by a change of microenvironment in the vicinity of the ligand molecule. They further studied the ligand accessibility to the protein when the precipitation process just initiated and the

Table 1.5 Affinity thermoprecipitation of various proteins.

No.	Affinity ligand	Thermoprecipitating polymer	Recovered protein	Reference
1	Human monoclonal antibody	Poly(N-isopropyl acrylamide-co-N-acryloylsuccinimide)	Human antigen	Monji and Hoffman (1987)
2	Protein A	---"---"---	Human IgG	Chen and Hoffman (1990)
3	p-amino benzamidine	Poly(N-isopropyl acrylamide-co-glycidyl methacrylate or N-acryloyl succinimide)	Trypsin	Nguyen and Luong (1989)
4	p-amino benzamidine	Poly(N-isopropyl acrylamide)	Trypsin	Galaev and Mattiasson (1993b)
5	Soyabean trypsin inhibitor	Poly(N-vinyl caprolactam)	Trypsin	Galaev and Mattiasson (1992)
6	p-amino benzamidine	Poly(N-isopropylacrylamide-co-acryloyl spacer)	Trypsin	Vaidya et al (1999)
7	IgG	Poly(N-isopropylacrylamide-co-glycidyl methacrylate)	Protein A	Galaev and Mattiasson (1992)
8	p-aminobenzamidine	Poly(N-isopropylacrylamide)	Alkaline protease	Pecs et al (1991)
9	Cibacron blue 3GA and Cu-iminodiacetic acid	Poly(N-isopropylacrylamide) and Poly (N-vinylcaprolactam)	Lactate dehydrogenase	Galaev and Mattiasson (1993b)
10	Cibacron blue	Eudragit S-100	Lactate dehydrogenase and Pyruvate Kinase	Guoqiang et al (1994)
11	Cu(II)-1,vinyl imidazole complex	Poly(N-vinyl caprolactam) and poly (N-isopropyl acrylamide)	Kunitz soybean trypsin inhibitor	Galaev et al (1997)
12	Cysteamine-maltose	Poly(N-acryloyl piperidine)	α-glucosidase and Con.A	Hoshino et al (1998)
13	Cu(II)-1,vinyl imidazole complex	Poly(N-isopropyl acrylamide)	α-amylase inhibitor	Kumar et al (1998)
14	Psoralen	---"---"---	Eco. R. I	Umeno et al (1998b)
15	Single stranded DNA	---"---"---	Oligonucleotides	Umeno et al (1998a)

precipitate was in the micro-dispersed form. It was found that 40-80 % of STI was still accessible to trypsin binding when poly (N-vinyl caprolactam) conjugated with STI is in micro-dispersed form. Also, the amount of trypsin affinity precipitated by poly (NIPAM) linked to PABA was independent of whether the trypsin was added before or after the microdispersion was formed. This shows that in the microdispersion form of ligand-polymer conjugate most of the affinity ligand remained accessible for the protein.

The credit goes to Monji and Hoffman (1987) for describing the first bioseparation process based on affinity thermoprecipitation. The authors conjugated monoclonal antibody to acryloyl group via the reaction of the antibody with N-acryloxysuccinimide. The monomer so synthesized was copolymerized with NIPAM. The resulting polymer was used in developing a new immunoassay system termed as PRECIPIA, which was used for recognition and subsequently, separation of human IgG from a mixture. The steps involved in this assay are –

1. An antibody (Ab1) specific for one epitope of an antigen (Ag) was conjugated to the backbone of poly (NIPAM).

2. Then a second antibody (Ab2) specific to a second epitope of same Ag, was attached to a signal molecule e.g. horseradish peroxidase or a fluorophore.

3. Both these conjugates were mixed with biological fluid containing Ag to form a sandwich type immune complex below LCST of the polymer.

4. Raising the temperature above LCST of the polymer precipitated the polymer-immune sandwich complex.

5. Signal determination from the precipitate to quantify Ag.

The advantages of this assay are manifold. It is a fast method, reduces the non-specific binding or entrapment and the signal obtained from the precipitate can be concentrated to detect very small amount of Ag, thereby increases the sensitivity of the assay. The same approach was used for affinity thermoprecipitation of human immunoglobulin by replacing monoclonal Ab with protein A [Chen and Hoffman (1990)].

Later, Nguyen and Luong (1989) copolymerized NIPAM with N-acryloxy succinimide (NASI) or glycidyl methacrylate (GMA). The resulting copolymers have reactive succinimide and epoxy groups respectively. Copolymers of NIPAM-NASI has a cloud point at 42 ^{0}C whereas, NIPAM-GMA copolymer has 34 ^{0}C. These polymers are also sensitive to ionic strength of the solution. The ammonium sulfate concentration of 38 g/lit and 27 g/lit. were sufficient to precipitate NIPAM-NASI and NIPAM-GMA respectively. These polymers were conjugated with IgG and PABA separately. The resulting macroligand comprising PABA was used to separate trypsin from a synthetic equimolar mixture of trypsin-chymotrypsin. Trypsin was eluted from the precipitated complex by reducing pH of the solution to 2.0 whereas; increasing the ionic strength of the solution i.e. salination precipitate the affinity macroligand. Thus, 85 % recovery of trypsin and 95 % recovery of macroligand were obtained by this procedure. Also, NIPAM-GMA was conjugated with IgG and it was found that this polymer could precipitate 100 % protein A from the solution.

Recently, Pecs et al (1991) copolymerized N-acryloyl-PABA with NIPAM and the resultant copolymer was used for affinity precipitation of alkaline protease from a multi-component protease mixture called as Maxatase. The precipitation of the complex

was achieved by increasing both salt concentration and temperature. At 50 ^{0}C using 2.25 % sodium tripolyphosphate gave 91 % precipitation efficiency. The precipitated complex was recovered and the protease was dissociated at pH 5.0 to yield 51.6 % protease activity. The authors attributed this decreased efficiency of separation to overcrowding of PABA on surface of the polymer or to the loss in affinity *per se.*

Trypsin separation from a synthetic mixture containing BSA was achieved using hydrolyzed polymer of poly (N-vinyl caprolactam) conjugated to STI. The affinity macroligand-enzyme complex was precipitated at 45 ^{0}C and the precipitate obtained was dissolved in 0.1 M KCl at room temperature. Trypsin was eluted from this complex by lowering the pH to 2.5 and the macroligand was thermo-precipitated by increasing the temperature to 45 ^{0}C. The authors observed that conjugation of STI to poly (N-vinyl caprolactam) increases the LCST marginally (by 2 ^{0}C) but, when this affinity polymer was complexed with trypsin the LCST increases considerably. The enzyme recovery was 54 %, which is comparable with the recoveries obtained by conventional affinity chromatography technique. A remarkable feature of this study was that non-specific precipitation of BSA was totally absent [Galaev and Mattiasson (1992)].

Shuji et al (1994) synthesized an affinity macroligand by reacting Poly (NIPAM) with p-aminophenylphosphorylcholine exhibiting LCST of 32 ^{0}C. This macroligand was used for the selective precipitation of C-reactive protein from rabbit serum in the presence of Ca^{+2} ions. The protein was eluted by cold EDTA solution with 80 % recovery.

The most interesting application of thermoprecipitation has been reported recently by Stayton et al (1995). They exhibited that a molecular "gate" was created by

conjugating a temperature sensitive [poly (NIPAM)] polymer to a genetically engineered site on streptavidin, which was involved in biotin binding. This modified streptavidin was immobilized on a solid support. Above LCST of the polymer i.e. at 37 ^{0}C this protein exhibit 84 % drop in the binding of biotin relative to its binding at 4 ^{0}C i.e. below LCST. When the polymer is below LCST it is fully hydrated and is in random coil conformation thereby it interferes minimally with the biotin binding pocket of the streptavidin. On the other hand above LCST, the polymer is in collapsed state which blocks the "gate" for easy access of biotin. Thus, such recombinant proteins provide environmentally sensitive "gates" or "switches" to make ligand on-off during binding with its ligate (in this case biotin).

The first report on the use of metal affinity precipitation integrated with thermoprecipitation was given by Galaev and Mattiasson (1993b). In this study poly (N-vinylcaprolactam) was conjugated to Cu-iminodiacetic acid (Cu-IDA). It was found that the thermoprecipitation of LDH in the presence of Cu-IDA-poly (N-vinylcaprolactam) conjugate resulted in 35-40 % recovery of enzyme activity. However, an unexpected result was obtained when an excess of iminodiacetic acid solution was added as an eluent to enhance the recovery of LDH. This yielded only 3-5 % recovery of activity. The reason for this low recovery of LDH is that during precipitation enzyme molecules along with it Cu^{+2} ions are stripped off which causes LDH deactivation. This illustrates again the demands of selection of right kind of polymer and affinity ligand for any affinity precipitation system to be productive.

Galaev et al (1997) reported exactly opposite trend when more than 95 % Kunitz STI was recovered using excess of imidazole as an eluent. In this report Cu (II) loaded

copolymer of vinyl imidazole and NIPAM was synthesized, which was used for the precipitation of Kunitz STI under mild conditions i.e. pH 7.0, 32 ^{0}C and 0.6 M NaCl. The efficiency of imidazole in affinity thermoprecipitation was attributed to the flexibility of water soluble polymer below LCST, which brings several imidazole ligands sufficiently close enough to interact efficiently with the same Cu (II)-ion. This provides sufficient strength to polymer-Cu (II) interactions unlike in immobilized metal affinity chromatography in which the polymer in rigid matrix did not possess the desired flexibility. In tentacle bio-chromatography the multipoint interactions observed in affinity precipitation could be realized [Chrombook and Merk (1996)]. In this technique the affinity ligand was linked to a long flexible polymer tail which provided the flexibility for multi-point protein interactions.

Recently, a single step purification of α-amylase inhibitor from the seeds of ragi was reported by Kumar et al (1998). Cu (II) loaded thermosensitive metal chelate copolymer of NIPAM and 1-vinyl imidadazole (VI) was used. The recovery was 84 %. As discussed already, the interaction between affinity ligand attached to the polymer and enzyme should be strong enough to obtain the desired results in affinity thermoprecipitation. Eggert et al (1998) proposed a method to enhance the interactions between m-aminophenylboronic acid and serine protease. In this report the macroligand was synthesized from N,N'-diethylacrylamide comprising free carboxylic acid groups on either ends of the low molecular weight (less than 15 monomeric units per molecule) polymer conjugated with m-aminophenylboronic acid by carbodiimide coupling. The inhibition constant of this macroligand was 10 μM/lit. for serine protease obtained from *Substilisin carsberg*. The affinity constant was brought down by an order of magnitude

by the addition of 5 % (v/v) of ethylene glycol for successful affinity thermoprecipitation. Ethylene glycol forms a cyclic (five membered) ester with phenylboronic acid which in turn exhibit higher binding ability with the enzyme.

Hoshino et al (1998) synthesized conjugates of cysteamine and poly (N-substituted acrylamide)s obtained from alkylamines such as N,N-diethylamine, N-propylamine, isopropylamine, pyrrolidine and piperidine having a range of LCSTs between 0 to 52 ^{0}C. Amongst these poly (N-acryloylpiperidine)-S-$(CH_2)_2$-NH_2 exhibited LCST of 4 ^{0}C. To free amino groups of this polymer, the affinity ligand maltose was conjugated to yield affinity adsorbent. This macroligand was used for the recovery of thermolabile enzyme such as α-glucosidase (using temperature cycles between 4 and 10 ^{0}C) from cell free extract of *Saccharomyces cerevisiae* with 68 % of activity recovery. Also, it was used to recover Con. A from crude extract of jack bean meal with 80 % recovery.

In the following examples affinity thermoprecipitation was used elegantly to isolate oligonucleotide and restriction enzyme in two independent studies. Methacryloyloxy succinimide was conjugated to 5'-amino-terminal of $(dT)_8$ (oligomer of thymidine), this vinyl derivative of $(dT)_8$ was copolymerized with NIPAM to yield the polymer having LCST of 14 ^{0}C. This affinity macroligand was allowed to hybridize with $(dA)_8$ (oligomer of adenine) by incubation at 0 ^{0}C for 6 hours and then hybridized macroligand was precipitated at 15 ^{0}C. It gives 84 % precipitation of $(dA)_8$. About 99 % of the precipitated oligonucleotide was eluted when the precipitate was suspended in deionized water and centrifuged at 40 ^{0}C. Also, the affinity ligand selectively coprecipitated $(dA)_8$ from a mixture of $d(A)_8$ and $(dA)_3dT(dA)_4$ [Umeno et al (1998a)].

In another case the same group synthesized the copolymer of NIPAM containing terminal psoralen group. Psoralen is known to intercalate DNA double strands in dark and form covalent double bonds at 3,4 and 4',5' of pyrimidines upon near UV irradiation. If both sides of psoralen were reacted, an interstrand DNA cross-linking occurs thereby connecting both strands of DNA to each other. This method of cross-linking was applied to attach pBR322 DNA to psoralen conjugated with poly (NIPAM). The resulting macroligand was used to precipitate restriction endonuclease Eco RI, which recognizes the 5'-GAATTC-3' unique sequence in pBR322. The procedure used was as follows – In the absence of magnesium ions the macroligand was incubated with Eco RI. After specific recognition of the DNA sequence on the pBR322, the enzyme binds to it. The complex of enzyme bound macroligand was centrifuged at 40 ^{0}C to collect the precipitate. Almost all enzyme activity could be precipitated by this method. The enzyme was eluted by the addition of 1.5 M NaCl. But, if the 5'-GAATTC-3' recognition sequence was interrupted by using Sty I enzyme, pBR 322 DNA was unable to bind to EcoRI and no precipitation of the enzyme was observed. The finding demonstrates that double stranded DNA bearing a recognition site for EcoRI acts as an affinity ligand for EcoRI and the presence of this site is exclusively responsible for the separation of the enzyme [Umeno et al (1998b)].

Thus, affinity thermoprecipitation is extensively used so far for the isolation of various biomolecules.

1.8.4 Addition of Low M.W. Substances

Dextran, galactomannan and alginate are natural polysaccharides, which are not stimuli sensitive on their own. But, the addition of certain small M.W. substances such as

metal ions, Con. A or salts, results in the formation of polycomplexes which precipitate out from the solution. The following examples illustrate the use of such polysaccharides in affinity precipitation of proteins.

A] Blue dextran consists of two parts e.g. dextran which is a polysaccharide which can be precipitated by addition of precipitating aid such as Con. A, which crosslink different dextran units (due to its inherent affinity for sugar residues on dextran) and there by precipitates the polymer without changing the external environmental conditions of the solution. The blue color of dextran is due to cibacron blue attached to it, which acts as an affinity ligand for LDH.

With regard to the precipitation, this system resembles homo-bifunctional affinity precipitation in that there must be a specific ratio between the blue dextran and Con. A to achieve an optimum recovery which is approximately 4.5 mg Con. A/mg of blue dextran. But, once this ratio is reached, one can theoretically affinity precipitate any kind of protein, even a monomeric one, irrespective of its concentration. In this regard it differs from homo-bifunctional affinity precipitation. This is because the affinity ligand (cibacron blue) *per se* does not take part in precipitation process. Two different fragments present on blue dextran bring about the precipitation and affinity complex formation. In this respect it resembles heterogeneous affinity precipitation. This system was used to precipitate LDH from pig heart extract. The complex formed between LDH and Con. A-blue dextran was dissociated by 1.5 M KCl and blue dextran was again made soluble by addition of glucose, acting as a competitive inhibitor for Con. A [Senstad and Mattiasson (1989)].

B] Galactomannan was oxidised by enzyme D-galactose oxidase to generate the free aldehyde groups (at C-6 position) in the polymer. The sodium cyanoborohydride was reacted with protein A to reduce schiff's base to alkyl amine. This is called as reductive amination. This base was further utilized to covalently link protein A to galactomannan. The resulting macroligand was precipitated by the addition of potassium tetraborate (precipitate remain insoluble above pH 8.0) and was used for the purification of human IgG as follows. The galactomannan-protein A macroligand was allowed to form complex with IgG. After precipitation, the IgG was subsequently eluted from the complex by potassium thiocyanate. The galactomannan-protein A remains insoluble as the borate complex. The disaggregation of galactomannan-protein A complex was carried out by reducing the pH of the solution to 4.0. The IgG obtained was 90 % pure. Further it was shown that a three-fold increase in protein A loading on galactomannan resulted in a 1.5 fold increase in the level of binding with IgG [Bradshaw and Sturgeon (1990)].

C] Alginate, a marine algae is a copolymer of mannuronic acid and guluronic acid. Linne et al (1992) used this polymer to crosslink to STI by carbodiimide coupling. It was used to precipitate trypsin. The alginate-STI-trypsin complex was precipitated by addition of calcium ions. Primarily, Ca^{+2} interact with guluronic acid units. These ions were quenched using EDTA to dissolve the complex and trypsin was dissociated by decreasing pH (by addition of HCl) or by displacement with arginine. The trypsin recovery was only 50 % and affinity macroligand could be recovered to 60 %. The disadvantage of using this polymer is that it is highly charged polymer, which induces coprecipitation of other proteins by non-specific adsorption. In the same group alginate was used for the affinity precipitation of endo-polygalacturonase from *Aspergillus niger*. The elution of this

enzyme was brought about by 0.5 M NaCl / 0.2 M Ca^{+2} as precipitating aid giving 10 fold purification [Gupta et al (1993)].

Tyagi et al (1996) used guar gum (affinity ligand specific for galactose binding lectin) conjugated to alginate for precipitation of peanut lectin. About, 63 % of added lectin could be precipitated.

1.8.5 Surfactant and Lipid Mediated Affinity Precipitation

Biotin was conjugated to dimyristylphosphatidylethanolamine and the non-ionic surfactant octaethyleneglycol mono-n-dodecyl ether. The resultant micelles were used to precipitate avidin below critical micelle concentration. By, this method greater than 90 % of avidin could be precipitated [Powers et al (1992); Guzman et al (1988)]. In continuation with this work Powers et al (1994) successfully demonstrated the selective precipitation of monoclonal antibiotin antibody using small micellar aggregates of biotin modified phospholipid and non-ionic ethoxylate alcohol surfactant. It was found that antibiotin antibody could be maximally precipitated with 60-70 % activity at an optimum ratio of dimyristylphosphatidylethanolamidobiotin to antobody which is 7:1. The addition of 0.5 M $(NH_4)_2SO_4$ increases the rate of precipitation by more than one order of magnitude.

Recently, Rockholm and Yamamoto (1996) purified Violaxanthin deepoxidase from Lettuce using anion-exchange chromatography integrated with lipid-affinity precipitation using monogalactosyldiacylglyderide. This enzyme specifically reacts with lipid at pH 5.2. The enzyme has pI of 5.4. Hence, affinity precipitation was carried out at pI of the enzyme and the enzyme was eluted using an anion-exchanger Mono. Q.

The example mentioned above deal with an integrated process of enzyme separation using two complementary techniques. In the next section we review briefly how affinity precipitation technique was integrated with other conventional techniques to provide successful alternative routes not only for the separation of proteins but also useful in different practical applications.

1.8.6 Affinity Precipitation – An Integrated Approach with other Techniques

1] Affinity precipitation integrated with two phase partitioning – As discussed already aqueous two phase system provides extraction of a desired protein in the top phase and cell debris remains in the bottom phase, but such a simple partitioning lacks the selectivity during separation. Integrating this technique with affinity precipitation could provide such selectivity. Moreover the separation of a target protein from the phase forming polymer could be achieved if the polymer is stimuli responsive. This is illustrated in following examples.

In the early development of such integrated techniques Hughes and Lowe (1988) provide an elegant solution by using acrylic acid based smart polymers as one of the phase forming polymer to separate various proteins. Harris et al (1991) further modified this approach. They used a random copolymer of ethylene and propylene oxide (50:50) with dextran or hydroxypropyl starch as two immiscible phases at 40 ^{0}C in presence of 0.2 M sodium sulfate. The 3-phosphogluco kinase enzyme from yeast homogenate could be preferentially partitioned in top hydrophobic phase while cell debris and other proteins accumulated in the bottom phase. This resulted in 60-80 % recovery of enzyme by double extraction, which is comparable to the results obtained by affinity chromatography.

It is well known that the triazine dye (Procion yellow HE-3G) has affinity for glucose-6-phosphate dehydrogenase. Alred et al (1992) conjugated this dye to ethylene oxide : propylene oxide copolymer and the resulting affinity polymer was added in trace quantities (0.5 %) to the top phase. Now, the enzyme could be selectively partitioned to top phase which otherwise could have been partitioned to bottom phase with cell debris in absence of affinity polymer. This yields 80 % recovery of enzyme from top phase.

To enhance the product yield further, a strong interaction between antigen and antibody was exploited. The human IgG was coupled to Eudragit S-100 and mixed with PEG 8000, Reppal PES 200, 0.4 M potassium phosphate buffer and recombinant *Escheria coli* cell homogenate (expressing protein A) to create a two phase aqueous system. The affinity macroligand complex with protein A (95 % of total present) moved to top phase along with PEG. The top phase was separated and Eudragit-IgG-protein A complex was recovered from this phase by precipitating at pH 4.5. Protein A was dissociated from the precipitated complex by glycine-HCl buffer (pH 2.5). The protein A obtained with 80 % yield. But, when the same polymer was reused in the next cycle only 80 % of protein A moved to the top phase. The 20 % loss was caused by the denaturation of IgG and/or incomplete elution of protein A. Thus, these results demonstrate that the use of strong affinity interactions will not always gives high yields [Kamihara et al (1992)]. The only advantage of this process is the feasibility of continuous extraction of protein A in presence of cell debris.

2] Affinity precipitation operated in chromatography mode – Poly (NIPAM) was chemically attached to the porous glass beads that were used for resolving different M.W. dextrans using gel permeation chromatography. With increase in temperature above

LCST the conformation of NIPAM changes from loose coil structure to compact polymer globule thereby it reduces the effective pore size of the glass beads, which changes the elution profile of dextran [Gewehr et al (1992)]. A similar temperature effect was seen when crosslinked poly (NIPAM) beads were used as a matrix for gel permeation chromatography [Park and Hoffman (1994)].

An interesting example in which the use of only temperature shift as an eluting agent without changing the buffer composition of the mobile phase is depicted in dye affinity chromatography column. The blue sepharose (sepharose chemically bound to cibacron blue) was used in affinity chromatography. Another polymer, poly (N-vinyl caprolactam) is known to interact efficiently with the cibacron blue. Cibacron blue exhibits affinity for the nucleotide dependent dehydrogenase enzymes. The LCST of poly (N-vinyl caprolactam) is 35 ^{0}C. Above this temperature it is in compact globule conformation, which reduces its effective surface area for binding with dye molecules. This leads to a reduction in number of dye molecules bound to the polymer on blue sepharose column. Then porcine muscle homogenate containing LDH was applied to the column. Now, LDH will have a better access towards cibacron blue and bind strongly to the column. The unwanted proteins are washed with 0.1 M KCl. Subsequently, the temperature of the column is decreased to room temperature, which converts poly (N-vinyl caprolactam) polymer from globule to expanded coil structure. The expanded coils will compete with LDH molecules for cibacron blue on the column. At a certain temperature the strength of interaction between expanded coils of the polymer with cibacron blue surpasses the interaction between LDH bound to the column. Thereby,

enzyme starts eluting from the column. It gives 90 % recovery of LDH [Galaev et al (1994)].

3] Affinity precipitation used for cell detachment – The changes in hydrophobicity of terminally grafted poly (NIPAM) with temperature was exploited for detaching the cultured mammalian cells from dishes. Bovine endothelial cells and rat hepatocytes cells were grown at 37 ^{0}C on polystyrene dishes with poly (NIPAM) grafted on it. After 2 days, the temperature was lowered at 10 ^{0}C for 30 minutes without changing the medium, the cells gradually detached from the surface. Cells recovered by this method maintained substrate adhesion, growth and secretion activities identical to those found in primary cultured cells, whereas, the conventional trypsinization method used to detach cells exhibit compromised functions [Okano et al (1993); Yamada et al (!990)].

1.8.7 Pre-requisite for an "Ideal Polymer" for Affinity Precipitation

After discussing the various possibilities of using affinity precipitation in conjunction with other techniques used for different applications. It would be appropriate to summarize the prerequisites for design and applications of new polymeric ligands to be used in affinity precipitation –

a) They must contain reactive functional groups to which affinity ligand could be attached.

b) The binding efficiency of the affinity ligand to the target protein should be high when conjugated with the polymer as well.

c) The polymer should not nonspecifically interact and coprecipitate impurities thus lowering the affinity ligand population available for interaction with the target protein.

d) The phase separation of the polymer should be complete and precipitation of polymer should be quantitative. The precipitated polymer should be in the form of small particles, which provides a large contact surface area with water, thereby increasing the chances of exposing bound protein to surrounding water. This is essential to maintain the enzyme in its active form during precipitation.

e) The phase separation should occur with a small change in external conditions (stimuli) of the medium. As, synthetic polymers exhibit a narrower molecular weight distribution they are better suitable than natural polymers for this purpose.

f) The precipitating polymeric phase should not be too hydrophobic, because this results in an undesirable environment for the isolated protein molecules which may cause their inactivation or "push them out" from the complex with macroligand [Galaev and Mattiasson (1993b)]. In addition to this if polymer phase is too hydrophobic, it will result in significant non-specific uptake of contaminating proteins, leading to a lower degree of purification and the output of the desired protein [Linne-Larsson and Mattiasson (1994)].

g) The precipitated polymer should be compact for its easy separation by low-speed centrifugation or filtration. The entrapped impurities inside precipitated gel should be minimum.

h) Polymer should be easily solubilizable after the precipitate is formed.

i) The precipitation-solubilization cycles must be repetitive without losing much of the efficiency of polymer for protein purification.

j) Polymer should be available inexpensive [Galaev et al (1996); Galaev and Mattiasson (1997); Gupta and Mattiasson (1994)].

Hubert and Dellacherie (1980) made following pertinent observations concerning the design of hetero-bifunctional affinity polymers.

a) The conjugation of affinity ligands to polymer should not alter solubility of the latter substantially. However, if the affinity of the ligand is poor for the target protein, one may wish to conjugate a large number of ligands to the polymer. In this situation, if the affinity ligand is highly hydrophobic, care must be taken during conjugation of this ligand to polymer so that the polymer will not become completely insoluble in aqueous medium.

b) The binding constant of the affinity ligand to the protein should not be adversely affected when the ligand is conjugated to the polymer. If the binding strength of the ligand decreases after incorporation in the polymer, then one has to load more affinity ligand in the polymer or use a different affinity ligand with high binding constants for the desired protein. The latter alternative will lead to design of new affinity ligands whereas, the prior option will cause i) Steric hindrance that leads to inaccessibility of affinity ligand to desired protein and ii) Crowding of affinity ligand on polymer matrix that decreases its efficiency of binding to the protein.

So far we have reviewed various separation techniques that use the basic principle of affinity between ligand and protein for the separation. Now we describe a novel technique that has emerged as a powerful tool for separating biomolecules based on the shape and size of crosslinked polymer matrices used therein.

1.9.0 Molecular-Imprinting

In 1972, Wulff and Sarhan developed molecular imprinting approach based on a well-known concept in biology i.e. elicitation of monoclonal antibody against a specific

antigen is a highly specific and selective process. In this approach a polymeric recognition system was synthesized which provides selectivity for binding with different classes of molecules e.g. dyes, sugars, peptides and proteins/enzymes depending upon the initial template used during synthesis of imprinted polymer. The technique of molecular-imprinting involves three steps and is shown schematically in Figure 1.8.

1. Specific and definable interactions between the monomer(s) and the print molecule are formed. These interactions are responsible for the recognition of the print molecule by the molecularly imprinted polymer (MIP). These interactions can either be non-covalent e.g. ionic and hydrogen bonding or reversible covalent type as in the case of boronate esters. We will consider only the first type i.e. non-covalent interactions since these are mainly used in bioseparations. In non-covalent imprinting the polymerization was carried out by simply mixing the print molecule with a suitable mixture of monomers in an appropriate solvent [Wulff (1986)].

2. The mixture of print molecule and the monomer(s) is polymerized in the presence of a high percentage of crosslinker. The resulting polymer is rigid and insoluble. A high concentration of crosslinker is necessary to preserve the complementarity between the polymer and the print molecule that is created in the polymeric net work during polymerization reaction.

3. The print molecule is removed by extraction to create an "induced molecular memory" i.e. **imprints**, which are complementary in both shape and chemical functionality to the print molecule. This imprinted polymer is allowed to incubate with substrates of similar structure to the print molecule. It was found that the imprint will preferentially bind to the print molecule [Andersson et al (1993)].

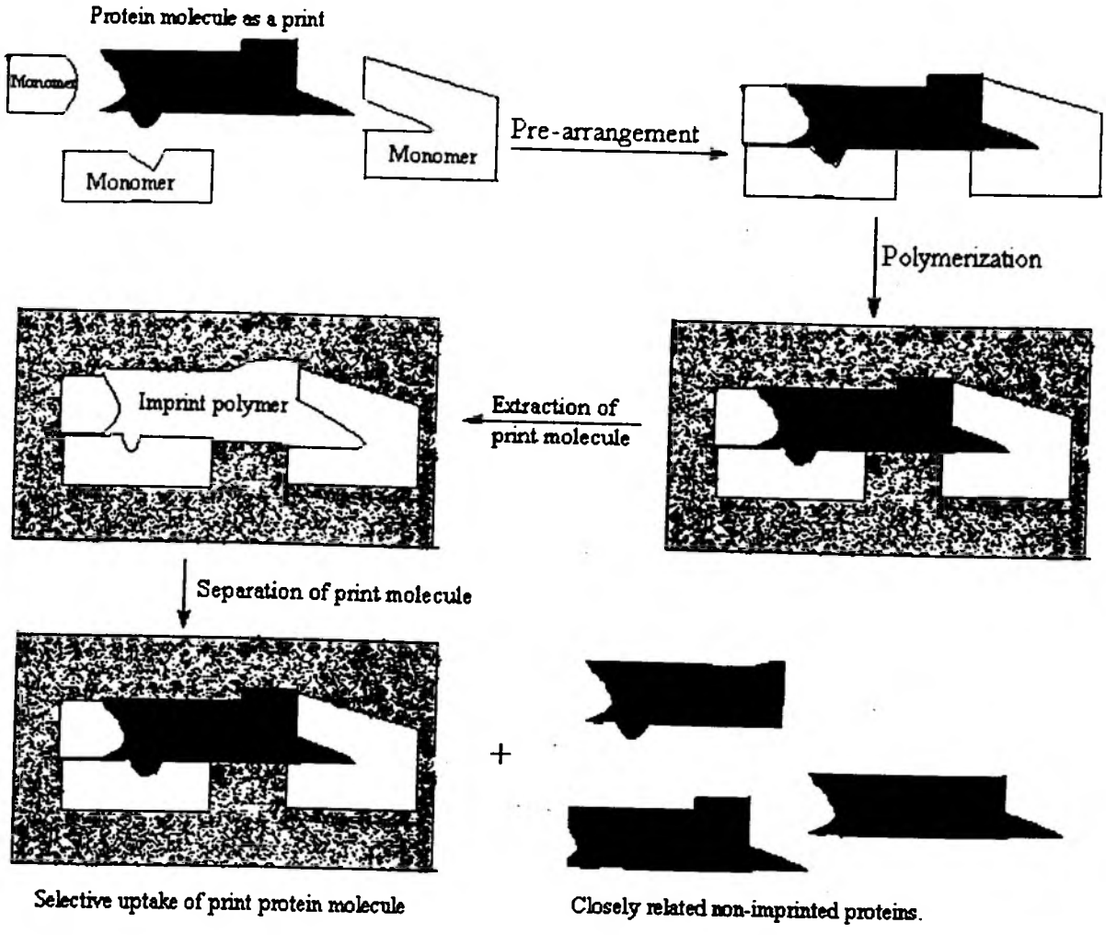


Figure 1.8: Schematic representation of molecular imprinting approach.

MIPs have been applied in a broad range of applications from structural studies on ligand-receptor interactions to use as selective binding matrices in detection, separation and purification of various organic as well as biomolecules [Andersson et al (1994)]. This discussion is confined only to protein/enzyme separations using molecularly imprinted polymers.

1.9.1 Selective Recognition of Proteins/Enzymes by MIPs

Glad et al (1985) demonstrated the separation of protein i.e. transferrin from BSA using non-covalent surface imprinting approach. It involved mixing a boronate silane monomer i.e. N-{2-hydroxy-3-[3-(tripropoxyxysilyl)propoxyl] propyl}-3-aminobenzene boronicacid propyl ester with other silane monomers e.g. phenyltriethoxysilane and N-2-aminoethyl-3-aminopropyltrimethoxysilane in presence of a crosslinker i.e. bis (2-hydroxy-ethyl) aminopropyltrimethoxysilane comprising the print molecule i.e. transferrin. The polymerization was carried on the surface of porous silica. It was found that the boronate derivative interacts with the glyco-moiety of the protein, which accounted for most of the observed recognition of transferrin by MIPs. The relative retention (elution volume for transferrin / elution volume for BSA) of transferrin was maximum (2.16) for the transferrin imprinted polymer containing boronate silane monomer. For the same polymer in the absence of boronate silane monomer, the relative retention decreased to 1.28. BSA imprinted polymer with or without boronate silane monomer exhibited no significant difference in relative retention (1.22 and 1.16 respectively). This demonstrates that incorporating an affinity ligand along with other monomers prior to polymerization contributes to increased affinity for the print molecule by resulting MIP as compared to polymer prepared in the presence of randomly

distributed affinity ligands. This method provides following advantages. The affinity polymers prepared by this method required minimum incorporation of affinity ligands thereby reduce the risk of non-specific adsorption of other proteins by the polymer. Secondly, this method saves costly affinity ligands normally used in affinity chromatography.

Recently, Venton and Gudipati (1995) synthesized the poly-siloxane polymer with entrapped urease and invertase respectively. Subsequently, the enzymes were removed from finely powdered polymers by contacting with pronase enzyme (a mixture of non-specific proteases), which could degrade the entrapped enzyme. The resulting polymers have specific cavities complementary to respective enzymes. These polymers were able to recognize preferentially their print enzyme molecules i.e. either urease or invertase. In this investigation no affinity ligand was incorporated in the polymer. These polymers recognizing enzymes have a potential use as affinity phases and biosensors for the isolation and detection of high-value bio-products.

Later Kempe et al (1995) synthesized an adsorbent for Rnase A enzyme using surface imprinting procedure based on the metal coordination approach. This enzyme has two surface exposed histidines capable of coordinating with Cu^{+2} ions. The porous silica support was derivatized with 3- (trimethoxysilyl) propyl methacrylate and mixed with Rnase A, Cu^{+2} and the metal chelating monomer N-(4-vinyl)-benzyl iminodiacetic acid which after polymerization yield the surface imprinted polymer. This polymer was used to separate lysozyme and Rnase A in high performance liquid chromatography mode. An increased Rnase A separation (K' = 5.79) was obtained on the Rnase A imprinted polymer as compared to the control polymer which was imprinted for the BSA. It was

also shown that the Rnase A imprinted polymer devoid of Cu^{+2} did not exhibit any separation. Thus it was demonstrated that not only the presence of imprint molecule but Cu^{+2} ions are also equally important for determining the selectivity of such MIPs.

Glucose oxidase (GO) was another enzyme, which was separated by the surface imprinting approach. A surface modified silica support was copolymerized by free radical polymerization in the presence of GO, acrylic acid derivatives, N, N'-1,2 dihydroxy-ethylene-bis(acrylamide), N,N' -(methylene)-bisacrylamide. The print molecule was extracted out. The resulting imprinted polymer exhibited uptake of 5.57 μg GO / g of polymer as compared to 3.00 μg GO / g of control polymer which was imprinted for BSA. Thus, the GO imprinted polymer showed an 18.5 % specific binding capacity for GO. Although, this value is considerably lower as compared to the theoretical maximum binding capacity which is 93 μg GO / g for the imprinted polymer, these results showed that there is a great scope to increase the binding specificity, strength and capacity of the surface imprinted polymers for the uptake of biomolecules [Burow and Minoura (1996)].

Recently, lightly crosslinked polyacrylamide gels comprising large pores were used to specifically adsorb the proteins in a column mode by molecular imprinting approach. By this method three different protein namely human growth hormone, ribonuclease and myoglobin from horse could be selectively separated. In this method affinity monomers were not used during preparation of the gels and a lower concentration of crosslinker was added during polymerization so as to obtain loosely crosslinked porous beads from which the high M.W. print molecules could be easily extracted. The rebinding of the protein molecule with MIPs was achieved by a large number of weak bonds instead of conventional few strong electrostatic bonds. The imprinted polymer exhibit

highest selectivity for the respective print molecule e.g. imprinted polymer for horse myoglobin exhibits selective uptake of horse myoglobin whereas the whale myoglobin did not picked up at all. This selectivity is note worthy as these proteins have an identical amino acid sequence and three-dimensional structure. Therefore, the columns prepared by these gels can be used to remove traces of different proteins contaminating a bulk protein sample i.e. negative purification. The problem with this system is that the gel synthesized is soft which restricts the flow rates. Also, the capacity of the column is very low in high pressure chromatography mode [Hjerten et al (1997)].

MIP approach provides many advantages such as easy recipe for polymerization, reusability and high storage life of the polymers, and can withstand the harsh external conditions such as high temperature, pH, ionic strength and pressure applied. However there is a considerable scope to improve their performance. The major lacunae are low capacity of the MIPs for separation of high M.W. biologicals and comparatively low selectivity to differentiate between closely related enzymes. Therefore, in the present investigation an attempt has been made to address these problems.

1.10.0 Concluding remarks

The above review on the bioseparations reveals that affinity based separations is most suitable as they exhibit high selective separation capacity for a given enzyme. Amongst affinity separation techniques, affinity precipitation demonstrates a number of advantages over affinity chromatography, affinity two phase separations and affinity ultrafiltration to name a few binding of enzyme to affinity macroligand occurs in homogeneous solution, easy separation, scale up of the process and the recyclability of specially tailored affinity polymers. The smart polymer plays an important role in affinity

precipitation. The success of the technique largely depends on the design of a suitable affinity polymer. Various smart polymers, although useful in affinity precipitation, suffer from the following limitations. 1) When an affinity ligand is covalently attached to the polymer, its affinity towards a given enzyme decreases many folds. This is due to the steric hindrance of long coiled polymer chains for the free access of enzyme towards the attached ligand. In affinity chromatography this problem has been partly circumvented by using a long flexible spacer between the ligand and the support. However in soluble polymers such studies have not been done. 2) Another problem with smart polymers is that when, a large number of ligands are attached to the same polymer chain it leads to the crowding effect i.e. a significant fraction of the ligands remain unutilized for binding with enzyme molecules. This problem needs to be addressed by designing novel polymers that will provide not only higher accessibility but also the enhanced efficiency of available ligands. 3) The stability of affinity ligands conjugated to the smart polymers also needs to be improved. In case of natural macromolecular ligands like soybean trypsin inhibitor and chitosan are prone to undergo denaturation and microbial attack which subsequently reduces their performance, efficiency and stability. Thus it is necessary to design stable small M.W. synthetic ligands and replace them in the place of above-mentioned natural macroligands. 4) It is also imperative to compare the efficacy of such new synthetic ligands with conventional natural ligands used in affinity precipitation of enzyme. This will logically assess the structure-property and performance relationship of the new ligands.

Thus any new investigation in affinity precipitation should be directed towards the design and synthesis of new smart polymers and polyligands that would help eliminate the above mentioned problems.

Molecular imprinting has also emerged as a new attractive technique for separating biomolecules. The MIPs are robust, particulate materials that are ideal for use in columns and beds, comprising different biologicals. However, MIPs rely on weak hydrogen bonding interactions with the imprinted protein molecules for their separation from the mixtures. Naturally, the selectivity and capacity of MIPs has been very poor so far. Thus one needs to employ the strong and exclusive affinity interactions in MIPs so as to prepare receptors that will have high capacity and specificity for the imprinted enzyme.

Chapter 2

Objectives and Scope of the work

The review of literature on affinity based separations has revealed the unresolved problems in this area. This work is directed towards the design, synthesis and evaluation of novel thermoprecipitating smart polymers and polyligands that will overcome some of the problems in affinity precipitation. Therefore the present investigation has been undertaken with the following objectives.

1) To design and synthesize new thermoprecipitating affinity polymers that will provide flexible spacers for the attachment of affinity ligands. To evaluate these polyligands for their binding efficacy with the enzymes and highlight the enhancement in binding due to the spacers.

2) To covalently attach increasing numbers of ligands to the above spacer containing polymers and evaluate them for the crowding effect. Due to the spacer between polymer and the ligand, the ligands in this case are expected to overcome the crowding effect that is observed in conventional smart polymers devoid of spacers.

3) To synthesize new stable monomeric ligands and polymerize them with suitable comonomers for the recovery of lysozyme, an industrially important enzyme.

4) To study the effect of hydrophilicity/hydrophobicity of the spacer on the enzyme binding.

5) To synthesize polyligands which exhibit enhanced binding to lysozyme as compared to their respective affinity ligands. These studies are expected to reveal the importance of microenvironment of the spacer and the enzyme active site in determining the final efficacy of the synthetic ligands as well as polyligands.

6) To evaluate the efficacy of the overall recovery process these new synthetic polyligands were compared with the natural ligand i.e. N-acetylglucosamine.

7) To compare the efficacy of these affinity polymers with acidic polymers used in lysozyme recovery and to assess the performances of exclusive affinity interactions with electrostatic interactions used in conventional ion-exchangers and build the framework for further research in designing new smart polymers and polyligands for affinity precipitation.

8) To synthesize molecularly imprinted polymers using affinity ligand as the functional monomer. To evaluate specificity and capacity of such affinity-imprinted polymers *vis-a-vis* imprinted polymers, which are synthesized using non-affinity based functional monomers exhibiting hydrogen bonding interactions only.

Chapter 3

ENHANCING LIGAND-PROTEIN BINDING IN AFFINITY THERMOPRECIPITATION: ELUCIDATION OF SPACER EFFECTS

3.0.0 Introduction

As mentioned in the previous chapter, the first objective of this work was to conceptualize and synthesize new thermoprecipitating polymers that would not adversely affect the binding efficiency between the affinity ligand and the enzyme, when the ligand was incorporated in the polymer. In this chapter the synthesis and evaluation of a new family of thermoprecipitating copolymers of N-isopropylacrylamide (NIPAM) and N-acryloyl amino acids is described. The pendant carboxyl group of the spacer was conjugated with the inhibitor specific for trypsin. These polymers are shown to exhibit features such as increased binding for trypsin, elimination of the crowding effect and ability to control the lower critical solution temperature (LCST). These polymers are expected to be useful for the recovery of a wide range of enzymes. Amongst various methods used in enzyme recovery, affinity - based separations offer high specificity and selectivity [Gupta and Mattiasson (1994)]. The affinity chromatography technique, although the most extensively investigated, suffers from a number of limitations as mentioned in chapter 1. To overcome these limitations, a number of affinity-based purification methods, such as affinity partitioning, affinity ultrafiltration and affinity precipitation have been developed [Mattiasson and Kaul (1993); Chen (1990); Galaev and Mattiasson (1993a); Pecs et al (1993)]. Of these, affinity precipitation appears most attractive, as it is easy to scale up and is the least equipment intensive [Morris et al (1992); Senstad and Mattiasson (1989)].

In affinity precipitation, the ligand used could be either homo-bifunctional or hetero-bifunctional. A detailed discussion of both types of ligands is already given in chapter 1. Amongst both hetero-bifunctional ligands offers better flexibility in operation as the enzyme-ligand binding takes place in a homogeneous solution [Morris et al

(1992)]. Therefore, it was suggested that spacers are not needed for hetero-bifunctional affinity precipitation [Luong et al (1988); Schneider et al (1981)]. Yet, an examination of the literature reveals that the inhibition constant (K_i) of free ligand increased i.e. para aminobenzamidine and trypsin binding decreased 30-200 fold when the ligand was bound to the polymers. K_i also increased with the crowding of the ligands on the polymer [Male et al (1987); Galaev and Mattiasson (1993b); Luong et al (1988); Pecs et al (1991)]. This was attributed mainly to the steric considerations, which limited the accessibility of the protein to the polymer bound ligand.

In order to enhance the yields in affinity precipitation, it is desirable to enhance the enzyme-ligand interactions. Incorporation of a spacer in thermoprecipitating polymers is expected to enhance these interactions [Mattiasson and Kaul (1993); Gupta and Mattiasson (1994)]. This has been demonstrated in affinity chromatography and homo-bifunctional affinity precipitation. But such a systematic study in the case of hetero-bifunctional affinity precipitation is not yet reported. In the present work we selected trypsin – para aminobenzamidine as a model system for investigation. The incorporation of a spacer resulted in decrease in K_i and enhanced the recovery and specific activity of trypsin. K_i decreased further with the incorporation of a hydrophobic comonomer in the polymer. The crowding effect was eliminated by the use of low molecular weight polymers. Finally, the recovery of activity of trypsin from a mixture of trypsin and chymotrypsin is shown.

3.1.0 Experimental section

3.1.1 Materials

Acrylic acid, 6-amino caproic acid (6ACA), para aminobenzamidine dihydrochloride (PABA.2HCl), 1-cyclohexyl -3 (2- morpholinoethyl) carbodiimide

metho-p-toluenesulfonate (CMC), N-isopropylacrylamide (NIPAM), and N-isopropyl methacrylamide (NIPMAM) were obtained from Aldrich. Trypsin (type III X, specific activity 1300 BAPNA units/mg trypsin), chymotrypsin (type II X, specific activity 500 BTPNA units/mg chymotrypsin), N-benzoyl-DL-arginine-para-nitroanilide (BAPNA) and N-benzoyl-L-tyrosine-para-nitroanilide (BTPNA) were obtained from Sigma. N-butylacrylamide was from Polysciences. 4-amino butyric acid (4-ABA), β-Alanine, glycine, thionyl chloride, ammonium per sulfate, tetramethylethylenediamine (TEMED), acrylamide, acetyl chloride, butyryl chloride, etc. were obtained from local suppliers. The solvents used in this work were of analytical grade.

3.1.2 Instrumentation

^1H NMR spectra were recorded on Varian 200 MHz spectrometer. IR spectra were recorded on Perkin Elmer 1600 FT-IR spectrometer. Electronic absorption measurements were done on Shimadzu UV1601 spectrophotometer. Melting points were recorded on Mettler melting point apparatus.

3.1.3 Methods

3.1.3.1 Synthesis of N- acryloyl para-aminobenzamidine (Ac. PABA)

Acryloyl chloride was synthesized by the reaction of thionyl chloride and acrylic acid [Lele (1997)]. N-acryloyl para aminobenzamidine (Ac. PABA) was synthesized by the reaction of acryloyl chloride and PABA. 2HCl [Male et al (1987); Pecs et al (1991)].

3.1.3.2 Synthesis of N-acryloyl 6 amino caproic acid (Ac. 6ACA) *(spacer)*

Ac.6ACA was synthesized as described below. In a 250 ml beaker equipped with dropping funnel and pH meter, 13.16 g 6ACA (0.1 M), 4 g NaOH (0.1 M) and 80 ml water were placed to obtain a clear solution (pH 13). This was stirred at 5-10 ^{0}C on a

magnetic stirrer. 9 ml acryloyl chloride (0.11 M) in 10 ml dichloromethane was added dropwise to the above solution over a period of 45-60 minutes. During this period the pH of the reaction mixture was maintained in the range 7.4-7.8 by adding 10 M NaOH solution. After the addition was over and the pH of the reaction mixture was steady, unreacted acid chloride was extracted in 100 ml ethyl acetate. The clear aqueous solution was acidified to pH 5.0 by adding concentrated HCl and the product was extracted in ethyl acetate (3 × 100 ml). The organic layer was dried on anhydrous sodium sulfate and concentrated under vacuum. The viscous liquid obtained was poured in 500 ml petroleum ether. This was kept in a refrigerator for 4 to 5 days with occasional scratching in order to obtain a solid product. Yield - 56 %. M.P. 77-78 ^{0}C.

IR (nujol)

1620 cm^{-1} (acrylic double bond), 1650 cm^{-1} (amide carbonyl stretching).

^{1}H NMR (D_2O)

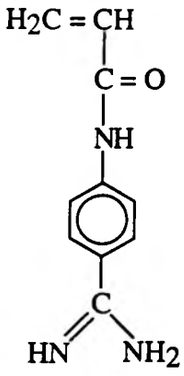
1.4 δ 2H multiplet [- $(CH_2)_2$ - $C\underline{H}_2$)], 1.7 δ 4H multiplet [(- $C\underline{H}_2$ -)$_2$], 2.3 δ 2H triplet (- $C\underline{H}_2COO$), 3.3 δ 2H triplet (- $C\underline{H}_2$ - NH), 5.6 δ 1H multiplet (- CH_2 - $C\underline{H}$ -), 6.2 δ 2H doublet (- $C\underline{H}_2$ - CH -).

All other N-acryloyl amino acids were similarly synthesized. Only in the case of N-acryloyl β-alanine and N-acryloyl glycine the products were obtained by precipitation of aqueous solutions in acetone and purified by reprecipitating from methanol into acetone [Lele (1997)]. The acryloyl monomers synthesized are shown in Figure 3.1.

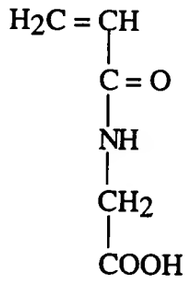
N-acryloyl 4 amino butyric acid (Ac. 4ABA)

Yield - 55 %, M.P. 90-91 ^{0}C.

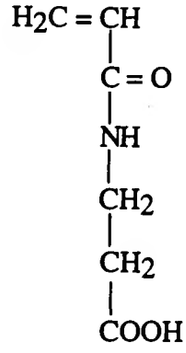
IR (nujol)



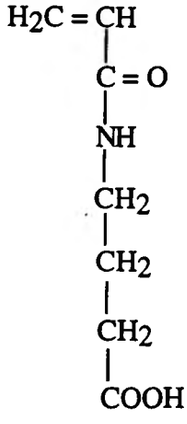
Ac. PABA



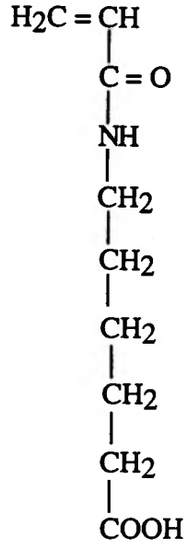
Ac. Gly.



Ac. β Ala



Ac. 4ABA



Ac. 6ACA

Figure 3.1 : Acryloyl spacer monomers.

1643 cm^{-1} (amide carbonyl stretching), 1700 cm^{-1} (carbonyl stretching of carboxyl), 3200 cm^{-1} (-NH and -OH stretching).

^{1}H NMR (DMSO d_6)

1.6 δ 2H triplet (- C<u>H</u>$_2$ - of C_3 of 4 ABA), 2.2 δ 2H triplet (- C<u>H</u>$_2$ - of C_4 of 4 ABA), 3.1 δ 2H triplet (- C<u>H</u>$_2$ - of C_2 of 4ABA), 3.8 δ 1H triplet (- N<u>H</u>), 5.5 δ 1H double doublet (- C<u>H</u> - CH_2), 6.2 δ 2H multiplet (- C<u>H</u>$_2$ - CH -), 8.1 δ 1H singlet (- COO<u>H</u>).

N-acryloyl - β alanine (Ac. β Ala)

Yield - 40 %, M.P. 128-130 ^{0}C.

IR (methanol)

1620 cm^{-1} (acrylic double bond), 1660 cm^{-1} (amide carbonyl), 1720 cm^{-1} (acid carbonyl).

^{1}H NMR (D_2O)

2.5 δ 2H triplet (- NH - C<u>H</u>$_2$ - of β Ala.), 3.3 δ 2H triplet (- C<u>H</u>$_2$ - COOH of β Ala.), 5.7 δ 1H double doublet (- C<u>H</u> - CH_2 -), 6.2 δ 2H doublet (- C<u>H</u>$_2$ - CH -).

N-acryloyl glycine (Ac. Gly)

Yield - 35 %, M.P.127-128 ^{0}C.

IR (methanol)

1623 cm^{-1} (acrylic double bond), 1658 cm^{-1} (amide carbonyl), 1722 cm^{-1} (acid carbonyl).

^{1}H NMR (D_2O)

3.3 δ 2H singlet (- NH - C<u>H</u>$_2$ - of Gly.), 5.7 δ 1H double doublet (- C<u>H</u> - CH_2 -), 6.2 δ 2H multiplet (-C<u>H</u>$_2$ - CH -).

3.1.3.3 Synthesis of NIPAM - SPACER copolymers

Typically, 0.01 M of the monomer comprising the spacer and 0.09 M of NIPAM were dissolved in 50 ml water. To this, 10 % w/w ammonium per sulfate was added and

the solution was purged with nitrogen for 15 min. Polymerization was initiated by adding 500 µl of TEMED and was allowed to proceed at 37 °C for 18 hours. The polymer was precipitated by increasing the temperature of the solution above its lower critical solution temperature (LCST). The polymer was washed twice with cold double distilled water and once with cold Tris-HCl buffer, pH 8.1 and dried under vacuum at room temperature. The same procedure was used for the synthesis of - 1) Poly (N-isopropylacrylamide –co- N-acryloyl glycine) i.e. poly (NIPAM -co- Ac. Gly.). 2) Poly (N-isopropylacrylamide – co- N-acryloyl β alanine) i.e. poly (NIPAM -co- Ac. β Ala.). 3) Poly (N-isopropylacrylamide –co- N-acryloyl 4 amino butyric acid) i.e. poly (NIPAM -co- Ac. 4ABA.). 4) Poly (N-isopropylacrylamide –co- N-acryloyl 6 amino caproic acid) i.e. poly (NIPAM -co- Ac. 6ACA.).

poly (NIPAM -co- Ac. Gly.).

^{1}H NMR (DMSO d_6)

1.0 δ singlet [$(C\underline{H}_3)_2$ - CH - of NIPAM], 1.5 δ singlet [- C$\underline{H}$ - $(CH_3)_2$ of NIPAM], 2.5 δ singlet (- NH - $C\underline{H}_2$ - of Gly.), 3.4 δ broad singlet (- $C\underline{H}_2$ - CH - of polymer), 3.9 δ singlet (- C$\underline{H}$ - CH_2 - of polymer).

poly (NIPAM -co- Ac. β Ala.)

^{1}H NMR (DMSO d_6)

1.0 δ doublet [$(C\underline{H}_3)_2$ - CH - of NIPAM], 1.4 δ multiplet [- C$\underline{H}$ - $(CH_3)_2$ of NIPAM], 2.0 δ triplet (- NH - $C\underline{H}_2$ - of β Ala.), 2.5 δ triplet (- $C\underline{H}_2$ - COOH of β Ala.), 3.4 δ broad singlet (- $C\underline{H}_2$ - CH - of polymer), 3.8 δ singlet (- C$\underline{H}$ - CH_2 - of polymer).

poly (NIPAM -co- Ac. 4ABA).

^{1}H NMR (DMSO d_6)

1.0 δ doublet [($C\underline{H}_3$)$_2$ - CH - of NIPAM], 1.5 δ multiplet [- $C\underline{H}$ - (CH_3)$_2$ of NIPAM], 2.0 δ multiplet (- $C\underline{H}_2$ - of C_3 of 4 ABA), 2.25 δ multiplet (- $C\underline{H}_2$ - of C_4 of 4 ABA), 2.4 δ triplet (- $C\underline{H}_2$ - of C_2 of 4 ABA), 3.5 δ broad singlet (- $C\underline{H}_2$ - CH - of polymer), 3.8 δ multiplet (- $C\underline{H}$ - CH_2 - of polymer), 7.0 δ singlet (- $N\underline{H}$), 8.0 δ singlet (- $COO\underline{H}$).

poly (NIPAM -co- Ac. 6ACA).

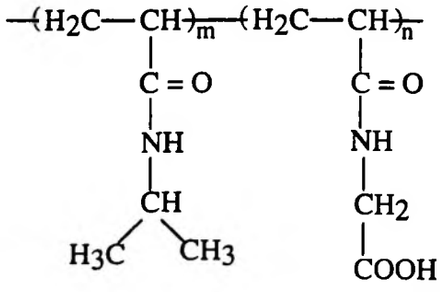
^{1}H NMR (DMSO d_6)

1.0 δ broad singlet [($C\underline{H}_3$)$_2$ - CH - of NIPAM], 1.5 δ multiplet [- $C\underline{H}$ - (CH_3)$_2$ of NIPAM], 2.0 δ singlet (- $C\underline{H}_2$ of C_4 of 6ACA), 2.2 δ singlet (- $C\underline{H}_2$ of C_5 of 6ACA), 2.4 δ singlet (- $C\underline{H}_2$ of C_3 of 6ACA), 2.5 δ singlet (- $C\underline{H}_2$ of C_6 of 6ACA), 2.7 δ singlet (- $C\underline{H}_2$ of C_2 of 6ACA), 3.5 δ broad singlet (- $C\underline{H}_2$ - CH - of polymer), 3.9 δ multiplet (- $C\underline{H}$ - CH_2 - of polymer), 7.3 δ singlet (- $N\underline{H}$), 8.0 δ singlet (- $COO\underline{H}$).

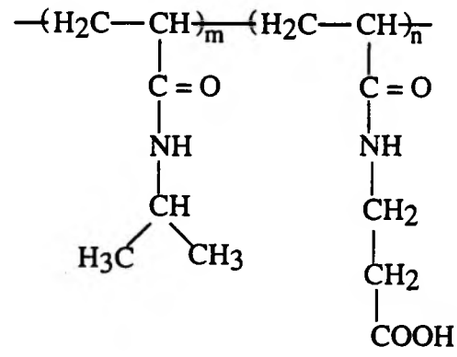
The polymers comprising spacers of varying chain length are shown in Figure 3.2.

3.1.3.4 Conjugation of para-aminobenzamidine to carboxyl groups of copolymers

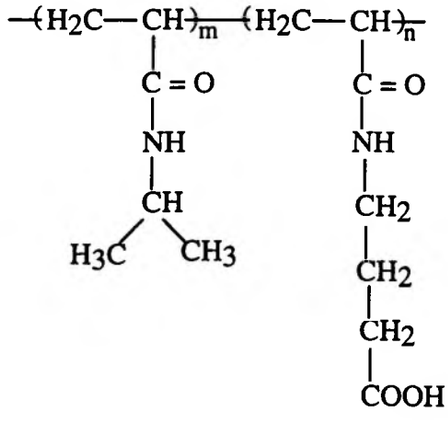
PABA was covalently linked to the pendant carboxyl groups of these polymers via amide bond formation using water soluble carbodiimide [Papisov et al (1985); Szajani et al (1991)]. PABA.2HCl was treated with 40-fold molar excess of sodium acetate in water to free the para-amino groups. Four grams of polymer was dissolved in 40 ml of double distilled water at 4 –10 ^{0}C, and CMC and PABA were added to it. The reaction mixture was stirred at 4 - 10 ^{0}C for 12 hours. The molar ratios of CMC:carboxyl groups and PABA:carboxyl groups were both 10:1. The polymer conjugated with PABA, was precipitated by increasing the temperature above its LCST. The polymer was washed three times with cold double distilled water and once with Tris-HCl buffer (pH 8.1) and



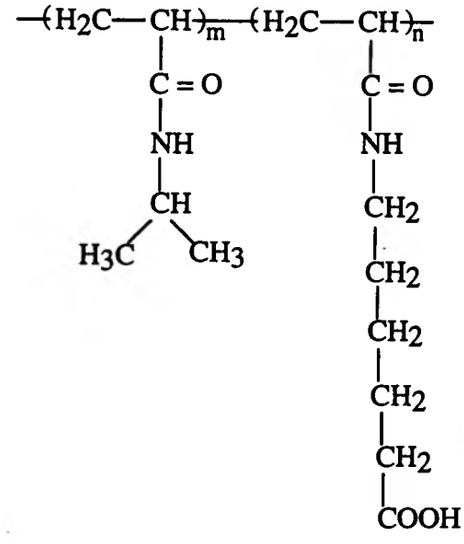
poly (NIPAM -co- Ac. Gly.)



poly (NIPAM -co- Acβ Ala.)



poly (NIPAM -co- Ac. 4ABA)



poly (NIPAM -co- Ac. 6ACA)

Figure 3.2 : Different polymers with spacers of varying chain length m and n are integers greater than 1.

dried under vacuum at room temperature. The structures of copolymers are schematically shown in Figure 3.3. 10 % w/v of stock solutions, of polymers in double distilled water were prepared. The loading of PABA was estimated spectrophotometrically, according to Nguyen and Luong (1989). The relevant data are listed in Table 3.1.

Poly (N-isopropylacrylamide –co- N-acryloyl para aminobenzamidine), poly (NIPAM -co- Ac. PABA).

^{1}H NMR (DMSO d_6)

1.0 δ singlet [$(C\underline{H}_3)_2$ - CH - of NIPAM], 2.5 δ singlet [- C$\underline{H}$ - $(CH_3)_2$ of NIPAM], 3.5 δ broad singlet (- C$\underline{H}_2$ - CH - of polymer), 3.8 δ (- C$\underline{H}$ - CH_2 - of polymer), 7.5 δ double doublet (ring protons of PABA), 7.7 δ double doublet (ring protons of PABA).

Poly (N-isopropylacrylamide –co- N-acryloyl glycyl para aminobenzamidine), poly (NIPAM -co- Ac. Gly. PABA).

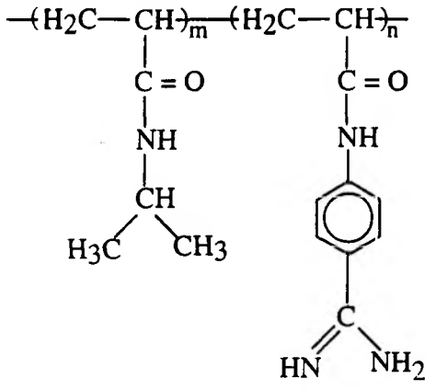
^{1}H NMR (DMSO d_6)

0.9 δ singlet [$(C\underline{H}_3)_2$ - CH - of NIPAM], 1.5 δ singlet [- C$\underline{H}$ - $(CH_3)_2$ of NIPAM], 1.9 δ singlet (- NH - C$\underline{H}_2$ - of Gly.), 3.4 δ singlet (- C$\underline{H}_2$ - CH - of polymer), 3.8 δ singlet (- C$\underline{H}$ - CH_2 - of polymer), 7.3 δ multiplet (ring protons of PABA).

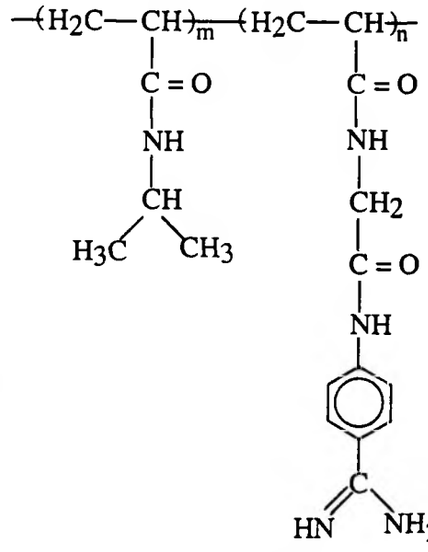
Poly (N-isopropylacrylamide –co- N-acryloyl β alaninyl para aminobenzamidine), poly (NIPAM -co- Ac. β Ala. PABA).

^{1}H NMR (DMSO d_6)

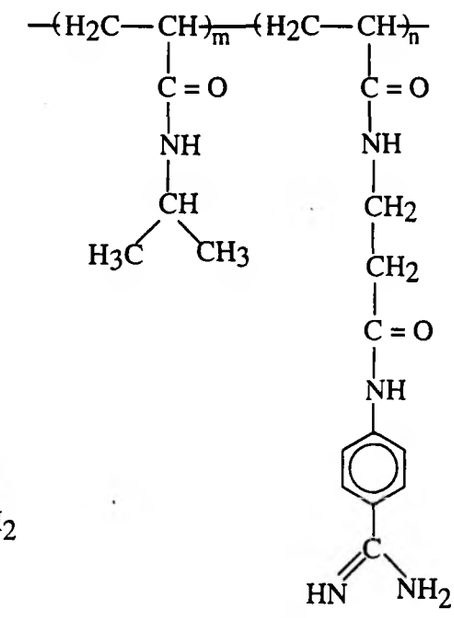
1.0 δ doublet [$(C\underline{H}_3)_2$ - CH - of NIPAM], 1.5 δ multiplet [- C$\underline{H}$ - $(CH_3)_2$ of NIPAM], 2.0 δ triplet (- NH - C$\underline{H}_2$ - of β Ala.), 2.5 δ triplet (- C$\underline{H}_2$ - COOH of β Ala.), 3.5 δ broad singlet (- C$\underline{H}_2$ - CH - of polymer), 3.9 δ singlet (- C$\underline{H}$ - CH_2 - of polymer), 7.18 δ double doublet (ring protons of PABA), 7.4 δ double doublet (ring protons of PABA).



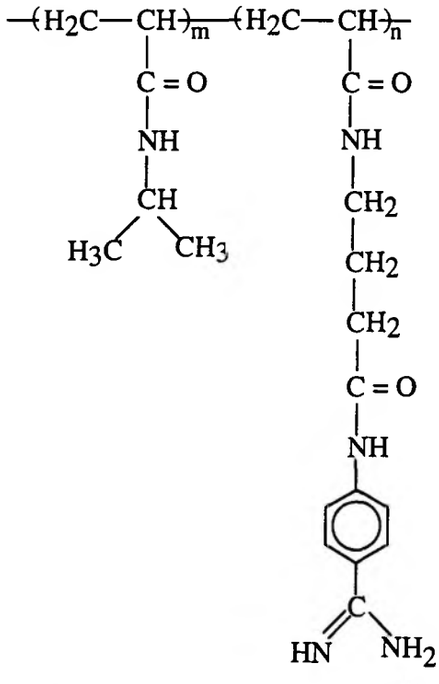
poly (NIPAM -co- Ac. PABA)



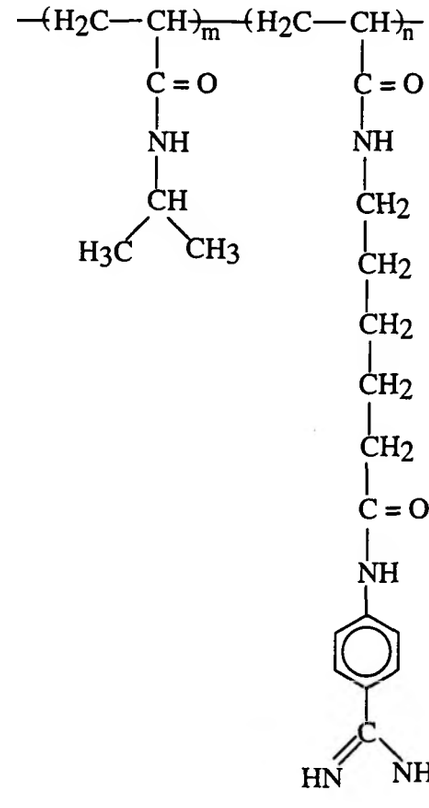
poly (NIPAM -co- Ac. Gly. PABA)



poly (NIPAM -co- Acβ Ala. PABA)



poly (NIPAM -co- Ac. 4ABA PABA)



poly (NIPAM -co- Ac. 6ACA PABA)

Figure 3.3 : Schematic representation of affinity thermoprecipitating polymers. m and n are integers greater than 1.

Table 3.1: Binding between trypsin and PABA conjugates : spacer effect.

No.	Polymer [a]	Moles of carboxyl groups / g polymer	Moles of PABA / g Polymer	LCST of PABA conjugated polymer (^{0}C)	M.W (M_n) [b]	K_i (Moles)[c]
1	Poly (NIPAM - co- Ac. PABA)	------	$2.75 * 10^{-4}$	53	940	$275\pm14 * 10^{-6}$
2	Poly (NIPAM - co- Ac.Gly.PABA)	$9.64 * 10^{-4}$	$2.25 * 10^{-4}$	34	800	$250\pm10 * 10^{-6}$
3	Poly (NIPAM - co- Ac. β Ala. PABA)	$4.10 * 10^{-4}$	$1.50 * 10^{-4}$	33	900	$225\pm12 * 10^{-6}$
4	Poly (NIPAM - co- Ac. 4ABA PABA)	$9.00 * 10^{-4}$	$2.50 * 10^{-4}$	48	960	$110 \pm6 * 10^{-6}$
5	Poly (NIPAM - co- Ac.6ACA PABA)	$9.82 * 10^{-4}$	$2.60 * 10^{-4}$	38	980	$25\pm3 * 10^{-6}$

[a] Molar feed ratio of NIPAM:acryloyl spacer monomer was 9:1 in all cases.
[b] Molecular weights were estimated by intrinsic viscosity method [Fujishige (1987)].
[c] K_i is the average value from three experiments.
(K_i of free PABA = $8 * 10^{-6}$ M and K_i of Ac. PABA = $120 \pm 7 * 10^{-6}$ M)

Poly (N-isopropylacrylamide –co- N-acryloyl 4 amino butyryl para aminobenzamidine), poly (NIPAM -co- Ac. 4ABA PABA).

^{1}H NMR (DMSO d_6)

1.0 δ doublet [$(C\underline{H}_3)_2$ - CH - of NIPAM], 1.7 δ multiplet [- C$\underline{H}$ - $(CH_3)_2$ of NIPAM], 2.0 δ multiplet (- C$\underline{H}_2$ - of C_3 of 4 ABA), 2.3 δ singlet (- C$\underline{H}_2$ - of C_4 of 4 ABA), 3.2 δ singlet (- C$\underline{H}_2$ - of C_2 of 4 ABA), 3.5 δ broad singlet (- C$\underline{H}_2$ - CH - of polymer), 3.9 δ multiplet (- C$\underline{H}$ - CH_2 - of polymer), 7.1 δ double doublet (ring protons of PABA), 7.4 δ double doublet (ring protons of PABA).

Poly (N-isopropylacrylamide –co- N-acryloyl 6 amino caproyl para aminobenzamidine) , poly (NIPAM -co- Ac. 6ACA PABA).

^{1}H NMR (DMSO d_6)

1.0 δ doublet [$(C\underline{H}_3)_2$ - CH - of NIPAM], 1.5 δ multiplet [- C$\underline{H}$ - $(CH_3)_2$ of NIPAM], 1.7 δ singlet (- C$\underline{H}_2$ of C_4 of 6ACA), 2.0 δ singlet (- C$\underline{H}_2$ of C_5 of 6ACA), 2.3 δ singlet (- C$\underline{H}_2$ of C_3 of 6ACA), 2.5 δ singlet (- C$\underline{H}_2$ of C_6 of 6ACA), 3.1 δ singlet (- C$\underline{H}_2$ of C_2 of 6ACA), 3.5 δ singlet (- C$\underline{H}_2$ - CH - of polymer), 3.9 δ singlet (- C$\underline{H}$ - CH_2 - of polymer), 7.1 δ double doublet (ring protons of PABA), 7.5 δ double doublet (ring protons of PABA).

3.1.3.5 Synthesis of polymers for the evaluation of crowding effect

Copolymers of NIPAM with increasing concentration of Ac.6ACA in the feed were synthesized and PABA was linked to the pendant carboxyl groups of these polymers. Similarly, copolymers of NIPAM, with increasing concentration of Ac. PABA in the feed, were synthesized. Data for the feed composition of monomers, PABA loading and K_i of the respective copolymers are summarized in Table 3.2.

Table 3.2: Binding between trypsin and PABA conjugates : crowding effect

No.	Polymer	Molar feed composition of NIPAM : Ac. Spacer	Moles of carboxyl groups / g polymer	Moles of PABA / g Polymer	M.W. (M_n)[a]	K_i (Moles)[b]
1	Poly (NIPAM -co-Ac. PABA)	30:1	------	$2.20 * 10^{-4}$	1180	$395 \pm 27 * 10^{-6}$
2	--,,----,,--	20:1	------	$2.52 * 10^{-4}$	1020	$350 \pm 24 * 10^{-6}$
3	--,,-----,,--	9:1	------	$2.72 * 10^{-4}$	940	$275 \pm 14 * 10^{-6}$
4	--,,----,,---	5:1	------	$2.90 * 10^{-4}$	965	$190 \pm 15 * 10^{-6}$
5	--,,----,,---	2:1	------	$3.20 * 10^{-4}$	960	$70 \pm 10 * 10^{-6}$
6	--,,----,,--	1:1	------	$3.38 * 10^{-4}$	930	$30 \pm 5 * 10^{-6}$
7	Poly (NIPAM -co-Ac. 6ACA. PABA)	30:1	$4.00 * 10^{-5}$	$3.70 * 10^{-5}$	3240	$38 \pm 5 * 10^{-6}$
8	---,,---,,--	20:1	$6.10 * 10^{-5}$	$3.70 * 10^{-5}$	3200	$38 \pm 5 * 10^{-6}$
9	---,,----,,--	9:1	$9.82 * 10^{-4}$	$2.60 * 10^{-4}$	980	$25 \pm 2 * 10^{-6}$
10	---,,----,,--	5:1	$1.49 * 10^{-3}$	$3.90 * 10^{-4}$	4200	$19 \pm 2 * 10^{-6}$
11	---,,----,,--	2:1	$2.19 * 10^{-3}$	$3.90 * 10^{-4}$	3900	$19 \pm 2 * 10^{-6}$
12	--,,----,,--	1:1	$2.12 * 10^{-3}$	$4.20 * 10^{-4}$	3100	$9 \pm 1 * 10^{-6}$

[a] Molecular weights were estimated by intrinsic viscosity method [Fujishige (1987)].

[b] K_i is the average value from three experiments.

3.1.3.6 Synthesis of model N-Acyl-para aminobenzamidine

The N-acetyl para aminobenzamidine was synthesized as follows. In a 250 ml beaker, 1 g PABA.2HCl, 17 g sodium acetate and 100 ml water were placed to obtain a clear solution. This was stirred at 4-8 ^{0}C. To this solution, 2 ml acetyl chloride in 2 ml dichloromethane was added dropwise, under stirring. The addition was completed in 10 minutes. The solution was stirred for 1 hour at 10 ^{0}C and acidified to pH 5.0 with concentrated HCl. The acidified solution was kept at - 4 ^{0}C overnight to precipitate the product. The isolated product was purified, by triturating with acetone. Similarly, N-butyryl para aminobenzamidine and N-hexanoyl para aminobenzamidine were synthesized by the same procedure. These model inhibitors are shown in Figure 3.4.

N-acetyl para aminobenzamidine

Yield - 50 %. M.P. 264-265 ^{0}C.

^{1}H NMR (DMSO d_6)

1.0 δ 3H singlet (- CO - C<u>H</u>$_3$), 7.8 δ 5H multiplet (ring protons of PABA), 9.0 δ 1H (- N<u>H</u>).

N-butyryl para aminobenzamidine

Yield - 55 %, M.P. 185 - 186 ^{0}C.

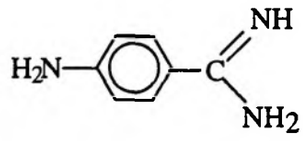
^{1}H NMR (DMSO d_6)

0.8 δ 3H triplet (- C<u>H</u>$_3$ of C_4 of butyryl), 1.6 δ 2H multiplet (- C<u>H</u>$_2$ - of C_3 of butyryl), 2.4 δ 2H triplet (- C<u>H</u>$_2$ - of C_2 of butyryl), 7.7 δ 5H multiplet (ring protons of PABA).

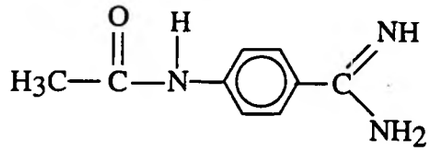
N - hexanoyl para aminobenzamidine

Yield - 65 %, M.P. 212 - 213 ^{0}C.

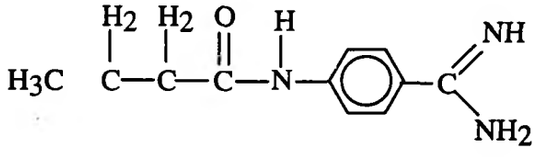
^{1}H NMR (DMSO d_6)



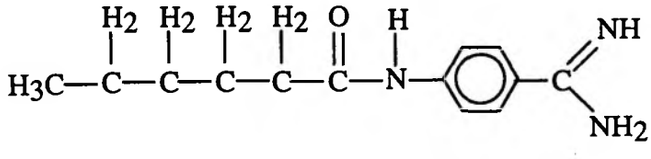
para-aminobenzamidine



N-acetyl para-aminobenzamidine



N-butyryl para-aminobenzamidine



N-hexanoyl para-aminobenzamidine

Figure 3.4 : Model N-acyl-para-aminobenzamidine inhibitors.

0.7 δ 3H triplet (- C<u>H</u>$_3$ of C_6 of hexyl), 1.1 δ 2H multiplet (- C<u>H</u>$_2$ - of C_5 of hexyl), 1.5 δ 2H multiplet (- C<u>H</u>$_2$ - of C_4 of hexyl), 1.9 δ 2H multiplet (- C<u>H</u>$_2$ - of C_3 of hexyl), 2.4 δ 2H triplet (- C<u>H</u>$_2$ - of C_2 of hexyl), 7.8 δ 2H double doublet (ring protons of PABA), 8.4 δ 2H double doublet (ring protons of PABA).

3.1.3.7 Synthesis of copolymers containing substituted acrylamides

In order to elucidate the effect of the nature of the polymer backbone on K_i, different copolymers were synthesized using 9:1 molar ratio of substituted acrylamide monomer:comonomer following the procedure described earlier. Poly (acrylamide -co- Ac.-4ABA. PABA), poly (N-butyl acrylamide -co- Ac.-4ABA. PABA), poly (acrylamide -co- Ac. PABA), poly (N-butyl acrylamide -co- Ac. PABA) and poly (N-isopropyl methacrylamide -co- Ac. PABA) were synthesized. Their structures are shown in Figure 3.5. Data for the feed compositions and K_i are listed in Table 3.3.

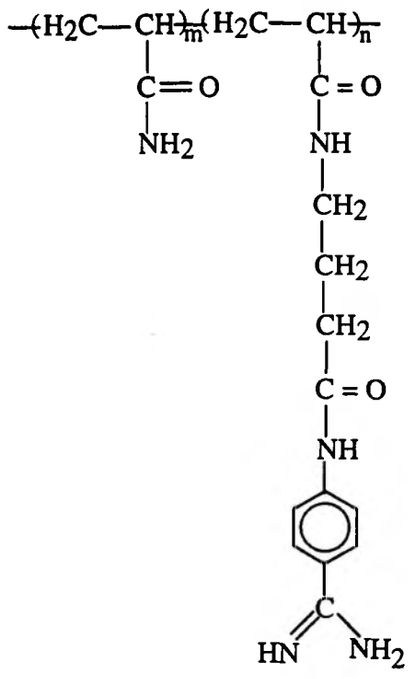
3.1.4 Characterization of copolymers

3.1.4.1 Estimation of carboxyl groups incorporated into the copolymers

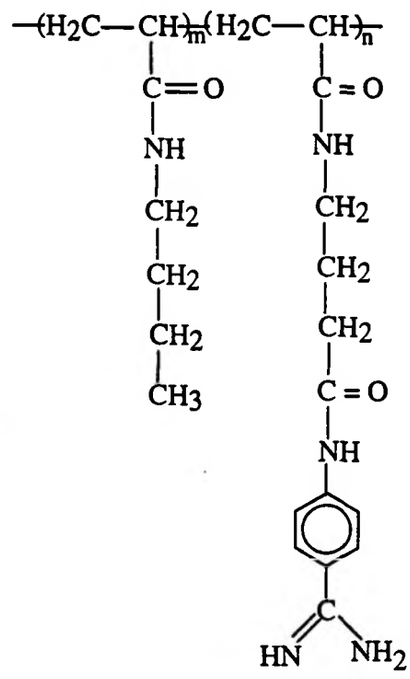
The pendant carboxyl groups incorporated into the copolymers were estimated as follows. 0.1 g copolymer was dissolved in 10 ml distilled ethanol and titrated against 0.1 M ethanolic KOH till colorless to faint pink end point, using phenolphthalein indicator. The carboxyl groups in the polymer were estimated from the amount of KOH consumed (See Tables 3.1 and 3.2).

3.1.4.2 LCST and molecular weight measurements for the copolymers

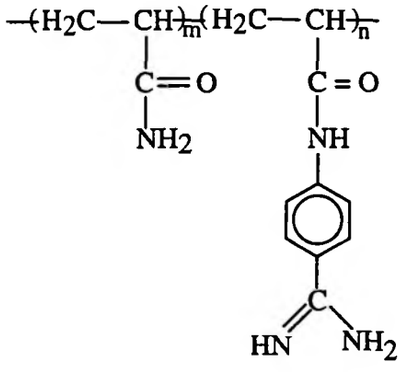
LCSTs were estimated by turbidometric method [Boutris et al (1997)]. Molecular weights were estimated by viscometry [Takezawa et al (1990); Fujishige (1987)] (See Tables 3.1 and 3.2). The molecular weights of the polymers were in the range 800 –



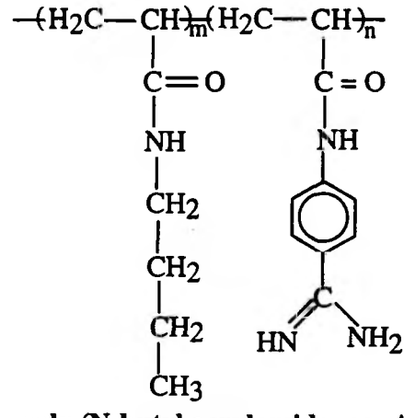
Poly (acrylamide -co- Ac.-4ABA. PABA)



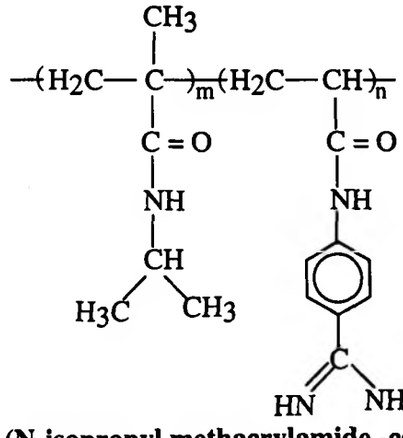
Poly (N-butylacrylamide -co- Ac.-4ABA. PABA)



poly (acrylamide -co- Ac. PABA)



poly (N-butyl acrylamide -co- Ac. PABA)



poly (N-isopropyl methacrylamide -co- Ac. PABA)

Figure 3.5 : Copolymers containing substituted acrylamides. m and n ar integers greater than1.

Table 3.3 : Effect of hydrophobicity of comonomer on K_i.

No.	Polymer[a]	Nature of comonomer	Type of spacer	K_i (Moles)[b]
P1	Poly (acrylamide -co- Ac. PABA)	Hydrophilic	No spacer	$295 \pm 16 * 10^{-6}$
P2	Poly (NIPAM -co- Ac. PABA)	Hydrophobic	No spacer	$275 \pm 14 * 10^{-6}$
P3	Poly (N-isopropyl methacrylamide -co- Ac. PABA)	Hydrophobic [c]	No spacer	$410 \pm 23 * 10^{-6}$
P4	Poly (N-butylacrylamide –co- Ac. PABA)	Linear and hydrophobic	No spacer	$20 \pm 2 * 10^{-6}$
P5	Poly (acrylamide -co- Ac. 4ABA PABA)	Hydrophilic	C_3	$35 \pm 5 * 10^{-6}$
P6	Poly (N-butylacrylamide –co- Ac. 4ABA PABA)	Linear and Hydrophobic	C_3	$0.1 * 10^{-6}$

[a] Molar feed ratio of acrylamide comonomer:acryloyl monomer was 9:1 in all cases.
[b] K_i values are the average from three experiments.
[c] Steric hindrance due to methyl group.

4000. Thus, low molecular weight affinity polymers comprising spacers were synthesized. Since the molecular weights were low, it was possible to prepare and work with 10 % w/v polymer solutions.

3.1.4.3 Estimation of K_i of copolymer-PABA conjugates

K_i was determined from Dixon plot [Luong et al (1988)]. 0.2 to 1 g polymer was dissolved in 1 ml water to obtain a range of inhibitor concentration. The substrate BAPNA was dissolved in dimethyl formamide to obtain the following concentrations- 0.6 mM, 0.48 mM, 0.36 mM, 0.24 mM and 0.12 mM. Trypsin solution (24 μg / ml) was prepared in 0.05 M Tris-HCl buffer, pH 8.1, containing 10 mM Ca^{+2}. The specific activity of this preparation was 31 units/ml. One BAPNA unit activity is the change in the optical density (ΔA_{410}) of 0.005 / min. at pH 8.1 at 25 ^{0}C in 3 ml reaction volume.

In a typical assay procedure, 1 ml polymer solution was mixed with 1 ml of trypsin solution. The mixture was vortexed, incubated at 25 ^{0}C for 15 minutes and added to 1 ml BAPNA solution placed in a 3 ml cuvette. The rate of substrate hydrolysis (V) was monitored by following the change in (ΔA_{410}) the absorbance at 410 nm for 1 minute. Dixon plot of 1/V vs. [I] at various substrate and inhibitor concentrations was plotted to estimate K_i. The K_i values are listed in Tables 3.1 and 3.2. A typical Dixon – plot for poly (NIPAM -co- Ac.6ACA.PABA) is shown in Figure 3.6.

3.1.5 Recovery of trypsin by affinity-thermoprecipitation

In a typical experiment, 1 ml of 10 % w/v polymer solution was mixed with 1 ml of trypsin solution (concentration 24 μg / ml). This was incubated at 25 ^{0}C for 15 minutes and then the temperature was raised above LCST of the polymer to precipitate the trypsin-polymer complex. The complex was separated by centrifugation at 10,000 rpm

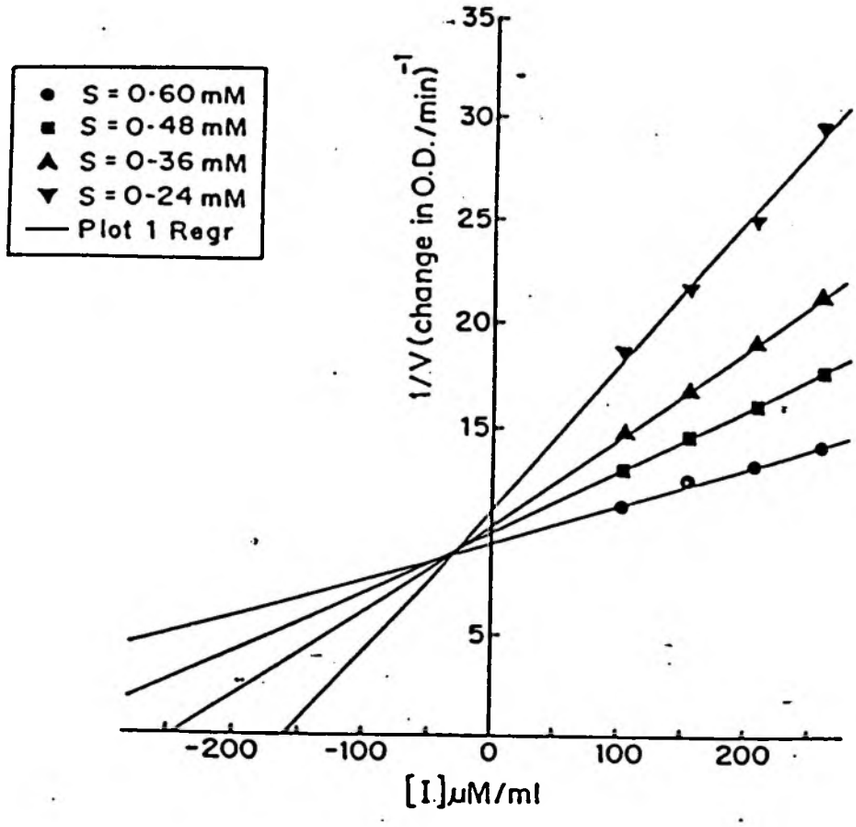


Figure 3.6: A typical Dixon plot for poly (NIPAM –co- Ac.6ACA PABA). S = Substrate concentration

for 20 minutes at 37 ^{0}C i.e. above LCST. Trypsin-polymer complex was resolubilized at lower temperature and the complex was dissociated by treatment with 0.05 M Glycine - HCl buffer, pH = 2.8, 10 mM Ca^{+2} [Senstad and Mattiasson (1989)]. The temperature of this solution was again raised above LCST and the precipitated polymer was separated by centrifugation. The clear filtrate containing trypsin was assayed for trypsin content, using Lowery's procedure. The activity was measured using BAPNA [Erlanger et al (1961)].

3.1.5.1 Separation of trypsin from chymotrypsin

For chymotrypsin, one BTPNA unit activity is the change in the optical density (ΔA_{410}) of 0.015/min. at pH 8.1 at 25 ^{0}C in 3 ml. reaction volume. In a typical experiment, 1 ml polymer solution (10 % w/v) was mixed with 1 ml of trypsin - chymotrypsin mixture of equal initial activities (trypsin 24 μg / ml and chymotrypsin 58 μg / ml). This solution was incubated at 25 ^{0}C for 15 minutes and trypsin was recovered by the thermoprecipitation method described above. The activities of trypsin and chymotrypsin were estimated using BAPNA and BTPNA respectively.

3.2.0 Results and Discussion

3.2.1 Choice of thermoprecipitating affinity copolymer and enzyme system

Polymers based on NIPAM exhibit LCST at ambient temperatures (30 – 40 ^{0}C) which makes them suitable for enzyme separations. Recently Badiger et al (1998) reported copolymers of NIPAM and N-acryloyl amino acids of varying chain length. The polymers so synthesized exhibited a wide range of LCSTs as a function of amino acid chain length. An important feature of these polymers is that they possess free carboxyl groups in the pendant chain, which can be coupled with various ligands of interest. Thus copolymers of NIPAM and N-acryloyl amino acids were selected. The recovery of

trypsin was investigated as it finds applications in medicine, beer haze removal and meat tenderization. Trypsin binds to the natural macromolecular ligand soybean trypsin inhibitor ($K_i = 2 * 10^{-10}$ M) and synthetic low molecular weight ligand PABA ($K_i = 8 * 10^{-6}$ M). The binding in both cases is weakened 30-200 fold when the ligand is conjugated with the polymer. Galaev and Mattiasson (1993b) concluded that for successful protein recovery by affinity precipitation, K_i should be around 10^{-5} M. Thus it is apparent that there is a greater need to restore the binding between PABA and trypsin. Moreover, PABA will have better recyclability and resistance towards denaturation than STI. We therefore selected the system NIPAM-spacer-PABA and trypsin for our investigations.

3.2.2 Choice of low molecular weight affinity copolymers

High molecular weight poly (acrylamide)s containing PABA were reported by Luong et al (1988) (M.W. 1,00,000) and Galaev and Mattiasson (1993b) (M.W. 2,90,000). These polymers exhibited higher K_i values as compared to free PABA. Such high molecular weight polymers are known to form highly coiled structures in solutions that hinder access to the macromolecules [Morawetz (1965)]. In affinity ultrafiltration, high molecular weight polymers are necessary to ensure appropriate molecular weight cut off and the selectivity of the process. In affinity precipitation, low molecular weight polymers can be conveniently used. This also helps in partly eliminating the crowding effect in polymers at higher ligand loading. Moreover, still better results are envisaged if a spacer is incorporated in such polymers.

Various low molecular weight polymers were synthesized and characterized as described in the experimental section. In the following sections their efficacy in lowering K_i is described.

3.2.3 Effect of spacer chain length on K_i

Table 3.1 shows that K_i increased fifteen fold when free PABA was derivatized to Ac. PABA and thirty-five folds when PABA was incorporated in the polymer. These results are consistent with those reported in the literature [Galaev and Mattiasson (1993b); Luong et al (1988); Sigmundsson and Filippusson (1996)]. But it is interesting to note that in the series of copolymers containing spacers, the value of K_i decreased from $275 * 10^{-6}$ M [poly (NIPAM -co- Ac. PABA)] to $25 * 10^{-6}$ M [poly (NIPAM -co- Ac. 6ACA. PABA)] as the spacer chain length increased from 0 to 5 carbon atoms. It may be noted here that 1,6 diaminohexane is routinely used as a spacer in affinity chromatography for enhancing the accessibility of ligand bound to the polymer matrix. Thus, the role of spacers in lowering the K_i of hetero-bifunctional affinity polymers has been demonstrated experimentally.

3.2.4 Investigation of the origin of spacer effect

Lowering of K_i values due to the spacers has been attributed to the spacer effect as well as to the change in the microenvironment around the ligand [Pecs (1991); Galaev and Mattiasson (1993b)]. In order to discern between the two contributions, novel inhibitors - N-acyl para aminobenzamidines comprising 0, 3 and 5 carbon atoms in the acyl chain length were synthesized. K_i values of these inhibitors were estimated. It was found that K_i increased from $8 * 10^{-6}$ M (free PABA) to $60 * 10^{-6}$ M (N-acetyl para aminobenzamidine). But, within the series of N-acyl benzamidines, K_i value decreased

with increase in the hydrophobicity of N-acyl group in the following order - N-acetyl para aminobenzamidine, $K_i = 60 * 10^{-6}$ M, N-butyryl para aminobenzamidine, $K_i = 48 * 10^{-6}$ M and N-hexanoyl para aminobenzamidine, $K_i = 35 * 10^{-6}$ M. In these cases, since PABA is not linked to a polymer chain, the steric effect does not influence K_i. On the other hand, the microenvironment around PABA has been modified by the hydrophobicity of N-acyl chain. It may be noted that the active site of trypsin is made up of a hydrophobic slit and a negatively charged pocket [Mares-Guia and Shaw (1965)]. Therefore, the results obtained here indicate that the hydrophobic groups linked to PABA exhibit enhanced hydrophobic interactions with the hydrophobic slit and thereby enhanced binding with trypsin. Thus the hydrophobicity of the spacer also influences K_i.

3.2.5 Effect of ligand concentration on K_i

As described earlier, the objective of using low molecular weight affinity polymers was to eliminate the crowding effect observed by Luong et al (1988). In order to demonstrate this, two sets of polymers were synthesized - 1) Poly (NIPAM –co- Ac. PABA) - Copolymers of N-isopropylacrylamide with increasing concentration of acryloyl PABA. 2) Poly (NIPAM -co- Ac. 6ACA. PABA) - Copolymers of N-isopropylacrylamide with increasing concentration of acryloyl 6ACA and PABA was linked to these polymers. Relevant data for the feed composition and K_i are listed in Table 3.2. In both cases, an increasing amount of PABA was incorporated in the polymers. The data reveal that despite the absence of spacers, poly (NIPAM –co- Ac. PABA) exhibited decrease in K_i with increase in the concentration of PABA. Thus, the low molecular weight polymers used in the present work exhibited higher accessibility of available PABA on the polymer chain. In other words, the crowding effect was

eliminated. In the case of poly (NIPAM -co- Ac. 6ACA. PABA) also, K_i values decreased. But the effect was ten-fold higher than in the case of poly (NIPAM –co- Ac. PABA). Thus even in the case of low molecular weight polymers, the spacer effect was highlighted.

3.2.6 Designing polymers for affinity thermoprecipitation

In the preceding sections, it was demonstrated that with the incorporation of spacer between the polymer backbone and ligand, it was possible to decrease the inhibition constants of the ligands bound to the polymers. Also, K_i values obtained for N-acyl para-aminobenzamidines showed that the microenvironment around the ligand plays an important role in governing K_i. To validate these findings further, various copolymers were synthesized and their inhibition constants were evaluated.

A brief description of the polymer characteristics and K_i values is given in Table 3.3. Polymers P1 to P4 were devoid of spacers. Therefore, in these cases, the effect of comonomers is expected to be highlighted. It was observed that with increase in the hydrophobicity of comonomer from acrylamide (P1, $K_i = 295 * 10^{-6}$ M) to N-isopropyl acrylamide (P2, $K_i = 275 * 10^{-6}$ M), K_i value decreased slightly. K_i again increased from $275 * 10^{-6}$ M to $410 * 10^{-6}$ M when the comonomer was N-isopropylmethacrylamide (P3). This can be attributed to the bulky methyl group in the polymer side chain, which is known to exhibit steric hindrance. But more interestingly, for linear hydrophobic comonomer N-butylacrylamide, K_i value was found to be substantially lowered (P4, $K_i = 20 * 10^{-6}$ M). The polymer P5 containing hydrophilic comonomer acrylamide, exhibited $K_i = 35 * 10^{-6}$ M. On the other hand, for P6, containing linear hydrophobic comonomer N-butylacrylamide, K_i was $0.1 * 10^{-6}$ M. This value was substantially lower than those

exhibited by polymers P1 to P5. Although P6 did not exhibit LCST behavior, it illustrated the methodology for the synthesis of polymers that can exhibit enhanced binding with a given biomolecule.

The low K_i value exhibited by the polymer P6 can be attributed to the synergistic effect of spacer and enhanced hydrophobic interactions of the polymer with trypsin. The above results show that K_i is governed not only by spacer effect but also by the microenvironment around the ligand.

3.2.7 Recovery of trypsin by affinity thermoprecipitation

The objective of introducing the spacer between the polymer and PABA was to enhance the recovery of trypsin from solutions. As described in the experimental section, after incubating polymer-trypsin solution below LCST of the polymer, the temperature shift was effected and the polymer-trypsin complex isolated. A control experiment was run without adding trypsin and the amount of thermoprecipitated polymer was estimated on dry weight basis. It was found that the polymer recovery was 90 %. Data in Table 3.4 show that with increase in the spacer chain length from 0 to 5, specific activity of trypsin recovered increased from 36 % to 65 %. Incorporation of spacers also resulted in polymers which exhibit a wide range of LCST (53 ^{0}C to 33 ^{0}C). But care was taken to precipitate polymers possessing higher LCSTs at ambient temperature (37 ^{0}C) by adding a small amount of sodium chloride. Thus loss in enzyme activity due to precipitation at high temperature was avoided. In this context, poly (NIPAM -co- Ac. 6ACA. PABA) offers an advantage of enhanced recovery at ambient temperature without adding salt.

In the above examples, only 36 % to 65 % recovery was possible since the contact time between polymer and trypsin was short, i.e. 15 minutes. For the quantitative

Table 3.4: **Trypsin recovery by affinity thermoprecipitation.**

No.	Polymer	Trypsin recovery [a]			Trypsin - chymotrypsin separation [b]	
		Protein (μg / g polymer)	Unit activity / μg protein	% specific activity	Unit trypsin activity recovered	Unit chymo-trypsin activity recovered
1	Poly (NIPAM - co- Ac. PABA)	2.33 ± 0.001	1.08 ± 0.008	36.43	10.61± 0.02	0.48 ± 0.01
2	Poly (NIPAM - co- Ac. Gly. PABA)	7.20 ± 0.03	2.88 ± 0.02	31.00	12.11± 0.03	0.45 ± 0.01
3	Poly (NIPAM - co- Ac. βala. PABA)	11.86± 0.05	6.85 ± 0.03	44.78	15.51± 0.05	0.63 ± 0.01
4	Poly (NIPAM - co- Ac. 4ABA. PABA)	13.44± 0.06	8.65 ± 0.03	49.89	16.85± 0.09	0.82 ± 0.03
5	Poly (NIPAM - co- Ac. 6ACA. PABA)	14.1 ± 0.05	11.90± 0.04	65.40	21.34± 0.09	1.03 ± 0.04

[a] Trypsin solution of concentration 24 μg/ml was used. Initial activity of this solution was 31 units. One unit activity is defined in the text.

[b] Trypsin-chymotrypsin mixture of identical initial activities was used (trypsin 24 μg/ml and chymotrypsin 58 μg/ml). The activity units have been defined in the text.

All the values are the average from three experiments.

recovery of trypsin, an additional experiment with poly (NIPAM -co- Ac. 6ACA. PABA) was done wherein the contact time was increased to 18 hours. In this case 83 % recovery was achieved. This value compared favorably with the 90 % recovery reported by Luong et al (1988) and 83 % recovery reported by Kumar and Gupta (1994). No attempt was made to optimize the minimum time needed for 83 % recovery.

3.2.8 Separation of trypsin and chymotrypsin

Trypsin and chymotrypsin are very closely related enzymes. To determine the selectivity of separation, a mixture of chymotrypsin (58 μg / ml) and trypsin (24 μg / ml) exhibiting identical initial activities was used. Data in Table 3.4 show that the recovery of trypsin activity increased from 10 to 21 units of BAPNA with increase in the spacer chain length from 0 to 5. Also, these affinity polymers exhibited very low nonspecific recovery of chymotrypsin activity (0.5 to 1 units of BTPNA). This nonspecific binding of PABA with chymotrypsin is consistent with the reports in the literature [Luong et al (1988); Schneider et al (1981)]. Very high purity trypsin can be recovered by cascading the operation.

3.3.0 Conclusions

Thermoprecipitating copolymers of N-isopropylacrylamide and N-acryloyl amino acids were synthesized and conjugated with para aminobenzamidine. The inhibition constant for trypsin decreased with increase in the spacer chain length. It was shown that the microenvironment around the ligand and the polymer also govern K_i. Incorporation of spacer resulted in enhanced recovery of trypsin and also lowered the LCST of the polymer. This is an added advantage for the recovery of thermosensitive enzymes. The

use of low molecular weight polymers eliminated the crowding effect. The methodology developed is generic and can be used for a wide range of biomolecule - ligand systems.

Chapter 4

NEW SYNTHETIC LIGANDS FOR LYSOZYME THERMOPRECIPITATION

4.0.0 Introduction

In the previous chapter, the rationale behind the synthesis of a new family of thermoprecipitating copolymers of N-isopropylacrylamide (NIPAM) and N-acryloyl amino acids was discussed. The terminal carboxyl groups of these polymers can be conjugated with a variety of affinity ligands e.g. in the prior work, p-aminobenzamidine, a synthetic inhibitor for trypsin was conjugated to the polymer. Apart from exhibiting the enhanced binding resulting from the spacer effect and eliminating the crowding effect, these polymers offer more latitude for the synthesis. Alternatively, this approach can also be modified to synthesize monomers bearing pendant amino groups. It has been realized that the effectiveness of the natural ligands in the recovery of enzymes by affinity precipitation is limited by the deactivation of these ligands. Chitosan undergoes microbial degradation and is insoluble in many solvents and is therefore of limited use in the recovery of lysozyme [Tyagi et al (1996); Hirano et al (1991)]. We therefore looked into the possibility of conceptualizing and synthesizing alternative ligands, which will be as efficient as N-acetylglucosamine (NAG) and will be more stable. In this chapter the synthesis and evaluation of such ligands, polymers comprising such ligands and their evaluation for binding and recovery of lysozyme is described. It has been shown that these ligands are far more efficient than their natural counterpart i.e. NAG, present in Chitosan.

Lysozyme is an industrially important enzyme, which finds diverse applications as infant food additive, as a preservative in cheese making, in formulations used for wound healing, as an antibiotic activator and largely as a bacteriolytic agent [Ruckenstein and Zeng (1997)]. Egg white, which contains about 3.5 % lysozyme, is routinely used for the recovery of lysozyme on industrial scale [Godfrey and West (1996)]. So far, a wide range

of techniques have been developed for the recovery of lysozyme which range from conventional salt precipitation [Alderton and Fevold (1946)] to modern ultrafiltration [Bozzano and Glatz (1991); Ehsani et al (1997); Iritani et al (1997)]. Amongst these, affinity based techniques, such as affinity membrane filtration, [Ruckenstein and Zeng (1997)] and affinity chromatography [Hirano et al (1991); Junowicz and Charm (1975); Liapis et al (1989); Mayes et al (1990); Safarik and Safarikova (1993); Yamasaki et al (1985)] provide high selectivity, but are beset with several practical difficulties. In affinity membrane filtration, the fractionation is carried out on the basis of molecular size. Selection of the membrane, which offers precise molecular weight cut off, is often difficult. At high pressures, the denaturation of enzyme, and the fouling of the membrane lead to poor product quality and productivity [Ehsani et al (1997)]. The affinity chromatography method suffers from slow binding, low capacity and flow rate limitations due to pressure drop etc [Chern et al (1996a)].

Affinity precipitation eliminates many of the above problems. It offers ease of scale up, amenability to continuous operation and recycling of the affinity ligand [Chern et al (1996a)]. There are few reports on the affinity precipitation for lysozyme recovery. For instance, Chern et al (1996 a and b) reported the use of pH sensitive submicron acrylic latex and Eudragit L 100. Sternberg and Hershberger (1974) reported the use of polyacrylic acid as a polyelectrolyte precipitant for lysozyme recovery. Tyagi et al (1996) used chitosan as an affinity macroligand for lysozyme precipitation. Except for chitosan, all other acidic polymers exhibit electrostatic/ionic interactions with lysozyme, which being non-specific, often result in lower recovery [Chern et al (1996a)] and/or lower selectivity [Sternberg and Hershberger (1974)].

The ligands containing glucose e.g. NAG, N-acetylmuramic acid, chitosan, chitin etc. are susceptible to microbial attack, hydrolytic degradation, poor stability etc. [Hirano et al (1991)]. Chitin and chitosan can undergo transglycosylation and mutarotation, which complicate the kinetic interpretation of inhibition data [Davies et al (1969); Neuberger and Wilson (1967)]. It is desirable to replace glucose in the affinity based synthetic ligands for lysozyme separation. Blake et al (1967) reported that NAG inhibited lysozyme but glucosamine (devoid of N-acetyl group) did not. Thus, N-acetyl groups are crucial for inhibiting lysozyme. On the contrary, the type of sugar did not influence binding significantly [Rupley (1967a)]. It therefore, appears that synthetic ligands containing N-acetyl groups could be used for lysozyme separation.

The synthesis of a series of ligands based on N-acetylated amino acids was therefore undertaken. The binding efficiency, as estimated by lysozyme inhibition, increased with the chain length and the hydrophilicity of the amino acid, which is further enhanced when the ligand is incorporated into a thermoprecipitating polymer. The protein and activity of lysozyme recovered from aqueous solution as well as from lysozyme-ovalbumin mixture exceed the corresponding values obtained for NAG. The synthetic ligands are also more stable.

4.1.0 Experimental section

4.1.1 Materials

6 amino caproic acid (6ACA), 2 hydroxy ethyl methacrylate (HEMA), N-isopropyl acrylamide (NIPAM), acrylic acid, 1,hydroxy benzotriazole, dicyclohexylcarbodiimide (DCC) etc. were purchased from Aldrich. Lysozyme (3x crystallized, activity 47,000 units/mg solid). One unit activity of lysozyme corresponds to

a change in the absorbance (ΔA_{450}) of 0.001 per minute at pH 6.2 at 25 ^{0}C using a suspension of *Micrococcus lysodeikticus* (78 μg/ml) as a substrate in a 2.7 ml reaction mixture (1 cm light path). *Micrococcus lysodeikticus* (*Micrococcus luteus* ATCC No. 4698), N-acetyl glucosamine (NAG), ovalbumin (grade II) etc. were purchased from Sigma. 4-(Dimethylamino)-pyridine (DMAP) was from Merck-Schuchardt Germany. Glycine, β alanine, 4 aminobutyric acid (4ABA), glycylglycine, acrylamide, acetyl chloride, acetic anhydride, thionyl chloride, sodium bicarbonate, sodium hydroxide, pyridine, azobisisobutyronitrile (AIBN), ammonium per sulfate, tetramethylethylenediamine (TEMED) etc. were supplied by local suppliers. All chemicals and solvents were of analytical grade and were used as received.

4.1.2 Instrumentation

^{1}H NMR spectra were recorded on a Bruker superconducting FT NMR AC 200 operating at 200 MHz. N-acetyl content in the polymer was determined using Bruker MSL-300 FT NMR spectrometer operating at proton larmor frequency of 300 MHz. Deconvolution was done using Bruker's WINNMR software. IR spectra were recorded on Shimadzu 8300 FT-IR spectrometer. Electronic absorption measurements were done on Shimadzu UV 1601 spectrophotometer. Melting points were recorded on Mettler melting point apparatus.

4.1.3 Methods

4.1.3.1 Synthesis of O-acryloyl N-acetyl glucosamine (Ac.NAG)

Acryloyl chloride was synthesized by the reaction of thionyl chloride and acrylic acid [Lele (1997)]. O-acryloyl N-acetyl glucosamine was synthesized as follows. In a 250 ml beaker equipped with dropping funnel and pH meter, 11.1 g (0.05 M) NAG and 4.2 g

(0.05 M) sodium bicarbonate was dissolved in 100 ml water to obtain a clear solution (pH 8-9). This was stirred at 5-10 ^{0}C on a magnetic stirrer. 5 ml (0.06 M) acryloyl chloride in 5 ml dichloromethane was added dropwise to the above solution over a period of 20-30 minutes. During this period the pH of the reaction mixture was maintained in the range 7.4-7.8 adding saturated sodium bicarbonate solution. After the addition of acryloyl chloride was over and the pH of the reaction mixture was steady, unreacted acryloyl chloride was extracted in 100 ml ethyl acetate. The clear aqueous solution was separated and acidified to pH 5.0 by concentrated HCl. Then the product was precipitated in acetone and maintained at 4 ^{0}C overnight to maximize the precipitation. The precipitated product was filtered. The product was reprecipitated from water into acetone. Yield - 80 %. M.P. 170 ^{0}C.

IR (nujol)

1560 cm $^{-1}$ (acrylic double bond), 1635 cm $^{-1}$ (amide carbonyl), 3323 cm $^{-1}$ (- OH, - NH stretching).

^{1}H NMR (D_2O)

2.0 δ 3H singlet (- CO - C<u>H</u>$_3$), 2.7 δ 2H doublet (- O - C<u>H</u>$_2$ -), 3.5 δ 2H singlet (<u>H</u> at C_3 and C_5 of glucose ring), 4.0 δ 3H singlet (<u>H</u> at C_1, C_2 and C_4 of glucose ring), 5.2 δ 1H triplet (H_2C = C<u>H</u> -), 5.7 δ 1H doublet (<u>H</u>$_a$C = CH -), 6.1 δ 1H doublet (<u>H</u>$_b$C = CH -).

4.1.3.2 Synthesis of acetamido acrylamide

In 1 liter capacity round bottom flask, 21.3 g (0.3 M) acrylamide, 0.5 g DMAP and 24.2 ml (0.3 M) pyridine was dissolved in 800 ml HPLC grade tetrahydrofuran (THF). The reaction mixture was stirred at 8-10 ^{0}C to obtain a clear solution. Then 21.3 ml (0.3 M) acetyl chloride in 22 ml THF was added dropwise to the above reaction

mixture over 1 hour. After the addition of acetyl chloride was over, a sticky yellow precipitate of pyridine-HCl was obtained. This precipitate was filtered under vacuum to obtain a clear THF solution, which was concentrated under vacuum at 40 ^{0}C to obtain a semisolid mass. This was precipitated in petroleum ether to get a low melting semisolid product. Yield - 40 %.

IR (neat)

1635 cm^{-1} (acrylic double bond), 1674 cm $^{-1}$ (acrylamide carbonyl), 3365 cm $^{-1}$ (- OH, - NH stretching).

^{1}H NMR (D_2O)

2.5 δ 3H singlet (- CO - C$\underline{H}_3$), 5.9 δ 1H triplet (H_2C = C$\underline{H}$ -), 6.3 1H doublet ($\underline{H}_a$C = CH -), 6.4 δ 1H doublet ($\underline{H}_b$C = CH -).

4.1.3.3 Synthesis of acetamido ligands containing spacers

6-acetamido caproic acid was synthesized as described below. In a 250 ml beaker equipped with dropping funnel and pH meter, 13.1 g (0.1M) 6ACA, 4 g (0.1 M) sodium hydroxide and 80 ml water was placed to obtain a clear solution (pH 13.0). This was stirred at 5-10 ^{0}C on a magnetic stirrer. Then, 9.5 ml (0.1 M) acetic anhydride was added to it over a period of 30 minutes. During this addition the pH of the reaction mixture was maintained in the range 7.4-7.8. After the addition was over, this reaction mixture was stirred for two hours and acidified to pH 2.0 by adding concentrated HCl. It was maintained at - 4 ^{0}C overnight. This reaction mixture was allowed to thaw and the product was filtered. The product was reprecipitated from water into acetone. Yield - 90 %. M.P.105 ^{0}C.

IR (nujol)

1620 cm^{-1} (amide carbonyl), 1705 cm^{-1} (carboxyl carbonyl), 3350 cm^{-1} (- OH, - NH stretching).

^{1}H NMR (DMSO d_6)

1.3-1.5 δ 6H multiplet (HOOC - H_2C - ($\underline{CH_2}$)$_3$ - CH_2 - NH -), 1.76 δ 3H singlet (- CO - $C\underline{H_3}$), 2.97 δ 2H triplet (H_2C - $C\underline{H_2}$ - NH - $COCH_3$), 2.17 δ 2H quartet (- $(CH_2)_3$ - $C\underline{H_2}$ - COOH), 7.90 δ 1H triplet (- CH_2 - N$\underline{H}$ - CO - CH_3), 12.02 1H singlet (- COO$\underline{H}$).

All other ligands were similarly synthesized from their respective amino acids. Only in the case of acetamido glycylglycine synthesis, the sodium hydroxide was replaced by equimolar amount of sodium bicarbonate and pH was maintained at 7.4-7.8 by the addition of saturated solution of sodium bicarbonate. These ligands are shown in Figure 4.1.

4-acetamido butyric acid

Yield - 70 %, M.P. 125 ^{0}C.

IR (nujol)

1610 cm^{-1} (amide carbonyl), 1705 cm^{-1} (carboxyl carbonyl), 3350 cm^{-1} (- OH, - NH stretching).

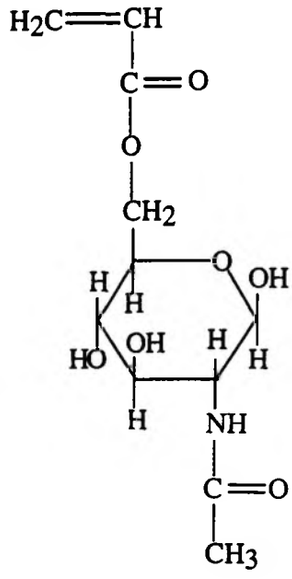
^{1}H NMR (DMSO d_6)

1.58 δ 2H quintet (HOOC - H_2C - $C\underline{H_2}$ - CH_2 - NH -), 1.77 δ 3H singlet (- CO - $C\underline{H_3}$), 2.19 δ 2H triplet (- $C\underline{H_2}$ - COOH), 3.02 δ 2H quartet (- H_2C - $C\underline{H_2}$ - NH - $COCH_3$), 7.83 δ 1H triplet (- CH_2 - N$\underline{H}$ - CO - CH_3), 12.08 1H singlet (- COO$\underline{H}$).

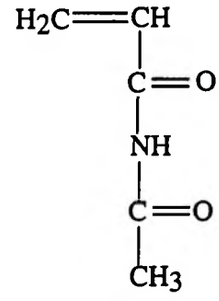
2-acetamido glycine

Yield - 81 %, M.P. 210 ^{0}C.

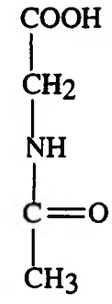
IR (nujol)



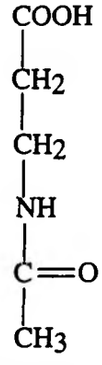
Ac. NAG



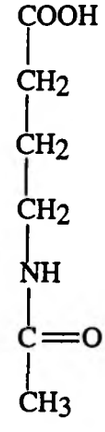
Acetamido acrylamide



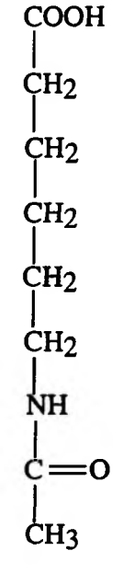
2- acetamido glycine



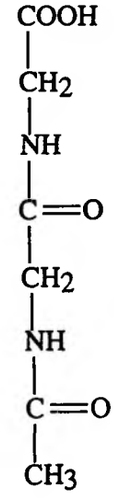
3- acetamidoβ alanine



4 - acetamido butyric acid



6-acetamido caproic acid



Acetamido glycylglycine

Figure 4.1: Schematic representation of various affinity ligands.

1630 cm^{-1} (amide carbonyl), 1715 cm^{-1} (carboxyl carbonyl), 2800 cm^{-1} (-CH stretching), 3300 cm^{-1} (- NH stretching), 3700 cm^{-1} (- OH stretching).

^{1}H NMR (DMSO d_6)

1.83 δ 3H singlet (- CO - $C\underline{H}_3$), 3.72 δ 2H doublet (- $C\underline{H}_2$ - NH - $COCH_3$), 8.20 δ 1H triplet (- CH_2 - $N\underline{H}$ - CO - CH_3), 9.0 1H singlet (- $COO\underline{H}$).

3-acetamido β alanine

Yield - 56 %. M.P. 250 ^{0}C.

IR (nujol)

1658 cm^{-1} (amide carbonyl), 1724 cm^{-1} (carboxyl carbonyl), 2854 cm^{-1} (-CH stretching), 3400 cm^{-1} (- NH stretching), 3762 cm^{-1} (- OH stretching).

^{1}H NMR (DMSO d_6)

1.22 δ 2H triplet (- $C\underline{H}_2$ - COOH), 1.74 δ 2H triplet (- $C\underline{H}_2$ - NH - $COCH_3$), 1.87 δ 3H singlet (- CO - $C\underline{H}_3$), 8.5 δ 1H triplet (- $N\underline{H}$ - CH_2 -), 10.0 1H singlet (- $COO\underline{H}$).

Acetamido glycylglycine

Yield - 84 %. M.P. 160 ^{0}C.

IR (nujol)

1660 cm^{-1} (amide carbonyl), 1710 cm^{-1} (carboxyl carbonyl), 2922 cm^{-1} (-CH stretching), 3305 cm^{-1} (- NH stretching), 3762 cm^{-1} (- OH stretching).

^{1}H NMR (DMSO d_6)

1.8 δ 3H singlet (- CO - $C\underline{H}_3$), 3.72 δ 4H doublet (- $C\underline{H}_2$ - NH - CO - $C\underline{H}_2$ - NH -), 8.14 δ 2H triplet (- CH_2 - $N\underline{H}$ - CO - CH_2 - $N\underline{H}$ -), 9.0 δ 1H singlet (- $COO\underline{H}$).

4.1.3.4 Synthesis of 2-(methacryloyl)oxyethyl derivatives of N-acetamido spacers

2-(methacryloyl)oxyethyl 6-acetamidocaproate (ME6Ac.CAP) was synthesized as described below. In a 250 ml round bottom flask 6.1 g (0.035 M) 6-acetamido caproic acid, 4.3 ml (0.035 M) HEMA, 4.8 g (0.035 M) 1,hydroxy benzotriazole (as activator) were dissolved in 300 ml dry THF. This reaction mixture was stirred at 10-15 ^{0}C under dry conditions to obtain a clear solution. Then, 7.2 g (0.035 M) DCC dissolved in 10 ml of THF was added to the above reaction mixture. It was allowed to stir at 10-15 ^{0}C for 48 hrs. A heavy white precipitate of dicyclohexyl urea salt was filtered and the clear filtrate was concentrated under vacuum at 40 ^{0}C to obtain a low melting solid product. This was purified by precipitation from THF into cold water. The product obtained was dried and preserved under hexane at 4 ^{0}C till further use. Yield - 50 %. M.P. 60-65 ^{0}C.

IR (nujol)

1651 cm^{-1} (acrylic double bond), 1680 cm^{-1} (N-acetyl carbonyl), 1715 cm^{-1} (methacryloyl carbonyl), 3300 cm $^{-1}$ (-NH stretching).

^{1}H NMR (DMSO d_6)

1.2-1.5 δ 6H multiplet (- OOC - CH_2 - ($C\underline{H}_2$)$_3$ - CH_2 - NH -), 1.77 δ 3H singlet (- CO - $C\underline{H}_3$), 1.88 δ 3H singlet (H_2C = $CC\underline{H}_3$ -), 2.23 δ 2H quartet (- CH_2 - $C\underline{H}_2$ - $NHCOCH_3$), 2.96 δ 2H triplet (- (CH_2)$_3$ - $C\underline{H}_2$ - COO -), 3.59 δ 2H triplet (CH_2 = CCH_3 - CO - O - $C\underline{H}_2$ - CH_2 - O -), 3.93 δ 2H triplet (CH_2 = CCH_3 - CO - O - CH_2 - $C\underline{H}_2$ - O -), 5.67 δ 1H singlet ($C\underline{H}_a$ = CCH_3), 6.06 δ 1H singlet ($C\underline{H}_b$ = CCH_3), 7.78 δ 1H triplet (- $N\underline{H}$ -).

All other 2-(methacryloyl)oxyethyl derivatives of acetamido spacers were similarly synthesized. Only in the case of 2-(methacryloyl)oxyethyl 3-

acetamidoβalaninate (MEAc.βALA) after the low melting solid was obtained, it was dissolved in minimum amount of dry ethyl acetate and this clear solution was precipitated in petroleum ether to obtain white solid, which was kept over petroleum ether till further use. The 2-(methacryloyl)oxyethyl derivatives of acetamido groups linked to spacers are shown in Figure 4.2.

2-(methacryloyl)oxyethyl 2-acetamidoglycinate (MEAc.GLY)

Yield - 90 %. M.P. 145 ^{0}C.

IR (nujol)

1610 cm^{-1} (methacrylic double bond), 1660 cm^{-1} (amide carbonyl), 1700 cm^{-1} (ester carbonyl), 2935 cm^{-1} (-CH stretching), 3425 cm^{-1} (-NH stretching).

^{1}H NMR (DMSO d_6)

1.83 δ 3H singlet (- CO - C$\underline{H}_3$), 2.27 δ 3H singlet (C$\underline{H}_3$ - C =), 2.49 δ 2H triplet (COO - C$\underline{H}_2$ - CH_2 - OOC - CH_2 - NH - CO - CH_3), 2.87 δ 2H triplet (= CCH_3 - CO - O - C$\underline{H}_2$ -), 3.71 2H doublet (- C$\underline{H}_2$ - NH - CO - CH_3), 5.66 δ 2H singlet (C$\underline{H}_2$ = CCH_3), 8.19 δ 1H triplet (- CH_2 - N$\underline{H}$ - CO - CH_3).

2-(methacryloyl)oxyethyl 3-acetamidoβalaninate (MEAc.βALA)

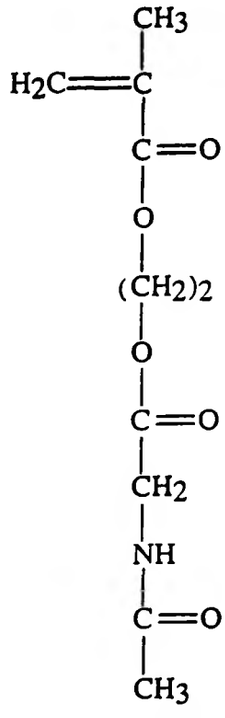
Yield - 80 %. M.P. 190 ^{0}C.

IR (nujol)

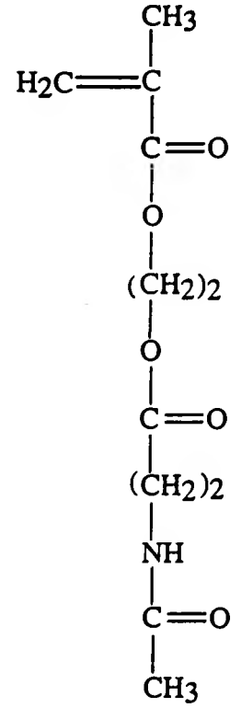
1620 cm^{-1} (methacrylic double bond), 1658 cm^{-1} (amide carbonyl), 1720 cm^{-1} (ester carbonyl), 2925 cm^{-1} (-CH stretching), 3415 cm^{-1} (-NH stretching).

^{1}H NMR (DMSO d_6)

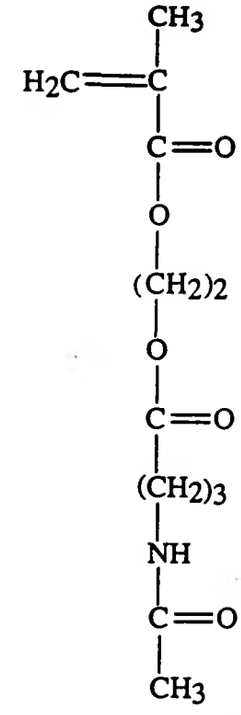
1.22 δ 2H triplet (- C$\underline{H}_2$ - COO -), 1.74 δ 2H triplet (- C$\underline{H}_2$ - NH - CO - CH_3), 1.87 δ 3H singlet (- CO - C$\underline{H}_3$), 2.0 δ 3H singlet (H_2C = CC$\underline{H}_3$ -), 3.59 δ 2H triplet (CH_2 = CCH_3 -



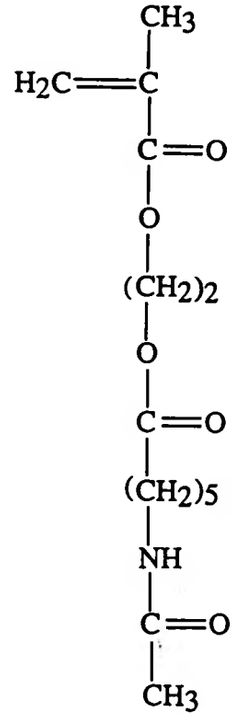
MEAc.GLY



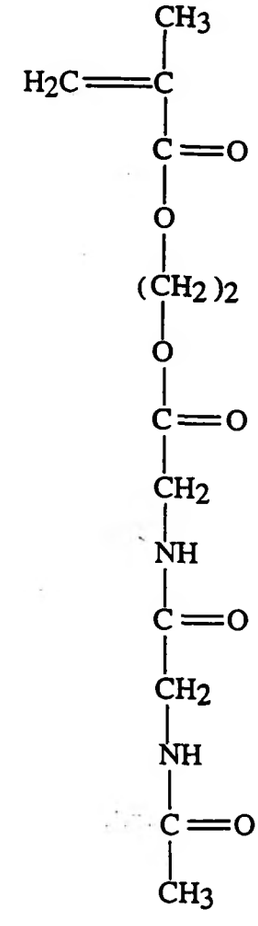
MEAc.βALA



ME4Ac.BUT



ME6Ac.CAP



MEAc.GLYGLY

Figure : 4.2 Schematic representation of various 2-(methacryloyl)oxyethyl derivatives of acetamido space

CO - O - $C\underline{H}_2$ -), 4.09 δ 2H triplet (CH_2 = CCH_3 - CO - O - CH_2 - $C\underline{H}_2$ -), 5.66 δ 1H singlet ($C\underline{H}_a$ = CCH_3), 6.05 δ 1H singlet ($C\underline{H}_b$ = CCH_3), 8.5 δ 1H triplet (- N$\underline{H}$ -).

2-(methacryloyl)oxyethyl 4-acetamidobutyrate (ME4Ac.BUT)

Yield- 60 %. M.P. 80-82 ^{0}C.

IR (nujol)

1650 cm^{-1} (methacrylic double bond), 1680 cm^{-1} (amide carbonyl), 1720 cm^{-1} (ester carbonyl), 3300 cm^{-1} (- NH stretching).

^{1}H NMR (DMSO d_6)

1.19 δ 2H quintet (- OOC - CH_2 - $C\underline{H}_2$ - CH_2 - NH -), 1.75 δ 3H singlet (- CO - $C\underline{H}_3$), 1.87 δ 3H singlet (H_2C = $CC\underline{H}_3$ -), 2.19 δ 2H triplet (- $\underline{H}_2C$ - COO -), 2.99 δ 2H quartet (- CH_2 - $C\underline{H}_2$ - NH - CO - CH_3), 3.60 δ 2H triplet (H_2C = CCH_3 - COO - $C\underline{H}_2$ - CH_2 - O -), 3.92 δ 2H triplet (H_2C = CCH_3 - COO - CH_2 - $C\underline{H}_2$ - O -), 5.66 δ 1H singlet ($C\underline{H}_a$ = CCH_3), 6.04 δ 1H singlet ($C\underline{H}_b$ = CCH_3), 7.8 δ 1H singlet (- N$\underline{H}$ -).

2-(methacryloyl)oxyethyl-acetamidoglycylglycinate (MEAc.GLYGLY)

Yield - 30 %, M.P. 120-123 ^{0}C.

IR (nujol)

1630 cm^{-1} (methacrylic double bond), 1658 cm^{-1} (amide carbonyl), 1724 cm^{-1} (ester carbonyl), 2954 cm^{-1} (-CH stretching), 3352 cm^{-1} (- NH stretching).

^{1}H NMR (DMSO d_6)

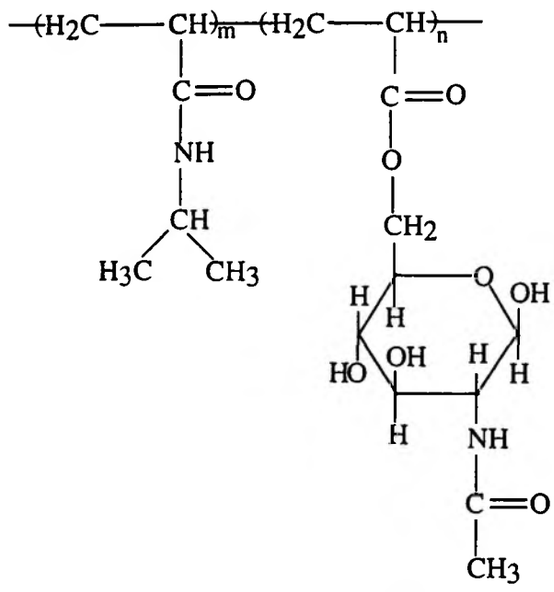
0.89 δ 3H singlet (H_2C = $CC\underline{H}_3$ -), 1.72 δ 2H triplet (- COO - $C\underline{H}_2$ - CH_2 - COO - CH_2 - NH -), 2.04 δ 2H triplet (- COO - CH_2 - $C\underline{H}_2$ - COO - CH_2 - NH -), 2.87 δ 3H singlet (- CO - $C\underline{H}_3$), 3.51 δ 2H doublet (- COO - $C\underline{H}_2$ - NH - CO - CH_2 -), 3.96 δ 2H doublet (-

COO - $C\underline{H}_2$ - NH - CO - $C\underline{H}_2$ - NH - CO - CH_3), 5.09 δ 2H singlet ($\underline{H}_2C$ = CCH3 -), 7.54 δ 2H triplet (- N$\underline{H}$ -).

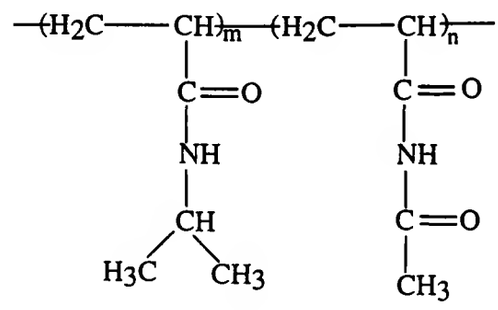
4.1.3.5 Synthesis of copolymers of NIPAM and 2-(methacryloyl)oxyethyl derivatives of ligands

0.7 M NIPAM and 0.3 M comonomer were dissolved in 50 ml of dry dimethylformamide (DMF) taken in a round bottom flask. This was stirred under continuous nitrogen purging to obtain a clear solution. Then, the solution temperature was slowly raised to 80 ^{0}C. Polymerization was initiated by adding 10 % (w/w) of AIBN dissolved in 1 ml DMF. The AIBN concentration was kept high in order to obtain the lower molecular weight polymer. This reaction mixture was maintained at 80 ^{0}C for 4-5 hours under continuous nitrogen purging. The mass was cooled to 37 ^{0}C and the polymer was precipitated in diethyl ether. The precipitated product was dried at 40 ^{0}C.

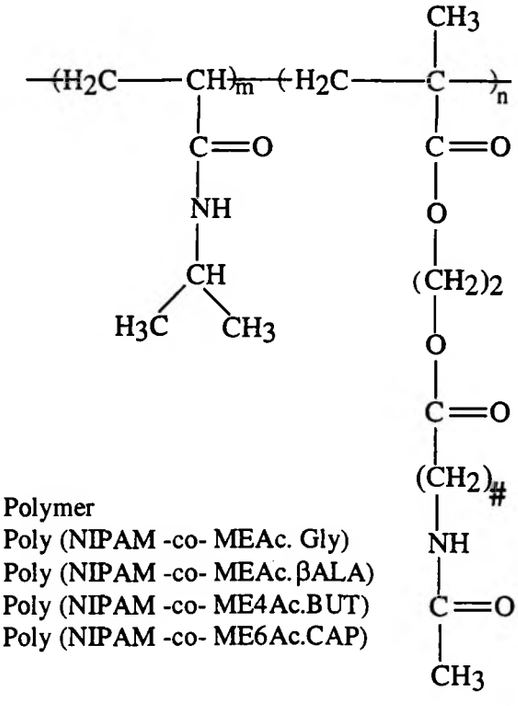
As Ac. NAG and acetamido acrylamide were soluble in water, the synthesis of the polymers comprising these monomers was carried out in water. 0.7 M of NIPAM and 0.3 M of Ac. NAG or acetamido acrylamide dissolved in 30 ml of water were taken in a round bottom flask. Polymerization was initiated at 37 ^{0}C under nitrogen purging by adding 10 % (w/w) of ammonium per sulfate (initiator) and 1 % v/w of TEMED (accelerator). Polymerization was allowed to proceed for 5-6 hours at 37 ^{0}C. Then, the polymer was precipitated by raising the temperature of the reaction mixture above lower critical solution temperature (LCST) of the polymer. The precipitated polymer was washed twice with cold double distilled water and once with cold 0.066 M phosphate buffer (pH 6.2) and dried under vacuum at room temperature. The polymers are shown schematically in Figure 4.3.



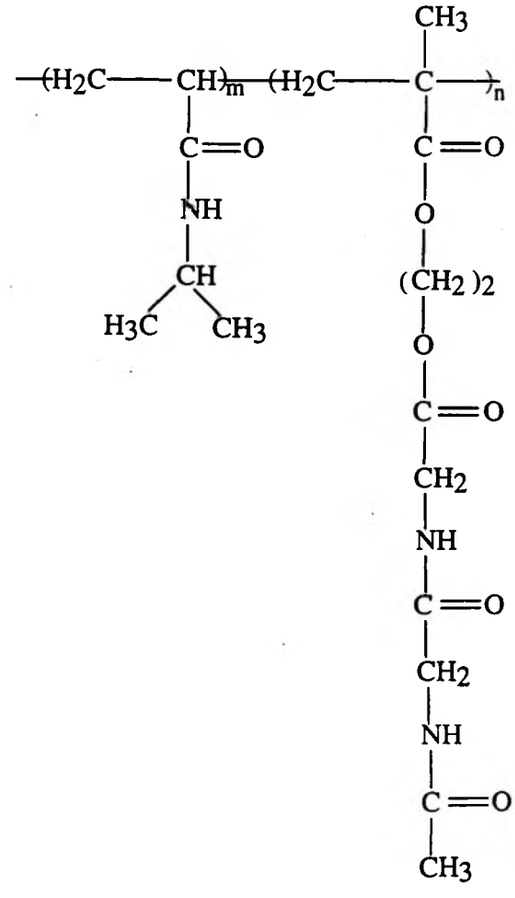
Poly(NIPAM-co-Ac.NAG)



Poly (NIPAM-co-acetamidoacrylamide)



Polymer
Poly (NIPAM -co- MEAc. Gly)
Poly (NIPAM -co- MEAc. βALA)
Poly (NIPAM -co- ME4Ac.BUT)
Poly (NIPAM -co- ME6Ac.CAP)



Poly (NIPAM -co- MEAc.GLYGLY).

Figure 4.3 : Schematic representation of polymers.
m and n are integers greater than 1.

Poly (N-isopropylacrylamide -co- O-acryloyl N-acetylglucosamine), Poly (NIPAM -co- Ac. NAG)

IR (KBr)

1550 cm $^{-1}$ (sugar ring), 1670 cm $^{-1}$ (amide carbonyl), 2875 cm $^{-1}$ (-CH stretching), 3680 cm^{-1} (-NH stretching).

^{1}H NMR (DMSO d_6)

1.0 δ [($C\underline{H}_3$)$_2$ - CH - of NIPAM], 1.5 δ [- C$\underline{H}$ - (CH_3)$_2$ of NIPAM], 1.9 δ (- NH - CO - $C\underline{H}_3$), 3.0 δ (- $C\underline{H}_2$ - O - of NAG), 3.4 δ (- $C\underline{H}_2$ - CH - of polymer), 3.9 δ (- CH_2 - C$\underline{H}$ - of polymer).

Poly (N-isopropylacrylamide -co- acetamido acrylamide), Poly (NIPAM -co- acetamido acrylamide)

IR (KBr)

1670 cm $^{-1}$ (amide carbonyl), 2930 cm $^{-1}$ (-CH stretching), 3680 cm^{-1} (-NH stretching).

^{1}H NMR (DMSO d_6)

1.0 δ [($C\underline{H}_3$)$_2$ - CH - of NIPAM], 1.5 δ [- C$\underline{H}$ - (CH_3)$_2$ of NIPAM], 2.1 δ (- NH - CO - $C\underline{H}_3$), 3.4 δ (- $C\underline{H}_2$ - CH - of polymer), 3.9 δ (- CH_2 - C$\underline{H}$ - of polymer).

Poly (N-isopropylacrylamide -co- 2-(methacryloyl)oxyethyl 2-acetamidoglycinate), Poly (NIPAM -co- MEAc.GLY)

IR (KBr)

1627 cm^{-1} (amide carbonyl), 1730 cm^{-1} (ester carbonyl), 2930 cm^{-1} (-CH stretching), 3325 cm^{-1} (-NH stretching).

^{1}H NMR (DMSO d_6)

1.0 δ [($C\underline{H}_3$)$_2$ - CH - of NIPAM], 1.5 δ [- $C\underline{H}$ - (CH_3)$_2$ of NIPAM], 1.7 δ (- $C\underline{H}_3$ of HEMA), 2.0 δ (- NH - CO - $C\underline{H}_3$), 2.5 δ (- $N\underline{H}$ - CH_2 - CO - of Gly.), 3.4 δ (- $C\underline{H}_2$ - CH - of polymer), 3.9 δ (- O - CH_2 - $C\underline{H}_2$ - O - of HEMA).

Poly (N-isopropylacrylamide -co- 2-(methacryloyl)oxyethyl 3-acetamidoβalaninate), Poly (NIPAM -co- MEAc.βALA)

IR (KBr)

1658 cm^{-1} (amide carbonyl), 1730 cm^{-1} (ester carbonyl), 2914 cm^{-1} (-CH stretching), 3327 cm^{-1} (-NH stretching).

^{1}H NMR (DMSO d_6)

0.8 δ (- $C\underline{H}_3$ of HEMA), 1.0 δ [($C\underline{H}_3$)$_2$ - CH - of NIPAM], 1.3 δ [- $C\underline{H}$ - (CH_3)$_2$ of NIPAM], 1.7 δ (- NH - CO - $C\underline{H}_3$), 2.5 δ (- CH_2 - $N\underline{H}$ - of β Ala.), 2.7 δ (- $C\underline{H}_2$ - COO - of β Ala.), 3.3 δ (- $C\underline{H}_2$ - CH - of polymer), 3.5 δ (- CH_2 - $C\underline{H}$ - of polymer), 4.0 δ (- O - CH_2 - $C\underline{H}_2$ - O - of HEMA).

Poly (N-isopropylacrylamide -co- 2-(methacryloyl)oxyethyl 4-acetamidobutyrate), Poly (NIPAM -co- ME4Ac.BUT).

IR (KBr)

1680 cm^{-1} (amide carbonyl), 1720 cm^{-1} (ester carbonyl), 2950 cm^{-1} (-CH stretching), 3500 cm^{-1} (-NH stretching).

^{1}H NMR (DMSO d_6)

0.9 δ (- $C\underline{H}_3$ of HEMA), 1.0 δ [($C\underline{H}_3$)$_2$ - CH - of NIPAM], 1.4 δ [- $C\underline{H}$ - (CH_3)$_2$ of NIPAM], 1.9 δ (- NH - CO - $C\underline{H}_3$), 2.7 δ (- $C\underline{H}_2$ - COO - of 4 ABA), 2.8 δ (- $C\underline{H}_2$ - CH_2 - COO - of 4ABA), 3.0 δ (- $C\underline{H}_2$ - NH - CO - CH_3 of 4 ABA), 3.3 δ (- $C\underline{H}_2$ - CH - of polymer), 3.5 δ (- CH_2 - $C\underline{H}$ - of polymer), 3.9 δ (- O - CH_2 - $C\underline{H}_2$ - O - of HEMA).

Poly (N-isopropylacrylamide -co- 2-(methacryloyl)oxyethyl 6-acetamidocaproate), Poly (NIPAM -co- ME6Ac.CAP).

IR (KBr)

1641 cm^{-1} (amide carbonyl), 1724 cm^{-1} (ester carbonyl), 2875 cm^{-1} (-CH stretching), 3680 cm^{-1} (-NH stretching).

^{1}H NMR (DMSO d_6)

0.9 δ (- C<u>H</u>$_3$ of HEMA), 1.0 δ [(C<u>H</u>$_3$)$_2$ - CH - of NIPAM], 1.2-1.5 δ [(C<u>H</u>$_2$)$_3$ - CH_2 - NH - of 6 ACA), 1.8 δ [- C<u>H</u> - $(CH_3)_2$ of NIPAM], 2.0 δ (- NH - CO - C<u>H</u>$_3$), 2.7 δ (- C<u>H</u>$_2$ - NH - CO - CH_3 of 6 ACA), 2.9 δ (- C<u>H</u>$_2$ - COO - of 6 ACA), 3.3 δ (- C<u>H</u>$_2$ - CH - of polymer), 3.6 δ (- CH_2 - C<u>H</u> - of polymer), 3.9 δ (- O - CH_2 - C<u>H</u>$_2$ - O - of HEMA).

Poly(N-isopropylacrylamide-co-2-(methacryloyl)oxyethyl-acetamido glycylglycinate), Poly (NIPAM -co- MEAc.GLYGLY).

IR (KBr)

1630 cm^{-1} (amide carbonyl), 1728 cm^{-1} (ester carbonyl), 2927 cm^{-1} (-CH stretching), 3327 cm^{-1} (-NH stretching).

^{1}H NMR (DMSO d_6)

0.8 δ (- C<u>H</u>$_3$ of HEMA), 1.0 δ [(C<u>H</u>$_3$)$_2$ - CH - of NIPAM], 1.5 δ [- C<u>H</u> - $(CH_3)_2$ of NIPAM], 2.4 δ (- NH - CO - C<u>H</u>$_3$), 3.1 δ (- C<u>H</u>$_2$ - CH - of polymer), 3.7 δ (- CH_2 - C<u>H</u> - of polymer), 4.0 δ (- O - CH_2 - C<u>H</u>$_2$ - O - of HEMA), 4.5 δ (- C<u>H</u>$_2$ - NH - CO - of GlyGly.), 6.5 δ (- N<u>H</u> -).

4.1.4 Characterization

4.1.4.1 Estimation of acetamido groups incorporated in thermoprecipitating polymers

Twenty milligram polymer was dissolved in 500 µl of dimethylsulfoxide (DMSO (d_6)) and acetamido groups were estimated by bruker MSL-300 FT NMR spectrometer operating at 300 MHz for protons. A 45 ^{0} pulse of 8 µsec was used to obtain the quantitative information from the proton resonances. A recycle delay of 15 seconds was kept during each accumulation. All samples were probed under identical conditions for 16 scans. The data were then processed and deconvoluted using WINNMR software. Deconvoluted spectra were taken into account to calculate exact area assigned by the acetamido group and vinyl methyl group of NIPAM as shown in Figure 4.4. The ratios of these areas were used to estimate the percentage incorporation of acetamido group in the thermoprecipitating polymer.

4.1.4.2 LCST and molecular weight measurements of thermoprecipitating polymers

LCST was estimated by turbidometric method [Boutris et al (1997)]. Molecular weights were estimated by intrinsic viscosity [Takezawa et al (1990); Fujishige (1987)]. Molecular weights of all the polymers were in the range of 4000 to 8000.

4.1.4.3 Estimation of relative inhibition of lysozyme by ligands and polymers

The procedure for estimating relative inhibition of 6-acetamido caproic acid and other ligands was as follows. 0.5 M stock solution of sodium salt of ligand (carboxyl groups were neutralized by sodium bicarbonate solution) was prepared in 0.066 M

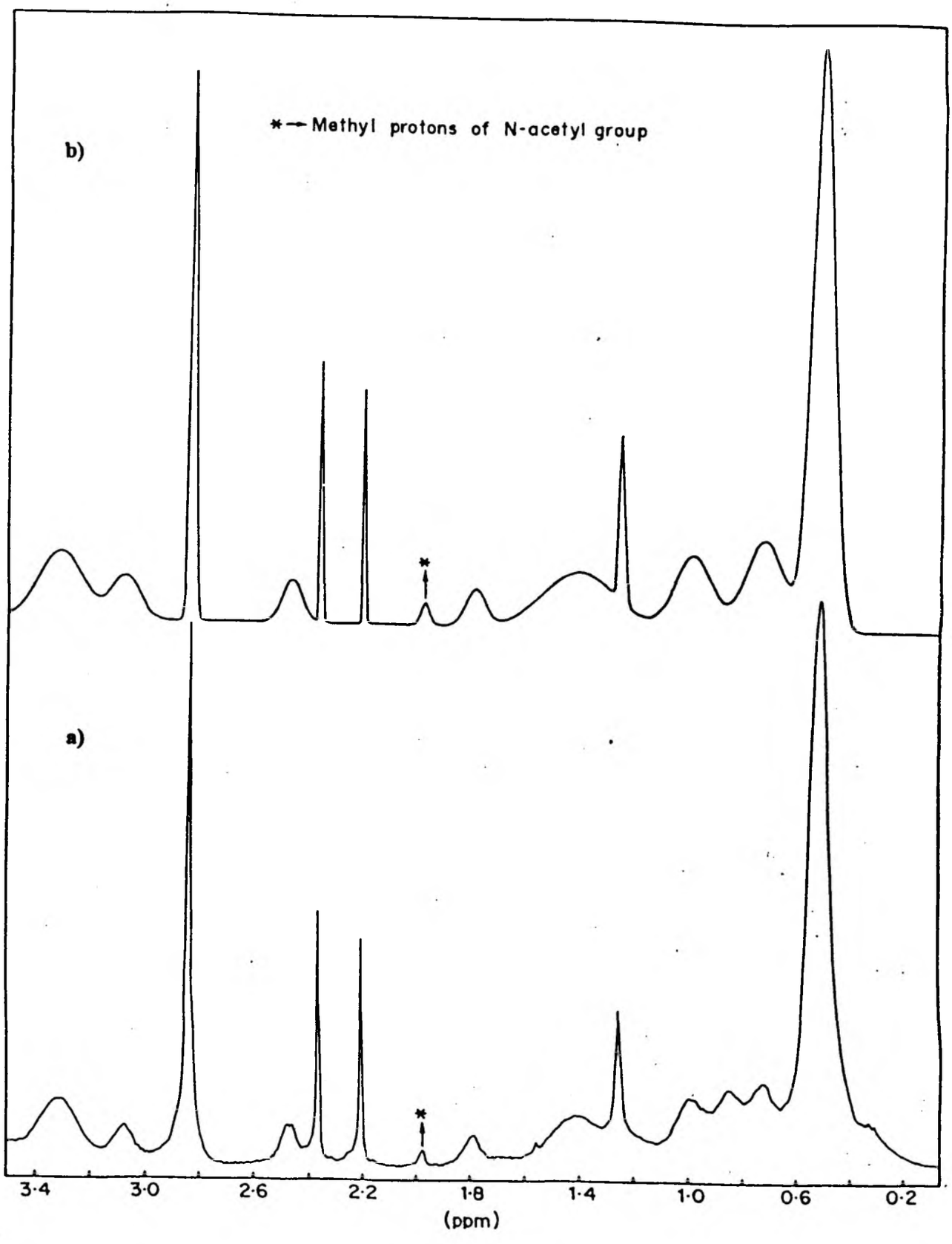


Figure 4.4: Quantification of acetamido groups in poly (NIPAM –co- ME6Ac.CAP)
a) normal spectra; b) deconvoluted spectra.

phosphate buffer pH 6.2, containing 0.0154 M sodium chloride and 0.008 M sodium azide. One milliliter of this solution containing different ligand concentration (prepared by serially diluting the stock solution) was mixed with 1.6 ml of 78 μg/ml of *Micrococcus lysodeikticus* in a 3 ml capacity glass cuvette. It was incubated for 5 minutes at 20 ^{0}C. To this mixture 0.1 ml of lysozyme (27 μg/ml) was added and mixed thoroughly. The decrease in the absorbance at 450 nm (ΔA_{450nm}) was read for 30 seconds. Similarly, a blank without an inhibitor was run and the change in the absorbance per second was calculated. From this the relative inhibition was calculated [Neuberger and Wilson (1967)]. For thermoprecipitating polymer Poly (NIPAM -co- ME6Ac.CAP), 1 % w/v solution was prepared in 0.066 M phosphate buffer pH 6.2, 0.0154 M sodium chloride and 0.008 M sodium azide. The relative inhibition of this polymer was calculated following the same procedure as described above.

The values of concentration of acetamido groups required to obtain 50 % relative inhibition for various synthetic ligands are summarized in Table 4.1. It was found that because of the solubility limitations of the polymers No.1 to 4 in Table 4.2 it was not possible to achieve 50 % inhibition using 1 % w/v solution under standard assay conditions. Since the purpose of this exercise was to study the effect of spacer and its hydrophilicity on the binding, we chose to compare I_{35} values for these polymers. For the other polymers (No.5 to 7 in Table 4.2) both I_{35} and I_{50} values have been quoted.

In Figure 4.5 a) the relative inhibition of lysozyme vs. the concentration of 6-acetamido caproic acid is shown. In Figure 4.5 b) the relative inhibition of lysozyme by Poly (NIPAM -co- ME6Ac.CAP) containing different concentration of acetamido groups is shown.

Table 4.1: Binding efficiency of synthetic ligands with lysozyme.

No.	Ligand	Milimoles of acetamido groups to achieve I_{50}[a]	$\Delta D_{293.5nm}$[b]
1	Ac. NAG	35 ± 2	0.006
2	Acetamido acrylamide	444 ± 12	N.D.
3	2-acetamido glycine	34 ± 4	0.006
4	3-acetamido βalanine	32 ± 2	0.007
5	4-acetamido butyric acid	19 ± 2	0.010
6	6-acetamido caproic acid	12 ± 1	0.015
7	Acetamido glycylglycine	8 ± 2	0.022

[a] Concentration (mM) of respective ligand to obtain 50 % relative inhibition. The values represent average from three experiments.

[b] $\Delta D_{293.5nm}$ was measured at ligand concentration at I_{50}.

N.D. not determined because ligand solution absorbed at 260-320 nm.

Table 4.2: Characterization of thermoprecipitating polymers.

No.	Polymer[a]	mM acetamido groups/g of polymer[b]	M.W (M_n)[c]	LCST (^0C)[d]	mM acetamido groups for I_{35}[e]	mM acetamido groups for I_{50}[f]
1	Poly (NIPAM –co-Ac.NAG)	2.241	8000	38	0.0050 ± 0.0004	N.D.
2	Poly (NIPAM –co-acetamido acrylamide)	2.414	6500	45	0.0160 ± 0.002	N.D.
3	Poly (NIPAM -co-MEAc.GLY)	2.759	6700	36	0.0120 ± 0.001	N.D.
4	Poly (NIPAM –co-MEAc.βALA)	3.621	7000	33	0.0120 ± 0.001	N.D.
5	Poly (NIPAM –co-ME4Ac.BUT)	1.379	4000	26	0.0032 ± 0.0004	0.0070 ± 0.0004
6	Poly (NIPAM –co-ME6Ac.CAP)	3.276	7800	21	0.0024 ± 0.0002	0.0050 ± 0.0003
7	Poly (NIPAM –co-MEAc.GLYGLY)	1.724	5000	42	0.0022 ± 0.0001	0.0030 ± 0.0001

[a] Molar feed ratio of NIPAM:acetamido ligand was 7:3 in all cases.

[b] Acetamido groups were determined by ^1H NMR technique as described in the text.

[c] Molecular weights were estimated by intrinsic viscosity method [Fujishige (1987)].

[d] LCST was determined by turbidometric method [Boutris et al (1997)].

[e] Milimoles of acetamido groups in polymer required to obtain 35 % inhibition (I_{35}). In these polymers due to solubility limitations it was not possible to achieve I_{50} using 1 % w/v polymer solution under standard assay conditions.

[f] Milimoles of acetamido groups in polymer required to obtain 50 % inhibition (I_{50}).

N.D. Not determined.

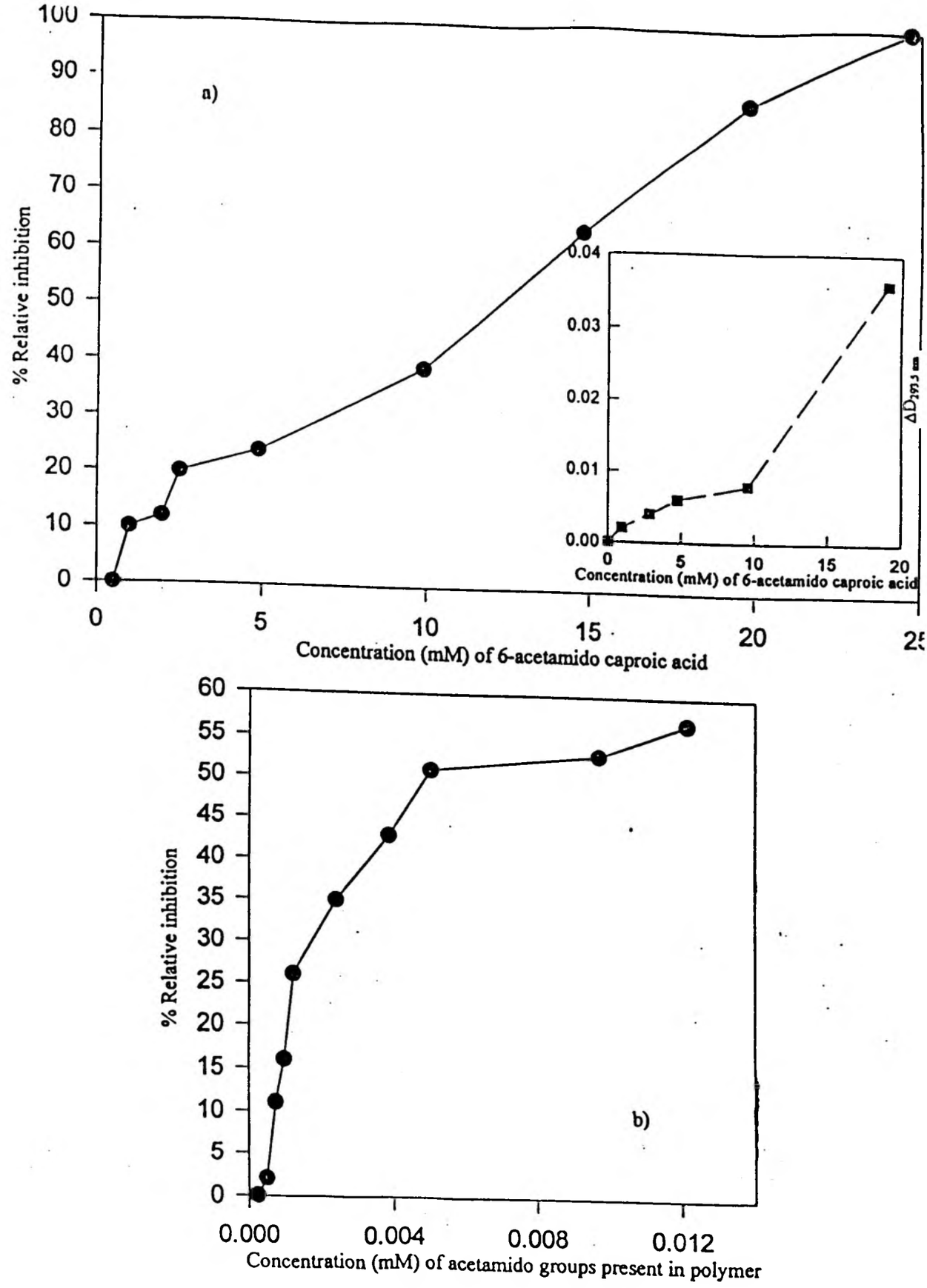


Figure 4.5: a) Percent relative inhibition of lysozyme by 6-acetamido caproic acid Inset graph of $\Delta D_{293.5nm}$ vs. concentration (mM) of 6-acetamido caproic acid; b) Percent relative inhibition of lysozyme by poly (NIPAM –co- ME6Ac.CAP)

In order to demonstrate the binding between the affinity ligand and lysozyme by differential spectrometry the following experiment was performed [Dahlquist et al (1966); Hayashi et al (1963); Rupley et al (1967a,b)]. In 1.5 ml capacity silica cuvette, 0.7 ml of varying concentrations of 6-acetamido caproic acid was mixed with 0.7 ml of lysozyme (1 mg/ml) and the absorbance of this solution was recorded at 293.5 nm, which is λ_{max} for tryptophan [Hayashi et al (1963); Neuberger and Wilson (1967)]. Similarly, the corresponding blank without the inhibitor ligand was recorded. The difference between the two readings i.e. $\Delta D_{293.5nm}$ was recorded. A plot of $\Delta D_{293.5nm}$ vs. concentration of 6-acetamido caproic acid is shown as an inset in the Figure 4.5 a).

4.1.5 Recovery of lysozyme from aqueous solution

Ten milliliter of 1 % w/v polymer solution was mixed with lysozyme solution (27 μg/ml). This was incubated at 20 ^{0}C for 16 hours with continuous shaking at 200 rpm. Then, the temperature of the mixture was raised above LCST of the polymer to precipitate the lysozyme-polymer complex. The complex was separated by centrifuging at 10,000 rpm for 20 minutes above LCST. The precipitated polymer was dissolved in dilute acetic acid solution (pH 2.0) [Ruckenstein and Zeng (1997)] to dissociate the complex. The temperature of this dissociated complex was raised above LCST and the precipitated polymer was separated by centrifugation. The clear filtrate was assayed for protein content using Lowery's procedure. The activity was measured by using *Micrococcus lysodeikticus* (78 μg/ml).

4.1.5.1 Separation of lysozyme from ovalbumin

Ten milliliter of 1 % w/v polymer solution was mixed with lysozyme and ovalbumin (27 μg/ml respectively) synthetic mixture. This solution was incubated at 20

^{0}C for 16 hrs and lysozyme was recovered by the thermoprecipitation method described above. Lysozyme recovery was estimated in terms of unit activity recovered/mM of acetamido groups present in the polymer.

4.1.6 Stability studies of thermoprecipitating polymers containing synthetic ligands and NAG

Ten milliliter of thermoprecipitating polymer (1 % w/v) was mixed with 27 μg/ml lysozyme solution. It was allowed to incubate at 20 ^{0}C for 16 hrs. Lysozyme was recovered by the procedure described earlier. The plot of specific activity recovered vs. number of cycles is shown in Figure 4.6. The weight of thermoprecipitating polymer before and after each cycle was measured. Almost 90 % of the polymer could be recovered after every cycle. Accordingly, in the next cycle, appropriate amount of lysozyme solution was added to maintain the polymer/lysozyme ratio constant.

4.2.0 Results and Discussion

The objective of the present work was to develop new, efficient, stable N-acetylated ligands, which can be used in place of NAG for the recovery of lysozyme. Rupley et al (1967b) reported that about 50-70 % of the total free energy of NAG binding to lysozyme is contributed by N-acetyl group. Glucose moiety contributes only upto 20 %.

Thermoprecipitating affinity copolymers of NIPAM and N-acryloyl straight chain amino acids of the formula CH_2=CH-CO-NH$(CH2)_n$COOH; where n varied from 1 to 5, were reported in previous chapter. When trypsin inhibitor, para aminobenzamidine

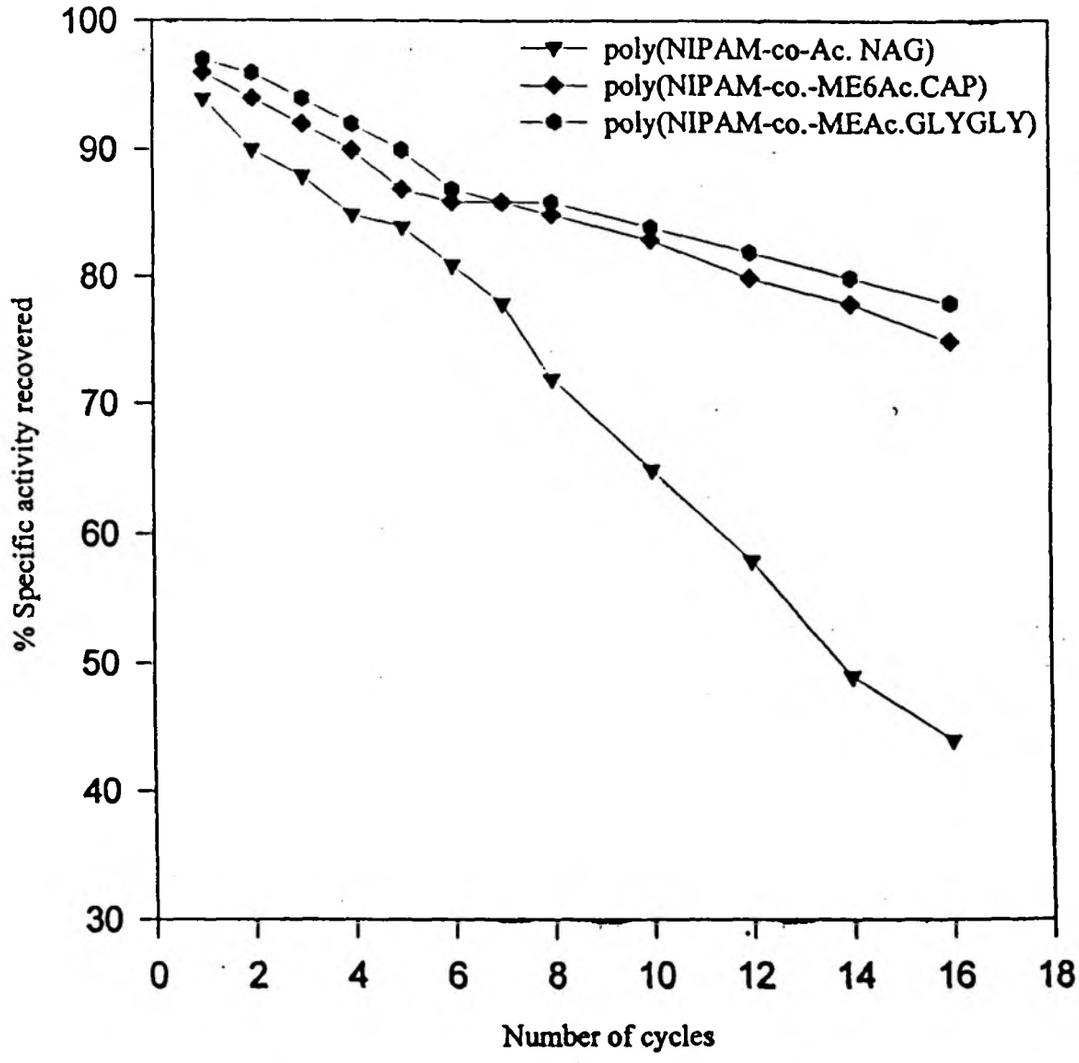


Figure 4.6: Comparative stability of different affinity thermoprecipitating polymers.

(PABA) was linked to carboxyl groups of these polymers, increasing inhibition of trypsin was observed, with increasing spacer chain length (Vaidya et al, 1999). Thus straight chain amino acids provided two advantages-1) Derivatization at both -NH_2 and -COOH terminal and 2) Enhancement in the ligand-active site interactions as a result of the incorporation of the spacers. Similar approach was used to synthesize ligands and thermoprecipitating polymers as described in the following sections.

4.2.1 Lysozyme binding with acetamido group

A number of ligands containing acetamido groups were synthesized by N-acetylating a series of amino acids of increasing chain length and hydrophilicities (Figure 4.1). These were then reacted with acryloyl chloride/HEMA so that these could be incorporated into the polymer structure and which further enhanced the spacer chain length as well as hydrophilicity (Figure 4.2). The efficiency of binding of these ligands with lysozyme was evaluated by the lysozyme inhibition test using *Micrococcus lysodeikticus* as a substrate. The values of the concentration of acetamido groups required to achieve 50 % inhibition of lysozyme are summarized in Table 4.1. The value reported for Ac. NAG (35 mM) is comparable to that of NAG (50 mM) reported by Neuberger and Wilson (1967). In these cases, since the acetamido group is not a part of the polymer chain, the steric considerations do not influence the binding. Yet when the glucose moiety in NAG is eliminated (as in acetamido acrylamide), binding with lysozyme is significantly weakened as reflected in increase of I_{50} from 35 mM to 444 mM. Similar effect was observed when we synthesized the ligand 4-acetamido phenyl acrylate, in which glucose was replaced by phenyl ring. In fact the polymer comprising this ligand exhibited very weak lysozyme binding (I_{35} = 100 mM). The binding efficiency of this

ligand in the monomeric form could not be estimated, as it was insoluble in aqueous solution. These two results suggested that ligand microenvironment should be hydrophilic as in the case of NAG. Moreover, there should be a spacer between the ligand i.e. acetamido group and the polymer backbone. Incorporation of ethylene glycol, a hydrophilic spacer between the acryloyl group and the acetamido group significantly enhances the binding with lysozyme as described in the following section.

-COOH groups in all ligands were converted to –COONa by treatment with stoichiometric amounts of sodium bicarbonate so as to prevent nonspecific interactions of acidic groups in the ligands with lysozyme. This ensured that any inhibition obtained by ligands was only due to the acetamido groups. The values of I_{50} in the case of 2-acetamido glycine (34 ± 4 mM) and 3-acetamido βalanine (32 ± 2 mM) are practically the same as for Ac. NAG (35 ± 2 mM). With increase in the chain length of amino acid to 4-acetamido butyric acid and 6-acetamido caproic acid the I_{50} value decreased further to 19 ± 2 mM and 12 ± 1 mM respectively. Incorporation of a more hydrophilic amino acid viz. glycylglycine leads to a further decrease in I_{50} value to 8 ± 2 mM. These results are consistent with the results reported in earlier chapter for binding between N-acylbenzamidines and trypsin. In these cases the microenvironment around PABA was modified by the hydrophobicity of the acyl chain. This led to enhance hydrophobic interactions within the hydrophobic slit in the active site of trypsin [Mares-Guia and Shaw (1965)]. In lysozyme, the active site is situated in a more hydrophilic environment [Ehsani et al (1997); Fujimoto et al (1993)]. Consequently, increased hydrophilicity around the acetamido group leads to enhanced binding with lysozyme. It is interesting to note that the values of I_{50} in these cases are substantially lower (25-50 %) than that of

NAG. Further, these values are substantially lower than those reported by Neuberger and Wilson (1967) for a series of synthetic D-glucosamine derivatives (50-230 mM). This confirms that the presence of the glucose residue is not as crucial for binding with lysozyme as the acetamido group. Creating a hydrophilic environment around the acetamido group can enhance the binding. Perhaps the role of glucose in chitin/chitosan is to act merely as a hydrophilic spacer between the macromolecular main chain and the acetamido group.

4.2.2 Similarity in lysozyme binding of new ligands and NAG

Hayashi et al (1963) and Neuberger and Wilson (1967) showed that the absorbance of lysozyme at 293.5 nm decreased sharply when it was bound to an inhibitor i.e. NAG. This is because one of the tryptophan residues in lysozyme, which absorbs at 293.5 nm, is removed from its solvent accessible and hydrophilic environment to a less polar hydrophobic environment [Dahlquist et al (1966)]. This reduction in absorbance at 293.5 nm was also found to correlate with the increase in the relative inhibition of lysozyme. Moreover, both these factors i.e. $\Delta D_{293.5nm}$ and relative inhibition were found to depend on the concentration of the ligand used. As shown in Table 4.1 the efficiency of binding of synthetic ligands with lysozyme increased with the spacer chain as well as hydrophilicity.

As an example, inhibition data for 6-acetamido caproic acid is shown in Figure 4.3 a). The decrease in the absorbance at 293.5 nm ($\Delta D_{293.5nm}$) was plotted as a function of concentration of 6-acetamido caproic acid (inset Figure 4.3 a). It was found that with an increase in the concentration of ligand, $\Delta D_{293.5nm}$ value increased. The nature of this graph is identical with the graph, which depicts the percent relative inhibition vs.

concentration of 6-acetamido caproic acid. This shows that as more and more tryptophanyl residues shift to a hydrophobic environment, the activity of lysozyme decreases correspondingly. It confirms that the synthetic ligands containing acetamido groups act in the same manner as NAG but exhibit more efficient binding with lysozyme than NAG.

4.2.3 Lysozyme binding with the thermoprecipitating polymers

Having thus established the efficacy of synthetic ligands in binding with lysozyme, thermoprecipitating copolymers containing these ligands were synthesized by copolymerization with NIPAM. The molecular weights of these copolymers as well as the LCST and I_{35}/I_{50} values are summarized in Table 4.2. Low molecular weight polymers were synthesized to avoid entanglement and crowding effects [Vaidya et al (1999); Luong et al (1988)]. The LCST of the thermoprecipitating polymer decreased as the hydrophobicity of the comonomer increased and LCST increased with the hydrophilicity of the comonomer (Table 4.2). These findings are consistent with those reported in literature [Badiger et al (1998)]. The acetamido group content in the polymer was estimated by proton NMR and it was found that the acetamido content (mM/g of polymer) in these polymers were different. To take into account these differences, I_{50} and I_{35} values were expressed in terms of mM of acetamido groups.

The most significant finding of these experiments is a sharp decrease in the values of I_{50} for the thermoprecipitating polymers containing the affinity ligands *vis a vis* their monomeric counter parts (Tables 4.1 and 4.2). The decrease is approximately three to four orders of magnitudes, which indicates tremendous enhancement in binding with lysozyme when the ligand is incorporated in the thermoprecipitating polymer. This

behavior was not observed when the ligands containing PABA were incorporated in the thermoprecipitating polymer and the binding with trypsin was examined. The enhancement in the binding observed in the present case can be explained on the basis of the nature of interaction between NAG units in chitin and lysozyme. Dahlquist et al (1966) reported that the binding of partial hydrolysis products of chitin increased markedly from monomer to tetramer. For chitotetraose dissociation constant (K_s) was 9.45 * 10^{-6}M whereas for chitotriose, chitobiose and NAG these values were 6.58 * 10^{-6}, 1.75 * 10^{-4} and 4-6 * 10^{-2} M respectively. Thus, from monomer to trimer the binding strength increased by four orders of magnitude. But, there was no significant difference in K_s values of chitotriose and chitotetraose. Therefore, it was concluded that minimum of three NAG units are involved in binding active site cleft of lysozyme. Neuberger and Wilson (1967) investigated the binding between lysozyme and various synthetic derivatives of D-glucosamine. The I_{50} value for NAG was 50 mM, which dropped in the case of N,N' diacetylchitobiose by almost two orders of magnitude (0.6 mM). This enhancement in binding was concomitantly reflected in enhanced value of $\Delta D_{293.5nm}$ from 0.069 for NAG to 0.085 for N,N' diacetylchitobiose.

It is therefore logical to expect that the binding efficiency will be considerably enhanced in the case of thermoprecipitating polymers, which contain multiple acetamido units attached through a spacer to the main chain polymer. This situation is analogous to chitin polymer except that the later polymer can be hydrolyzed by lysozyme, while the thermoprecipitating polymer synthesized in this work is not. Unfortunately, the experimental results obtained for the copolymers could not be complemented by the differential spectral measurements since NIPAM also absorbed in the same region i.e.

280-300 nm. It can therefore be reasonably concluded that very low I_{50} and I_{35} values obtained in the case of thermoprecipitating polymers result from enhanced binding between the multiple acetamido groups and lysozyme.

4.2.4 Recovery of lysozyme from aqueous solutions: the spacer effect

In comparison to poly (NIPAM -co- Ac. NAG) the recovery as well as the activity of lysozyme recovered was poor for poly (NIPAM -co- acetamido acrylamide). Since, the glucose unit, which acts as a hydrophilic spacer, is eliminated from the structure. The effect of type of spacer on the recovery of lysozyme from aqueous solutions was also studied (Table 4.3). With increase in the spacer chain length from glycine to glycylglycine, the recovery of protein was found to increase from 176 to 752 μg/mM of acetamido groups and the corresponding increase in the activity recovery was 6850 to 34455 units/mM of acetamido groups respectively. Thus, the increment in the specific activity recovery was 14.5 % when glycine was replaced by glycylglycine. Also, the later polymer comprising hydrophilic spacer glycylglycyl exhibited almost two fold higher protein and activity recovery as compared to the polymer containing NAG. In order to unequivocally demonstrate that the inhibition is due to the spacers containing acetamido groups, a control experiment was carried out using NIPAM homopolymer. It was found that NIPAM homopolymer devoid of acetamido groups did not inhibit the lysozyme activity. This demonstrates that the inhibition of lysozyme by the thermoprecipitating polymer is due to the spacer containing acetamido groups only.

In the case of poly (NIPAM -co- ME6Ac.CAP), the yield in terms of protein and activity recovery (420 μg/mM and 18956 units/mM of acetamido groups respectively) was lower as compared to poly (NIPAM -co- ME4Ac.BUT) (548 μg/mM and 23495

Table 4.3: Separation of lysozyme

No.	Thermoprecipitating polymer[a]	Lysozyme recovery[b]			Lysozyme activity recovery[c]
		Protein (μg/mM of acetamido groups)	Unit activity / mM of acetamido groups	% specific activity	
1	Poly (NIPAM -co-Ac.NAG)	410 ± 5	18072 ± 210	94	17590 ± 200
2	Poly (NIPAM -co-acetamido acrylamide)	168 ± 2	6711 ± 75	85	6352 ± 70
3	Poly (NIPAM -co-MEAc.GLY)	176 ± 1	6850 ± 70	83	6657 ± 72
4	Poly (NIPAM -co-MEAc.βALA)	201 ± 2	8202 ± 70	87	7978 ± 65
5	Poly (NIPAM -co-ME4Ac.BUT)	548 ± 5	23495 ± 240	91	22516 ± 220
6	Poly (NIPAM -co-ME6Ac.CAP)	420 ± 4	18956 ± 190	96	16484 ± 175
7	Poly (NIPAM -co-MEAc.GLYGLY)	752 ± 8	34455 ± 350	97.5	33672 ± 320

[a] Molar feed ratio of NIPAM:acetamido ligand was 7:3 in all cases.

[b] 10 ml polymer (1 % w/v) solution was mixed with lysozyme (27 μg/ml) and was equilibrated for 16 hrs. at 20 ^{0}C. Then the protein and activity of lysozyme was measured as given in experimental section. One unit activity is defined in the text.

[c] 10 ml polymer (1 % w/v) solution was mixed with Lysozyme-ovalbumin mixture with identical weights (27 μg/ml respectively) and equilibrated for 16 hrs. at 20 ^{0}C. Then activity of lysozyme recovered was measured as given in experimental section.

The values represent the average taken from three experiments.

units/mM of acetamido groups respectively). At present this difference can not be explained. More interestingly the lysozyme bound to poly (NIPAM -co- ME6Ac.CAP) could be recovered almost completely (96 % recovery of specific activity) because lysozyme being a hydrophilic enzyme [Ehsani et al (1997)] non-specific hydrophobic interactions with this polymer are negligible. The highest specific activity recovery was found in the case of poly (NIPAM -co- MEAc.GLYGLY) which is 97.5 %. Thus it shows that for the maximum recovery of lysozyme the acetamido ligand containing hydrophilic spacer is most suitable. Moreover, the lysozyme was found to be stable at the LCST temperature of this polymer i.e. at 42 ^{0}C. Chern et al. (1996 a) showed that Eudragit L 100 could precipitate 95 % of lysozyme, as compared to only 7 % precipitated by acrylic latex, but in Eudragit L 100 only 30 % of the precipitated lysozyme could be recovered as compared to the complete recovery by the latex particles.

4.2.5 Separation of lysozyme from ovalbumin

The selectivity of thermoprecipitating polymers in the separation of lysozyme and ovalbumin from aqueous solution was also evaluated. The activity of lysozyme recovered per mM of acetamido groups was found to increase from 6657 units for poly (NIPAM -co-MEAc.GLY) to 33672 units for poly (NIPAM -co- MEAc.GLYGLY). Thus, in a lysozyme-ovalbumin mixture thermoprecipitating polymers exhibit selectivity towards lysozyme. Comparing the activity recovered from the aqueous solution and the lysozyme-ovalbumin mixture (Table 4.3), it can be concluded that due to the slight hydrophobic nature of 6-acetamido caprylate, it binds nonspecifically with the ovalbumin, which is also a hydrophobic protein (average hydrophobicity per mole of amino acid residue i.e. H_ϕ is 980 cal/mole) as compared to lysozyme (with H_ϕ is 890 cal/mole). Therefore, these

sites were masked with bulky ovalbumin (partial specific volume 0.746 ml/g) and therefore were not available for binding with lysozyme (partial specific volume 0.714 ml/g [Ehsani et al (1997)]. This was reflected in the loss of about 2472 unit activity/mM of acetamido groups recovered from the mixture as compared to the activity recovered from the aqueous solution. In this respect also poly (NIPAM -co- MEAc.GLYGLY) was found to be a better choice which exhibits minimum loss of activity (783 units/mM acetamido groups) with the maximum recovery of activity (33672 units/mM acetamido groups) of lysozyme from a synthetic mixture of lysozyme and ovalbumin. Moreover, this polymer shows two-fold enhancement in recovery of activity/mM of acetamido groups as compared to poly (NIPAM -co- Ac. NAG). Thus poly (NIPAM -co- MEAc.GLYGLY) was found to be a better choice for the recovery of lysozyme from ovalbumin.

4.2.6 Thermoprecipitating polymers : Stability studies

One of the limitations of chitin in the recovery of lysozyme is its susceptibility to hydrolytic degradation. Hirano et al (1991) showed that the stability against hydrolysis could be enhanced by synthesizing N-lower fatty acyl derivatives of chitin. It was hoped that the synthetic ligands devoid of glucose residues would be more stable than NAG. As described in the experimental section, sixteen recovery cycles were run. The percent recovery in terms of specific activity as a function of number of cycles is shown in Figure 4.6. The data show that even after 16 cycles the decrease in percent recovery in terms of specific activity was 20 % and 21 % for poly (NIPAM -co- MEAc.GLYGLY) and poly (NIPAM -co- ME6Ac.CAP) respectively. But for poly (NIPAM -co- Ac. NAG), the decrease in the percent recovery in terms of specific activity was 50 %. The probable

reason for this difference is that glucose is a carbon source for many microbes. It also undergoes hydrolytic degradation [Hirano et al. (1991)]. Thus, after 16 cycles, some of the NAG molecules may have lost their efficiency for lysozyme binding. Also, the 20 % loss exhibited by synthetic polyligands is partly due to the actual polymer loss in each cycle of solubility/precipitation. This could be overcome by enhancing the molecular weight of the polymer. Thus, the synthetic thermoprecipitating polymers provide a more stable system for the recovery of lysozyme as compared to the polymer based on NAG.

4.3.0 Conclusions

Ligands containing acetamido group and a spacer were conjugated with an acrylic monomer and copolymerized with NIPAM. The binding of ligands with lysozyme was enhanced by increasing the spacer chain length and hydrophilicity as demonstrated by inhibition experiments as well as differential spectral studies. The synthetic ligands exceeded the binding exhibited by NAG itself. Thus, during the development of new affinity ligands for enzyme separation, it is crucial to take into consideration the hydrophilic/hydrophobic nature of the active site of enzyme. Incorporation of the synthetic ligands in thermoprecipitating polymers enhanced binding further by three orders of magnitude. The recovery of lysozyme and activity of lysozyme recovered from aqueous solution as well as lysozyme-ovalbumin mixture increases with the length and the hydrophilicity of the spacer. The synthetic ligands also exhibited better stability than NAG. Thus, the present investigations could lead to a new method for the recovery of lysozyme.

Chapter 5

ACIDIC THERMOPRECIPITATING POLYMERS FOR LYSOZYME RECOVERY

5.0.0 Introduction

In the previous chapter, the spacers containing amino acid functionalities and increasing methylene chain length were derivatized to obtain polymerizable synthetic ligands containing pendant acetamido groups. These monomers were copolymerized with N-isopropylacrylamide (NIPAM) to obtain thermoprecipitating polymers containing affinity ligands. It was demonstrated that one could synthesize affinity ligands devoid of glucose moiety, which were as efficient as N-acetylglucosamine (NAG). Since the lysozyme is a basic protein, cationic exchangers are used to recover lysozyme on commercial scales. These operations suffer from the same limitations as the affinity chromatography operations. Thermoprecipitation of lysozyme using acidic copolymers of NIPAM would overcome some of these problems. However, lysozyme recovery using thermoprecipitating polymers has not been reported so far. In this chapter recovery of lysozyme using copolymers of NIPAM and acidic comonomers which exploits electrostatic interactions for binding with lysozyme is discussed and the results compared with those obtained using affinity ligands discussed in the earlier chapter. Copolymers of NIPAM and strongly acidic monomers are superior to affinity polymers comprising both natural ligands such as NAG as well as synthetic affinity ligands developed in previous chapter.

Egg white contains a large number of proteins viz. lysozyme (3.5 % w/w), ovalbumin (54 % w/w), ovatransferin and ovomucoid (12 % w/w) and globulins (8 % w/w). Lysozyme is the only protein in the egg white which is positively charged at physiological pH (pI = 10) [Ruckenstein and Zeng (1997)] and exhibits strong electrostatic interactions with carboxylic and sulfonic acids at physiological pH. A

number of these have been used for the recovery of lysozyme by ion exchange [Shinano et al (1993); Vachier et al (1995); Shuichi et al (1994)]. Besides ion exchange columns, the other techniques used so far for the recovery of lysozyme are enlisted in Table 5.1. Recovery of lysozyme using ion exchange resins practiced commercially poses operational difficulties. Alternative methodologies such as ion exchange of lysozyme during permeation across a hollow fiber have been investigated to overcome these problems [Shinano et al (1993)].

Based on the results obtained for the separation of lysozyme by affinity thermoprecipitation technique reported in the previous chapter as well as recovery by ion exchange processes reported in the literature, it was envisaged that it should be possible to recover lysozyme selectively from aqueous solutions as well as from egg white by using thermoprecipitating polymers containing acidic functions.

In this chapter recovery of lysozyme using thermoprecipitating polymers containing acidic monomers is reported. Not only the polymers designed specifically for this purpose exhibited higher recovery of lysozyme, but they were also more stable than the polymers containing NAG.

5.1.0 Experimental section

5.1.1 Materials

Acrylic acid, methacrylic acid and N-isopropylacrylamide (NIPAM) were purchased from Aldrich. Lysozyme (3x crystallized, activity 47,000 units/mg solid). One unit activity of lysozyme is the change in the absorbance (ΔA_{450}) of 0.001 per minute at pH 6.2 at 25 ^{0}C using a suspension of Micrococcus lysodeikticus (78 μg/ml) as a substrate in a 2.7 ml reaction mixture (1 cm light path). *Micrococcus lysodeikticus*

Table 5.1: Different techniques used for the isolation of lysozyme.

Technique	Comments	Reference
Salt precipitation	First bulk separation method. The problems of salt removal and low purity product.	Alderton and Fevold (1946)
Reverse miceller extraction	Cibacron blue conjugated to lecithin was used for micelle formation.	Naoe et al (1995); Sun et al (1998).
Adsorption on zeolites and hydroxyapatites	Multilayer pH dependent adsorption of lysozyme on ultrastable zeolite was done. As well as phosphate ions was specifically used for lysozyme adsorption.	Kandori et al (1997); Klint and Eriksson (1997).
Hydrophobic perfusion chromatography	Lysozyme adsorbed on mild hydrophobic stationary phase at high salt concentration. Elution was achieved by reducing salt concentration.	Staby and Mollerup (1996).
Ultrafiltration	Membranes with covalently anchored polysulfone and polysulfite groups were used which exhibits different binding capacities. In an another investigation lysozyme was precipitated using polyacrylic acid and then the resulting complex separated on size using ultrafiltration membrane.	Bozzano and Glatz (1991); Ehsani et al (1997); Iritani et al (1997); Tsuneda et al (1994).
Gradiflow filtration	Combines membrane filtration and electrophoresis	Wringley and Manusu (1996)
Affinity chromatography	Chitin, chitosan and cibacron blue were used as the affinity ligands. The support used were cellulose or dextran. The capacity of such columns were high (3-5 mg/ml of resin).	Imoto and Yagishita (1973); Hirano et al (1991); Liapis et al (1989); Mayes et al (1990); Cherkasov and Kravchenko (1970); Safarik and Safarikova (1993); Junowicz and Charm (1975); Yamasaki and Eto (1981)
Affinity membrane filtration	Macroporous chitin membranes were used.	Ruckenstein and Zeng (1997)

(*Micrococcus luteus* ATCC No. 4698), N-acetylglucosamine (NAG) etc. were purchased from Sigma. Itaconic acid was supplied by Spectrochem Ltd; India. 2-acryloylamido-2-methyl-propane-sulfonic acid (Amps) was purchased from Fluka. Ammonium per sulfate, tetramethylethylenediamine (TEMED) etc. were supplied by local suppliers. All chemicals and solvents were of analytical grade and were used as received.

5.1.2 Instrumentation

^{1}H NMR spectra were recorded on Bruker superconducting FT NMR AC 200 operating at 200 MHz. IR spectra were recorded on Shimadzu 8300 FTIR spectrometer. Electronic absorption measurements were done on Shimadzu UV 1601 spectrophotometer. Melting points were recorded on Mettler melting point apparatus.

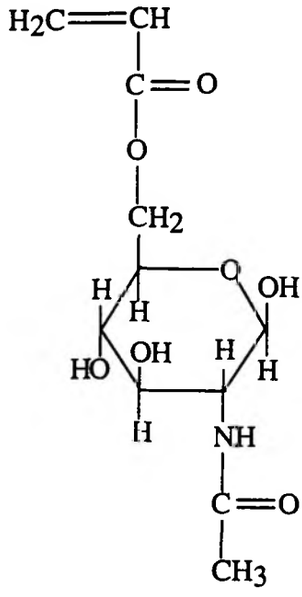
5.1.3 Methods

5.1.3.1 Synthesis of O-acryloyl N-acetyl glucosamine (Ac. NAG)

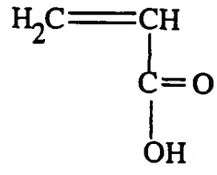
This monomer was synthesized by following the same procedure as described in the previous chapter. Yield-80 %. M.P. 170 ^{0}C. The IR and ^{1}H NMR characterization of this monomer is also given in the previous chapter. The structures of all the monomers are shown in Figure 5.1.

5.1.3.2 Synthesis of copolymers of NIPAM and acidic monomers

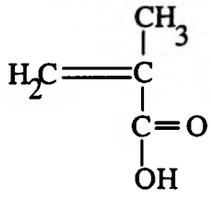
Thirty milliliters aqueous solution containing NIPAM (0.98 M) and acidic monomer (0.02 M) was polymerized at 37 ^{0}C under nitrogen purging by the addition of 10 % (w/w) of ammonium per sulfate and 1 % v/w of TEMED. Polymerization was allowed to proceed for 5-6 hours at 37 ^{0}C and then the polymer was precipitated by raising the temperature of the reaction mixture above the lower critical solution temperature (LCST) of the polymer. The precipitated polymer was washed twice with



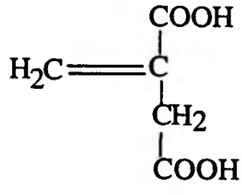
Ac. NAG



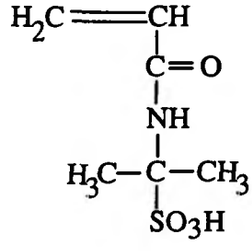
Acrylic acid



Methacrylic acid



Itaconic acid



Amps

Figure 5.1 : Schematic representation of all the monomers used for lysozyme separation.

cold double distilled water and once with cold 0.066 M phosphate buffer (pH 6.2) and dried under vacuum at room temperature. Polymer comprising NIPAM and Ac. NAG in the mole ratio 98:2 was synthesized following the same procedure. It was observed that this polymer did not exhibit inhibition of lysozyme. The polymer comprising NIPAM and Ac. NAG in the mole ratio 70:30, inhibited lysozyme. Hence in all subsequent experiments poly (NIPAM -co- Ac. NAG) comprising the two monomers in the mole ratio 70:30 was used. All polymers were characterized by ^{1}H NMR and the spectral data summarized below. Schematic representation of all the polymers are shown in Figure 5.2.

Poly (N-isopropylacrylamide -co- O-acryloyl N-acetylglucosamine), Poly (NIPAM-co-AC.NAG).

IR (KBr)

1550 cm $^{-1}$ (sugar ring), 1670 cm $^{-1}$ (amide carbonyl), 2875 cm $^{-1}$ (-CH stretching), 3680 cm^{-1} (-NH stretching).

^{1}H NMR (DMSO d_6)

1.0 δ (- ($C\underline{H}_3$)$_2$ - CH - of NIPAM), 1.5 δ (- $C\underline{H}$ - (CH_3)$_2$ - of NIPAM), 1.9 δ (- NH - CO - $C\underline{H}_3$ -), 3.0 δ (- $C\underline{H}_2$ - O - of NAG), 3.4 δ (- $C\underline{H}_2$ - CH - of polymer), 3.9 δ (- $C\underline{H}$ - CH_2 - of polymer).

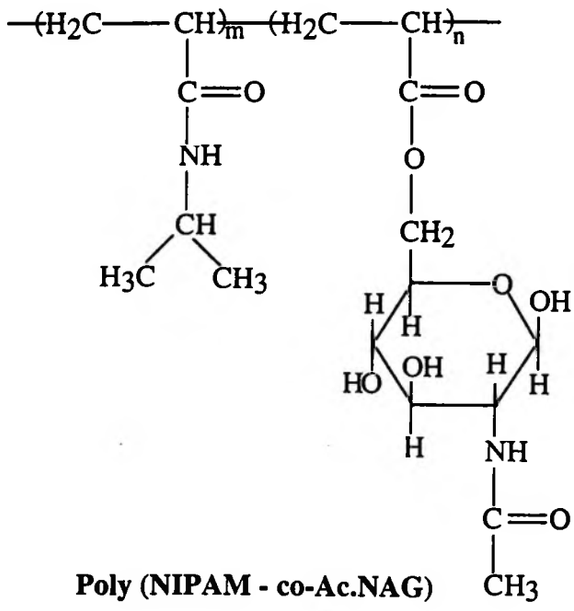
Poly (N-isopropylacrylamide -co- acrylic acid), Poly (NIPAM -co- acrylic acid).

IR (KBr)

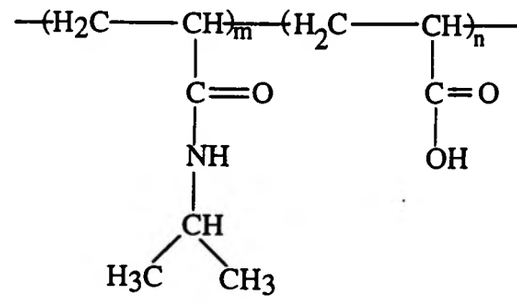
1675 cm $^{-1}$ (amide carbonyl), 1735 cm $^{-1}$ (acid carbonyl), 2930 cm $^{-1}$ (-CH stretching), 3680 cm^{-1} (-OH, -NH stretching).

^{1}H NMR (DMSO d_6)

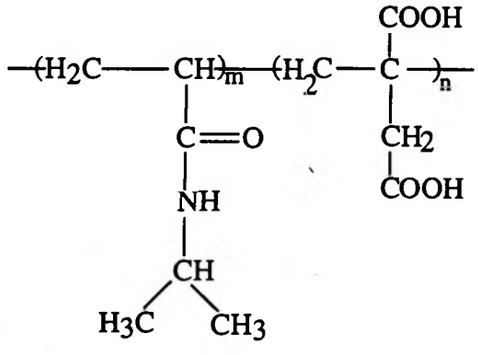
1.02 δ (- ($C\underline{H}_3$)$_2$ - CH - of NIPAM), 2.49 δ (- $C\underline{H}$ - (CH_3)$_2$ - of NIPAM), 2.72 δ



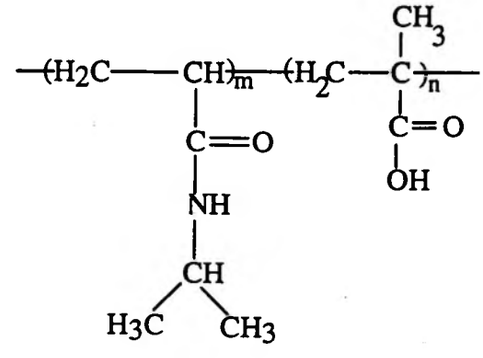
Poly (NIPAM - co-Ac.NAG)



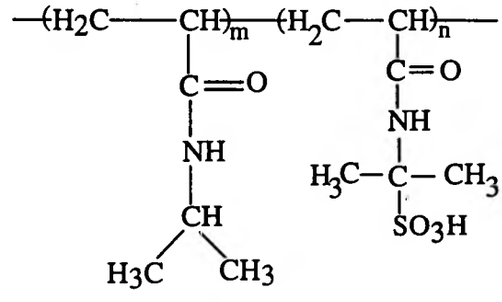
Poly (NIPAM -co- acrylic acid)



Poly (NIPAM -co- itaconic acid)



Poly (NIPAM -co- methacrylic acid)



Poly (NIPAM -co- Amps)

Figure 5.2 : Schematic representation of various acidic thermoprecipitating polymers. m and n are integers greater than 1.

(- C<u>H</u>$_2$ - CH - of acrylic), 2.88 δ (- CH$_2$ - C<u>H</u> - of acrylic), 3.35 δ (- C<u>H</u>$_2$ - CH - of NIPAM), 3.80 δ (- CH$_2$ - C<u>H</u> - of NIPAM), 7.2 δ (- N<u>H</u> -), 8.0 δ (- COO<u>H</u>).

Poly (N-isopropylacrylamide -co- methacrylic acid), Poly (NIPAM -co- methacrylic acid).

IR (KBr)

1654 cm $^{-1}$ (amide carbonyl), 1720 cm $^{-1}$ (acid carbonyl), 2935 cm $^{-1}$ (-CH stretching), 3315 cm^{-1} (-NH stretching), 3600 cm^{-1} (-OH stretching).

^{1}H NMR (DMSO d$_6$)

1.01 δ (- (C<u>H</u>$_3$)$_2$ - CH - of NIPAM), 2.48 δ (- C<u>H</u> - (CH$_3$)$_2$ - of NIPAM), 2.71 δ (- C - C<u>H</u>$_3$ of methacrylic), 3.37 δ (- C<u>H</u>$_2$ - CH - of NIPAM), 3.79 δ (- CH$_2$ - C<u>H</u> - of NIPAM), 7.29 δ (- N<u>H</u> -), 8.0 δ (- COO<u>H</u>).

Poly (N-isopropylacrylamide -co- itaconic acid), Poly (NIPAM -co- itaconic acid).

IR (KBr)

1650 cm $^{-1}$ (amide carbonyl), 1730 cm $^{-1}$ (acid carbonyl), 2950 cm $^{-1}$ (-CH stretching), 3450 cm^{-1} (-NH stretching), 3600 cm^{-1} (-OH stretching).

^{1}H NMR (DMSO d$_6$)

1.03 δ (- (C<u>H</u>$_3$)$_2$ - CH - of NIPAM), 2.51 δ (- C<u>H</u> - (CH$_3$)$_2$ - of NIPAM), 3.19 δ (- C<u>H</u>$_2$ - CH - of NIPAM), 3.85 δ (- CH$_2$ - C<u>H</u> - of NIPAM), 5.69 δ (- C<u>H</u>$_2$ - COOH of itaconic), 7.94 δ (- N<u>H</u> -).

Poly (NIPAM -co- 2-acryloylamino-2methyl-propane-sulfonic acid), Poly (NIPAM -co- Amps).

IR (KBr)

1638 cm $^{-1}$ (amide carbonyl), 2935 cm $^{-1}$ (-CH stretching), 3305 cm^{-1} (-NH stretching),

4356 cm^{-1} (-OH stretching).

^{1}H NMR (DMSO d_6)

1.0 δ (- (CH_3)$_2$ - CH - of NIPAM), 1.5 δ (- C - (CH_3)$_2$ of Amps), 2.5 δ (- CH - (CH_3)$_2$ - of NIPAM), 2.75 δ (- CH_2 - SO_3H of Amps), 4.0 δ (- CH_2 - CH - of NIPAM), 6.0 δ (- NH -), 7.5 δ (- OH).

5.1.4 Characterization

5.1.4.1 Estimation of acid value of polymers

The acidic groups (carboxyl or sulfonic) incorporated into the copolymers were estimated as follows [Vaidya et al (1999)]. 0.1 g polymer was dissolved in 10 ml distilled ethanol and titrated against 0.1 M ethanolic KOH till colorless to faint pink end point, using phenolphthalein indicator. The acid groups in the polymer were estimated from the amount of KOH consumed.

5.1.4.2 LCST and molecular weight measurements of polymers

LCST was estimated by turbidometric method [Boutris et al (1997)]. Molecular weights were estimated by intrinsic viscosity [Takezawa et al (1990); Fujishige (1987)]. Molecular weights of all the polymers were in the range 8000 to 14,000.

5.1.4.3 Estimation of relative inhibition of lysozyme

The procedure for estimation of relative inhibition of lysozyme using acrylic acid and polymer containing acrylic acid was as follows. 0.5 M stock solution was prepared in 0.066 M phosphate buffer pH 6.2, containing 0.0154 M sodium chloride and 0.008 M sodium azide. One milliliter solution, containing different concentrations of acidic groups (prepared by serially diluting the stock solution) was mixed with 1.6 ml of 78 μg/ml of *Micrococcus lysodeikticus* in a 3 ml capacity glass cuvette. It was incubated for 5 minutes

at 20 ^{0}C. 0.1 ml of lysozyme (27 μg/ml) was added and mixed thoroughly. Immediately, the decrease in the absorbance at 450 nm (ΔA_{450}) was read for 30 seconds. The change in absorbance per second was calculated. Similarly, a blank was run and the change in the absorbance per second for the blank was calculated. From this the relative inhibition was calculated [Neuberger and Wilson (1967)]. For thermoprecipitating polymer Poly (NIPAM -co- acrylic acid), 1 % w/v solution was prepared in 0.066 M phosphate buffer pH 6.2, 0.0154 M sodium chloride and 0.008 M sodium azide. The relative inhibition of this polymer was calculated following the same procedure as described above.

The concentration of acidic groups required to obtain 50 % relative inhibition for various monomers are summarized in Table 5.2. Because of the solubility limitations of the polymers 3 to 5 in Table 5.3, it was not possible to achieve 50 % inhibition using 1 % w/v solution under standard assay conditions. Since the purpose of this exercise was to study the effect of nature of acidic groups on the inhibition, we chose to compare I_{30} values for these polymers. For remaining polymers (No.1 and 2 in Table 5.3) both I_{30} and I_{50} values for polymers have been quoted.

5.1.4.4 Estimation of apparent total number of binding sites (q^{app}_{max}) and dissociation constant (K_d) of polymers

Following procedure was used for the estimation of binding sites and dissociation constant in Poly (NIPAM -co- acrylic acid). 100 mg of polymer was weighed and dissolved (below LCST) in 10 ml of phosphate buffer (0.066M and pH 6.2, containing 0.0154 M sodium chloride and 0.008 M sodium azide) containing different amounts of lysozyme. This was allowed to equilibrate at 20 ^{0}C for 16 hours with continuous shaking at 200 rpm. The polymer-lysozyme complex was precipitated by increasing the

Table - 5.2 : Binding efficiency of acidic monomers with lysozyme.

No.	Acidic ligand	mM of acidic groups to achieve I_{50} [a]
1	Acrylic acid	11 ± 0.2
2	Methacrylic acid	19 ± 0.5
3	Itaconic acid	17 ± 0.4
4	Amps	12 ± 0.4
5	Ac. NAG [b]	35 ± 2
6	6-acetamido caproic acid [c]	12 ± 1

[a] Concentration (mM) of respective ligand to obtain 50 % relative inhibition. The values represent average from three experiments.
[b] Affinity ligand synthesized from active site inhibitor NAG.
[c] Synthetic affinity ligand (reported in the previous chapter).

Table - 5.3 : Characterization of thermoprecipitating polymers.

No.	Polymer[a]	MM acidic groups /g polymer[c]	M.W. (M_n)[d]	LCST (^0C)[e]	mM acidic groups for I_{30}[f]	mM acidic groups for I_{50}[g]
1	Poly (NIPAM – co- acrylic acid)	0.263	9000	34	0.00019	0.00050
2	Poly (NIPAM – co- methacrylic acid)	0.351	10,500	33	0.00052	0.0013
3	Poly (NIPAM – co- itaconic acid)	0.438	12,000	35	0.0015	----
4	Poly (NIPAM – co- Amps)	0.351	14,000	33	0.0013	----
5	Poly (NIPAM – co- Ac. NAG)[b]	2.241	8000	38	0.0033	----

[a] Molar feed ratio of NIPAM:acidic monomer was 98:2 in all cases.
[b] In this polymer the molar feed ratio of NIPAM:Ac. NAG was 7:3.
[c] Acidic groups were determined by acid values and acetamido content was determined by ^1H NMR technique as described in the text.
[d] Molecular weights were estimated by intrinsic viscosity method [Fujishige (1987)].
[e] LCST was determined by turbidometric method [Boutris et al (1997)].
[f] milimoles of acidic/acetamido groups in polymer required to obtain 30 % inhibition (I_{30}). In the polymers due to solubility limitations it was not possible to achieve I_{50} using 1 % w/v polymer solution under standard assay conditions.
[g] milimoles of acidic groups in polymer required to obtain 50 % inhibition (I_{50}).

temperature above LCST and was separated by centrifugation at 10,000 rpm for 20 minutes. The protein in the filtrate was estimated by Lowery's method. The difference between initial protein added and that in the filtrate gave the amount of adsorbed protein. As shown in Figure 5.3 a) Langmuir isotherm for lysozyme adsorbed on poly (NIPAM - co- acrylic acid) was plotted. The Scatchard plot is also shown as an inset in Figure 5.3 a). The values of q^{app}_{max} and K_d were estimated both below and above LCST.

5.1.5 Recovery of lysozyme from aqueous solution

Ten milliliters of 1 % w/v polymer solution was mixed with lysozyme. The concentration of lysozyme in the resulting solution was 27 μg/ml. This was incubated at 20 ^{0}C for 16 hours with continuous shaking at 200 rpm. The temperature of the mixture was raised above LCST of the polymer to precipitate the lysozyme-polymer complex. The complex was separated by centrifugation at 10,000 rpm for 20 minutes above LCST. The precipitated polymer was dissolved in dilute acetic acid solution (pH 2.0) [Ruckenstein and Zeng (1997)] to dissociate the complex. The temperature of this dissociated complex was raised above LCST and the precipitated polymer was separated by centrifugation. The clear filtrate was assayed for protein content using Lowery's procedure. The activity was measured using *Micrococcus lysodeikticus* (78 μg/ml).

5.1.5.1 Separation of lysozyme from egg white

Egg white solution was prepared as follows [Ehsani et al. (1997)]. Twenty five milliliters of homogenized egg white was diluted four hundred times with distilled water containing 0.0154 M NaCl and 0.008 M sodium azide. It was maintained at 4 ^{0}C overnight and then filtered through 80 μM nylon filter to obtain transparent egg white solution. This was preserved at 4 ^{0}C till further use. The dilution was necessary to lower

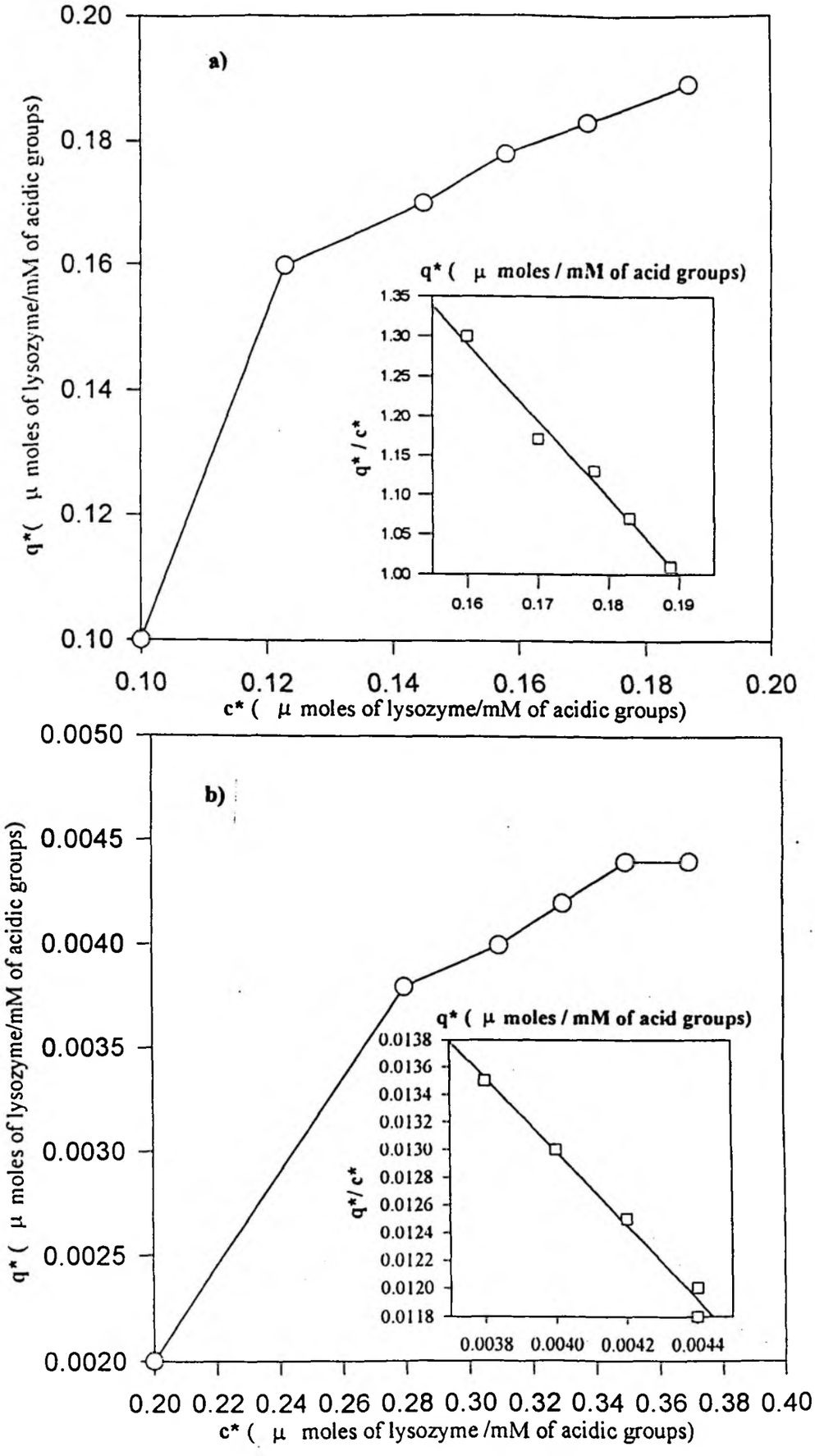


Figure 5.3: Langmuir isotherm and Scatchard plots (inset) a) below LCST; b) above LCST for poly (NIPAM –co- acrylic acid).

the viscosity of the egg white solution. The lysozyme activity of this diluted solution was 30 units/ml.

Fifty milligrams of the polymer was dissolved in 5 ml of egg white solution at 10 ^{0}C. This solution was incubated at 20 ^{0}C for 16 hrs. and lysozyme was recovered by the thermoprecipitation method described above. Recovery was estimated in terms of unit activity recovered/mM of acid groups present in the polymer.

5.1.6 Stability of synthetic polymers vs. poly (NIPAM -co- Ac. NAG)

Ten milliliters of thermoprecipitating polymer (1 % w/v) was mixed with lysozyme. The concentration of lysozyme in the resulting solution was 27 μg/ml. It was allowed to incubate at 20 ^{0}C for 16 hrs. Lysozyme was recovered by the procedure described earlier. The graph of specific activity recovered vs. number of cycles is shown in Figure 5.4. The weight of thermoprecipitating polymer before and after each cycle was measured. Almost 90 % of the polymer could be recovered after every cycle. Accordingly, in the next cycle, appropriate amount of lysozyme solution was added to maintain the polymer/lysozyme ratio constant.

5.2.0 Results and Discussion

Recovery of lysozyme using either affinity or acidic thermoprecipitating polymers has not been reported so far. In the previous chapter use of copolymers of NIPAM and synthetic affinity monomers bearing acetamido groups for thermoprecipitation of lysozyme was reported. The objective of the present work was to synthesize and evaluate thermoprecipitating copolymers of NIPAM and acidic comonomers and compare the efficacy of the two types of polymers. The essential prerequisites for thermoprecipitating polymer are listed in chapter 1. These were used as guidelines for synthesizing the

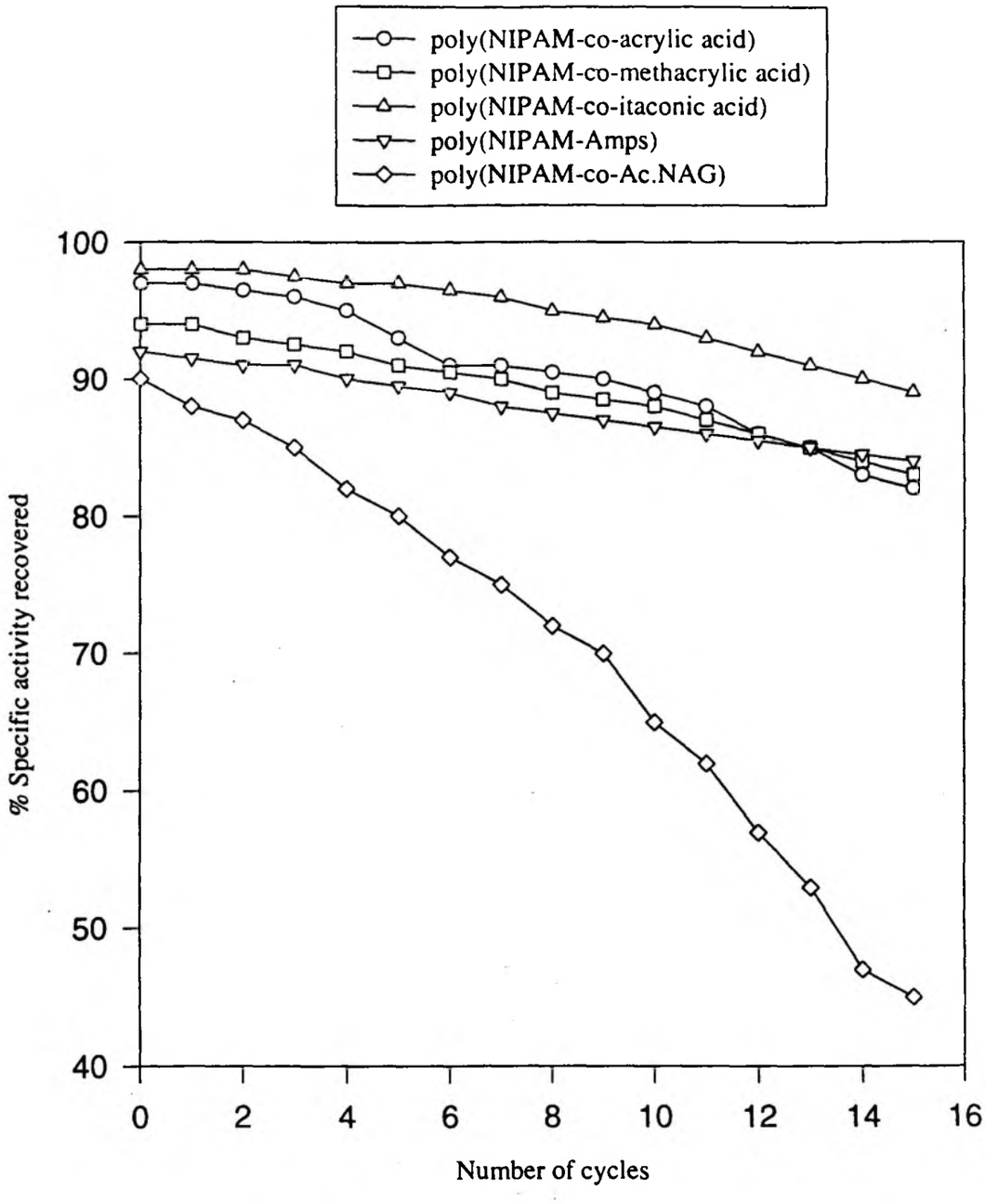


Figure 5.4: A comparison of stability of different thermoprecipitating polymers.

polymers in the present investigation. Copolymers of NIPAM with acidic monomers which exhibit LCST in the range of 30 – 32 ^{0}C were used. Homo-polymer of NIPAM did not show any binding to lysozyme. The M.W. and LCST of the polymers were determined and are summarized in Table 5.3. Low M.W. polymers were chosen to overcome crowding effect and also enhance ligand-protein interaction in solution [Luong et al (1988); Vaidya et al (1999)]. Hence, we purposely synthesized the low M.W. polymers (8,000 to 14,000). It can be seen from Table 5.3 that as the hydrophilicity of the acidic monomer increases, the LCST of the polymer increases. Poly (NIPAM -co- Ac. NAG) has the highest LCST (38 ^{0}C) because of the hydrophilic nature of NAG, while Poly (NIPAM -co- Amps) and Poly (NIPAM -co- methacrylic acid) exhibit lowest LCST (both at 33 ^{0}C) due to hydrophobic nature of bulky methyl groups. This trend is also consistent with our earlier work [Vaidya et al (1999)]. The extent of incorporation of acidic groups in the polymer was calculated from the acid value of the polymer. The N-acetyl groups incorporated in poly (NIPAM -co- Ac. NAG) were estimated by ^{1}H NMR deconvolution technique as described in the chapter 4.

5.2.1 Lysozyme binding with acidic monomers

The four monomers selected differ either in acidic functional groups or the environment around the acid functionality e.g. structurally the acrylic and methacrylic acid are identical except that the later contains a hydrophobic methyl group. Itaconic acid contains two carboxylic acid groups present in close vicinity. The Amps comprises sulfonic acid group attached to main chain via a hydrophobic bulky 2-methyl propane group. These monomers were copolymerized with NIPAM in the mole ratio 98:2. This feed ratio was particularly selected since these showed good lysozyme binding and also

with further increase in the concentration of acidic monomer in the feed, the LCSTs of the resulting polymers exceeded 80 ^{0}C.

The acidic monomers and polymers were tested for the relative inhibition of lysozyme using *Micrococcus lysodeikticus* as the substrate. For the monomers the values of concentration (mM) of acidic groups required to achieve 50 % inhibition of lysozyme are summarized in Table 5.2. From the data it can be seen that the acrylic acid (pK_a= 4.25) (I_{50} = 11 ± 0.2 mM) is better inhibitor than the methacrylic acid (pK_a= 4.65) (I_{50} = 19 ± 0.5 mM) even though their pK_a values are comparable. This difference in I_{50} values arises due to the hydrophilic nature of lysozyme [Ehsani et al (1997)], which interacts and binds favorably with acrylic acid than methacrylic acid. These findings are consistent with our earlier results, wherein we synthesized affinity ligands comprising pendant acetamido groups attached through spacers having different hydrophobicities. It was shown that the affinity ligand containing more hydrophilic spacer viz. glycylglycine exhibited enhanced binding to lysozyme (I_{50} = 8 ± 2 mM) than the affinity ligand comprising higher hydrophobic character viz. 6-acetamido caproic acid (I_{50} = 12 ± 1 mM).

Further, the monomer containing sulfonic acid group, Amps (pK_a = 1) [Chirai et al (1989)] exhibited almost identical inhibition capacity (I_{50} = 12 ± 0.4 mM) as acrylic acid (I_{50} = 11 ± 0.2 mM). This lower inhibition capacity of Amps, inspite of its lower pK_a value, is attributed to the fact that not only the pK_a value of the monomer but the hydrophobicity of the monomer (hydrophobic nature of 2, methyl propane group in Amps) also governs the binding of the monomer with the enzyme. Itaconic acid which consists of two carboxylic acid groups exhibits I_{50} = 17 ± 0.4 mM which is higher than

that for acrylic acid. This is probably because the second carboxylic group is not readily accessible after the first one is bound to lysozyme. The I_{50} value for Ac. NAG is 35 ± 2 mM which showed that acidic monomers have a better inhibition capacity than the monomer comprising natural affinity ligand NAG. Also, acrylic acid is as effective as 6-acetamido caproic acid. The reason for this difference lies in the mode of inhibition of acidic groups *vis a vis* NAG. Acidic monomers bind to lysozyme via electrostatic interactions with the primary amino groups of surface lysine and arginine residues. NAG acts as an active site inhibitor, which is required to bind at the active site buried in the interior of the lysozyme molecule.

5.2.2 Enhanced binding of lysozyme to thermoprecipitating polymers: cluster effect

Coupling of the ligand to a polymer results in a 100 – 1000 fold decrease in enzyme affinity towards ligand [Galaev and Mattiasson (1993)]. However, in the present investigation it was observed that when the monomers containing pendant acid groups were copolymerized with NIPAM, the binding efficiency of these polymers was enhanced by four to five orders of magnitude as shown in Table 5.3. A similar trend was reported by us when the affinity monomers containing acetamido groups were copolymerized with NIPAM. It should be noted here that the polymers containing acetamido groups contained 30 mole % acetamido monomer in the initial feed ratio, while the acidic monomer content in the polymers evaluated in the present work is only 2 mole %. This indicates much higher lysozyme recovery per unit mole of functional group in the present case *vis a vis* acetamido groups.

Recently, Yamada et al, (1999) showed that the interactions between wheat germ agglutinin lectin with NAG bound to synthetic polymer exhibited enhanced binding affinity compared to the corresponding mono- and oligosaccharides. The association constants (K_a) for these synthetic block copolymers containing NAG were in the range 1.6 - 3.3 * 10^5 M, whereas the K_a values for corresponding mono-, di- and trisaccharides of NAG were 6.8 * 10^2 M, 4.5 * 10^3 M and 2.0 * 10^4 M respectively. The three orders of magnitude increase in the association between polymeric NAG and lectin was attributed to the appropriate spatial arrangement and density of the NAG groups in these polymers. This induces a marked enhancement of binding affinity toward proteins due to the multivalent recognition, which is known as "cluster effect". Similar effect operating in the present case explains the enhancement in binding of acidic polymers to the lysozyme. An investigation of fouling of contact lenses showed that lenses containing acrylic acid adsorbed lysozyme in tear drops more rapidly and extensively due to self associating nature of lysozyme [Sassi et al (1996)]. Similar considerations also explain high capacity of the poly (NIPAM -co- acrylic acid) in the recovery of lysozyme.

At this stage it is worthwhile to compare our results with those reported by earlier investigators. Chern et al (1996), used lattices containing acidic groups for affinity precipitation of lysozyme by reducing the pH from 7 to 4.5. Thus the pH shift was exploited for the precipitation of latex - lysozyme complex and subsequently the recovery of lysozyme from the complex. These lattices exhibited only 7 % recovery of lysozyme. This is because protonation of carboxyl groups at acidic pH, resulted in significant dissociation of the latex-lysozyme complex. Tyagi (1996) et al used chitosan as a macroaffinity ligand and reported 70 % recovery of lysozyme activity using 2.5 M $MgCl_2$

to dissociate the enzyme-ligand complex. Yet the recovery of activity is considerably lower compared to the values obtained for ion-exchange resins and the method followed in the present investigation.

5.2.3 Estimation of q^{app}_{max} and K_d of thermoprecipitating polymers

To correlate binding efficiency with polymer structure, the binding parameters were calculated using Langmuir isotherm model [Segel, I. W. (1976)] to describe the adsorption of protein (lysozyme) on the polymer surface when the system was at equilibrium:

$$q^*/c^* = -q^* / K_d + q^{app}_{max} / K_d$$

where; q^* denotes the amount of protein adsorbed in μM/mM of acid groups; q^{app}_{max} denotes the apparent maximum amount (μM) of protein adsorbed/mM of acid groups; c^* is the amount of protein in μM present in filtrate/mM of acid groups; and K_d denotes the dissociation constant for the protein - binding site pair. Thus, the binding parameters K_d and q^{app}_{max} can be obtained from the slope and the intercept of the q^*/c^* vs. q^* curve as shown in Figure 5.3 for poly (NIPAM -co- acrylic acid).

For the thermoprecipitating polymers one would expect the binding parameters to differ above and below LCSTs of the respective polymers. The q^{app}_{max} values for poly (NIPAM -co- acrylic acid) are 0.190 and 0.00445 μmole/mM of acid groups below and above LCST, respectively. Below LCST, the polymer chains are expanded out considerably as the polymer is soluble and all the pendant acidic groups are well exposed encouraging favorable bridging interactions as described by Morris et al (1993). Therefore the efficiency of binding below LCST was found to be maximum. Precipitation of the polymer above LCST, reduces the effective surface area available for binding with

lysozyme also the acidic groups would be entrapped inside the coiled structure of the polymer chain. Hence, these groups are not available for binding with lysozyme. This favors the wrapping mechanism [Morris et al (1993)] for binding with fewer number of lysozyme molecules, above LCST. This was reflected in decrease in two orders of magnitude in q^{app}_{max} values above LCST. The K_d values for the same polymer are $10 * 10^{-6}$ M and $3 * 10^{-6}$ M below and above LCST, respectively. The slight reduction in K_d value above LCST might be due to the high local concentration of acidic groups in the collapsed polymer which enhances binding with lysozyme. As shown in Table 5.4 all polymers exhibited differences in the q^{app}_{max} and K_d values above and below their respective LCST's.

The most significant difference to be noted here is very high values of q^{app}_{max} for polymers comprising acidic groups at 2 mole % concentration *vis a vis* the polymer comprising Ac. NAG at 30 mole % concentration. Poly (NIPAM -co- acrylic acid) exhibits maximum q^{app}_{max} value (0.190 μM of protein/mM of acid groups) whereas Poly (NIPAM -co- Ac. NAG) exhibits lowest q^{app}_{max} (0.015 μM of protein/mM of acetamido groups). These differences could also be attributed to the differences in the mechanism of binding of the two ligands with lysozyme, discussed in the preceding section. The thermoprecipitating polymers containing acidic ligands could be a better choice for lysozyme separation by thermoprecipitation technique. Also, the strongest binding was provided by Poly (NIPAM -co- acrylic acid) with $K_d = 10 * 10^{-6}$ M whereas, the weakest binding ($K_d = 38 * 10^{-6}$ M) was provided by polymer containing Ac. NAG. This difference might be attributed to the fact that the Ac. NAG is an affinity ligand which binds to the active site of an enzyme, which is situated deep in the tertiary structure of

Table - 5.4 : Binding parameters of polymers above and below their respective LCSTs.

No.	Polymer[a]	Below LCST		Above LCST	
		$q^{app}{}_{max}$ [b]	K_d (M)[c]	$q^{app}{}_{max}$ [b]	K_d (M)[c]
1	Poly (NIPAM -co-acrylic acid)	0.190	$10 * 10^{-6}$	0.00445	$3 * 10^{-6}$
2	Poly (NIPAM -co-Methacrylic acid)	0.070	$17 * 10^{-6}$	0.00360	$14 * 10^{-6}$
3	Poly (NIPAM -co-itaconic acid)	0.032	$25 * 10^{-6}$	0.00300	$23 * 10^{-6}$
4	Poly (NIPAM -co-Amps)	0.040	$21 * 10^{-6}$	0.00330	$16 * 10^{-6}$
5	Poly (NIPAM -co-Ac. NAG)	0.015	$38 * 10^{-6}$	0.00200	$32 * 10^{-6}$

[a] Molar feed ratio of NIPAM:acidic monomer was 98:2 in all cases. But, for Poly (NIPAM -co- Ac. NAG) the molar feed ratio of NIPAM:Ac. NAG was 7:3. The reason for selecting these feed ratios is given in the text.

[b] $q^{app}{}_{max}$ is the apparent maximum number of binding sites of a given polymer. (μmole protein / mM acid or acetamido groups).

[c] K_d is the dissociation constant of a given polymer.

lysozyme, whereas acrylic acid interacts with easily accessible amino groups of enzyme on the surface. This reiterates that acidic ligands are a better choice for lysozyme separation by thermoprecipitation technique.

5.2.4 Recovery of lysozyme from aqueous solution and from egg white

After the evaluation of various kinetic parameters these thermoprecipitating polymers, containing acid groups were tested for the recovery of lysozyme from aqueous solution as well as from egg white, which is the natural source of lysozyme. The data for the recovery of lysozyme summarized in Table 5.5, indicate that very high specific activity of lysozyme recovered (97.6 %) by Poly (NIPAM -co- acrylic acid) results from the high protein recovery (2680 ± 10 μg/mM acid groups) and activity of lysozyme recovered (123,000 ± 400 units/ mM acid groups). In contrast, although specific activity of lysozyme recovered by Poly (NIPAM -co- Ac. NAG) is marginally lower (90.4 %), the protein recovery (200 ± 12 μg/mM acetamido groups) as well as the activity of lysozyme recovered (8500 ± 25 units/ mM acetamido groups) are at least one or two orders of magnitude lower. A comparison of these results with the results obtained for the recovery using synthetic polymers containing acetamido ligands reported earlier illustrates that polymers comprising acrylic acid exhibited almost three times enhanced recovery of protein as well as activity as compared to the poly (NIPAM -co- MEAc.GLYGLY). The later polymer recovered 752 ± 8 μg protein/mM of acetamido groups whereas the activity recovery was 34455 ± 350 units /mM of acetamido groups. This demonstrates that for the recovery of lysozyme, the acidic thermoprecipitating polymers are better suited than polymers comprising affinity ligand NAG.

Table - 5.5: Separation of lysozyme.

No.	Acidic polymer[a]	Lysozyme recovery[b]			Lysozyme recovery from egg-white[c]
		Protein (μg /mM of acid or acetamido groups)	Unit activity / mM of acid or acetamido groups	% specific activity recovery	
1	Poly (NIPAM -co-acrylic acid)	2680 ± 10	123000 ± 400	97.6	11000 ± 60
2	Poly (NIPAM -co-methacrylic acid)	950 ± 12	42000 ± 200	94.1	8000 ± 35
3	Poly (NIPAM -co-Itaconic acid)	420 ± 10	19500 ± 60	98.8	6750 ± 30
4	Poly (NIPAM -co-Amps)	522 ± 13	22700 ± 50	92.5	7850 ± 13
5	Poly (NIPAM -co-Ac. NAG)	200 ± 12	8500 ± 25	90.4	1200 ± 10
6	Poly (NIPAM -co-ME6Ac.CAP)	420 ± 4	18956 ± 190	96.0	----------

[a] Molar feed ratio of NIPAM:acid monomer was 98:2 whereas the feed ratio of NIPAM: Ac.NAG / ME6Ac.CAP was 7:3.

[b] 10 ml polymer (1 % w/v) solution was mixed with lysozyme (27 μg/ml) and was equilibrated for 16 hrs at 20 ^{0}C. Then the protein and activity of lysozyme was measured as given in experimental section. One unit activity is defined in the text.

[c] 50 mg of polymer was dissolved in 5 ml of egg white solution and equilibrated for 16 hrs at 20 ^{0}C. Then activity of lysozyme recovered (unit activity/mM of acid or acetamido groups) was measured as given in the text.

The values represent the average taken from three experiments.

These polymers were then tested for the recovery of activity of lysozyme from egg white. The data shown in Table 5.5 illustrate that all polymers exhibit greater than 90 % recoveries of activity of lysozyme from its natural source too.

5.2.5 Stability studies

As described in the experimental section, sixteen recovery cycles were run. The percent recovery in terms of specific activity as a function of number of cycles is shown in Figure 5.4. The data show that even after sixteen cycles the decrease in percent recovery in terms of specific activity was 15, 12, 9 and 8 % for Poly (NIPAM -co- acrylic acid), Poly (NIPAM -co- methacrylic acid), Poly (NIPAM -co- itaconic acid) and Poly (NIPAM -co-Amps) respectively. While, for Poly (NIPAM -co- Ac. NAG) the decrease in the percent recovery in terms of specific activity was 50 %. The probable reason for this difference is that glucose is a carbon source for many microbes. It also undergoes hydrolytic degradation [Hirano et al. (1991)]. Thus, after 16 cycles, some of the NAG molecules may have lost their efficiency for lysozyme binding.

In the case of polymers containing synthetic acetamido groups, after sixteen cycles the values for the decrease in the percent recovery in terms of specific activity were in the range of 20 % for Poly (NIPAM -co- MEAc.GLYGLY) and Poly (NIPAM -co- ME6Ac.CAP). Thus, in the present case the acidic polymers exhibit better stability as compared to affinity based polymers containing NAG or its synthetic analogues. Thus loss of 8 to15 % could be due to the loss of low molecular weight polymer in each cycle of dissolution/precipitation. To overcome this loss still high molecular weight polymers can be used.

5.3.0 Conclusions

A series of acidic monomers were copolymerized with NIPAM. The inhibition of lysozyme was enhanced by five orders of magnitude when the acidic monomers were incorporated in the thermoprecipitating polymers. The polymers were far more effective when the acidic monomer content was much lower (2 mole %) than the monomer containing natural inhibitor NAG (30 mole %). Below LCST the q^{app}_{max} values are almost ten times higher than the corresponding values above LCST. These polymers were used for the recovery of lysozyme from aqueous solution as well as from egg white. The polymer containing acrylic acid exhibited maximum recovery and high selectivity for lysozyme from aqueous solution as well as egg white. These polymers showed better stability than the polymer based on NAG. This approach provides an alternative route for the recovery of lysozyme.

Chapter 6

ENHANCED SELECTIVITY OF THE RECEPTOR BY AFFINITY-IMPRINTING TECHNIQUE

6.0.0 Introduction

The use of affinity ligands e.g. inhibitor, coenzyme or substrate analogues in the affinity precipitation technique provides selectivity during separation of the desired enzyme. These interactions are based on the forces involved in the bio-specific recognition process as discussed in the chapter 1. The cumulative strength of such forces leads to high levels of recognition and selectivity. So far the utility of such ligands was limited to the conventional affinity techniques used in the recovery of bio-molecules. The understanding of bio-recognition between antigen-antibody gave an impetus to the work on molecular-imprinting [Mosbach (1995); Wulff (1995)]. In this technique the rebinding of the template molecule occurs through weak hydrogen bonding/ionic interactions between the template and the functional monomers surrounding the imprint cavity. Eventhough these interactions closely resemble the bio-recognition process, they lack the selectivity observed in the later. In the present chapter, affinity interactions enhanced by molecular imprinting have been employed so as to enhance the selectivity as well as the capacity of the imprinted bio-receptors. Trypsin imprinted gels in which affinity monomer specific for trypsin has been used as functional monomer during synthesis of receptor. In this study various control gels were also synthesized to unequivocally establish the contribution made by the affinity and imprinting interactions. It has been shown that by fine-tuning this approach, one can obtain molecularly imprinted receptors for biomolecules having very high capacity and selectivity.

Over the past two decades, molecular imprinting has emerged as an attractive technique to separate variety of bioactive molecules [Wulff (1995); Mosbach (1995); Shea (1994); Steinke et al (1995)]. In this technique, functional monomers are pre-organized around the template molecules using covalent or non-covalent interactions between the two. This monomer-template assembly is polymerized in the presence of the crosslinker. Subsequently, the template is extracted out to obtain molecularly imprinted polymer (MIP).

The MIP comprises imprinted cavity of the shape and size of the template. MIPs preferentially adsorb substrate molecules that are structurally similar to the template. Amongst small bioactive molecules, MIP based receptors for β-adrenergic antagonists e.g. atenolol, acebutol [Nilsson et al (1997)], amino acids and peptides [Kempe and Mosbach (1995)], phenyl phosphonic acid [Sasaki et al (1999)], DNA and RNA bases [Spivak and Shea (1998)], theophylline [Baggiani et al (1997)], nicotine [Matsui and Takeuchi (1997)], herbicides e.g. prometryn [Matsui et al (1995a)], pesticides e.g. atrazine [Matsui et al (1995b)], sterols e.g. androst -5-ene-3 β, 17 β-diol, testesterone [Smith et al (1994); Cheong et al (1997)], cholesterol [Whitcomb et al (1995)] have been reported. In most of the above mentioned examples, methacrylic acid was used as a functional monomer that exhibited weak hydrogen bonding interactions with substrates during rebinding.

Hydrogen bonding has also been exploited in the synthesis of MIPs for proteins and enzymes e.g. Shi et al (1999) synthesized thin polymeric films comprising disaccharide molecules imprinted with proteins. The polymers exhibited selective recognition for imprinted proteins-albumin, IgG, lysozyme, RNAse and streptavidine through hydrogen bonding with disaccharides. Umeno et al (1998) reported Poly (N-isopropylacrylamide) grafted with DNA. The DNA on the polymer was imprinted with restriction endonuclease EcoRI for its re-recognition mediated by hydrogen bonding. Metal chelation of proteins was used in MIPs by Mallik et al (1994). In this, silica beads comprising Cu (II) iminodiacetate groups were imprinted with proteins having exposed surface histidine groups for their selective rebinding on the beads. Bayerl et al (1999) bound bovine trypsin inhibitor (BTI) vesicles of dimyrystoylphospahtidylcholine coated on silica. Template BTI was removed from silica at 4 ^{0}C i.e. below melting temperature of lipid bilayer to obtain imprinted beads. Hjerten et al (1997) synthesized polyacrylamide gels in the presence of human growth hormone, RNAse and myoglobin. The imprinted gels exhibited selective recognition of the

respective proteins through hydrogen bonding. Glad et al (1993) reported MIPs for RNAse and soybean trypsin inhibitor by mixing them with methacrylate silica, vinylimidazole, acrylamide etc. and crosslinking the assembly. Functionalized silica gels have also been imprinted with enzymes- urease, RNAse and transferrin to create macromolecular receptors [Glad et al (1985); Kempe et al (1995); Venton and Guddipati (1995); Burow and Minoura (1996)].

As mentioned earlier, hydrogen bonding interactions are weak non-specific secondary valence interactions. This often results in very low uptake (micrograms per gram of the polymer) of proteins or enzymes by MIPs. Selectivity is also limited by the fact that non-imprinted proteins can also exhibit hydrogen bonding with the polymers. In contrast to hydrogen bonding, electrostatic interactions between active site of an enzyme and inhibitor i.e. affinity ligand are very strong and selective for a given pair of enzyme and the ligand [Mares-Guia and Shaw (1965)]. Thus affinity chromatography, affinity precipitation and affinity ultrafiltration have been extensively used for the separation of enzymes. However, polymers or columns based merely on affinity ligands also exhibit some non-specific adsorption of proteins other than the desired ones [Luong et al (1988a); Schneider et al (1981); Sigmundsson and Filippusson (1996); Luong et al (1988b)]. Using the molecular imprinting concept, the selectivity of such affinity-imprinted polymers could be dramatically increase. Also, the uptake of enzymes or proteins will be much higher than in the case of MIPs based on only hydrogen bonding interactions.

To our knowledge, there is no previous report on use of enzyme-inhibitor interaction in MIP synthesis. In this communication, we report trypsin-imprinted receptor comprising trypsin-inhibitor para aminobenzamidine (PABA). This receptor exhibited exclusive uptake of trypsin over chymotrypsin, a closely related enzyme. It also exhibited linear Scatchard plot, a characteristic of true receptor. On the other hand, PABA containing non-imprinted polymer exhibited nonspecific uptake of chymotrypsin and did not obey the Scatchard plot.

6.1.0 Experimental section

6.1.1 Materials

Acrylic acid, para aminobenzamidine dihydrochloride (PABA.2HCl) were obtained from Aldrich. Trypsin (type II S: from bovine pancreas), α-chymotrypsin (type II: from bovine pancreas), N_{α} -benzoyl-DL-Arginyl- para nitroanilide (DL-BAPNA), N-benzoyl-L-Tyrosyl- para nitroanilide (L-BTPNA) were obtained from Sigma. Thionyl chloride, acrylamide, methylenebisacrylamide, ammonium per sulfate (Aps), tetramethyleneethylenediamine (TEMED) were obtained from local suppliers. All the chemicals were used as received. Acryloyl chloride was synthesized from the reaction between acrylic acid and thionyl chloride.

6.1.2 Instrumentation

Electronic absorption measurements were made on UV-1601PC Shimadzu spectrophotometer. Mean pore radii of imprinted polymers were determined on Quanta chrome mercury porosimeter SP-33B.

6.1.3 Methods

6.1.3.1 Synthesis of N-acryloyl para aminobenzamidine. hydrochloride (Ac. PABA. HCl)

This was synthesized according to the following procedure [Luong et al (1988a); Schneider et al (1981)]. In a 100 ml capacity beaker, 1 g PABA. 2HCl, 17 g anhydrous sodium acetate and 60 ml water was placed to obtain a clear solution. The solution was stirred at 5 – 10 ^{0}C (ice-water bath) and 2 ml acryloyl chloride was added dropwise to it. This reaction mixture was stirred at 5-10 ^{0}C for 30 minutes and then it was acidified to pH 4 with concentrated nitric acid. The precipitated white product was isolated and triturated with acetone to obtain dry powder. The product was purified by re-precipitation from methanol into acetone.

Yield - 50 %. M.P. - 237-239 ^{0}C (reported - 237 ^{0}C).

6.1.3.2 Synthesis of trypsin imprinted receptor gels

A typical procedure for the synthesis of the gel was as follows. In an air tight round bottom teflon tube, 50 mg Ac. PABA. HCl was dissolved in 3 ml of dilute NaOH so as to free the guanidine group from its hydrochloride form. To this solution, 50 mg trypsin was added; and the resulting solution was shaken gently on a shaker bath for 15 minutes to form trypsin-Ac. PABA complex. The formation of trypsin – Ac. PABA complex was monitored by estimating the activity of the inhibited trypsin against standard substrate DL-BAPNA. It was observed that 94 % trypsin activity was inhibited due to the formation of trypsin-Ac. PABA complex. (The rationale for the selection of 1:1 w/w ratio of Ac. PABA to trypsin is discussed later in Results and Discussion). Comonomer acrylamide 800 mg, crosslinker methylenebisacrylamide 100 mg, and initiator ammonium per sulfate 10 mg, were dissolved in 1 ml DMF and added to an aqueous solution containing trypsin-Ac. PABA complex. Nitrogen gas was purged through this solution for 15 minutes and 40 µl TEMED was added. Polymerization was carried out by keeping the teflon tube containing this solution in a water bath at 37 ^{0}C for 18 hrs. The gel so synthesized was treated with acetone to remove water. It was then crushed to fine particles.

The template trypsin was extracted from the particles by degrading it with alternative treatments of acetone and chloroform for 3-4 times [Fahien and Kmiotek (1979)]. From the absorbance of this eluate monitored at 280 nm it was concluded that more than 90 % of template trypsin was extracted out. The choice of using this method for extraction of the template is discussed in the Results and Discussion section. Imprinted gels so synthesized were dried in vacuum oven at 50 ^{0}C for 12 hrs. Dried gels were then sieved through standard test sieves and particles in the size range 250-500 µ were used for all further studies. Details of the composition of gels synthesized are listed in Table 6.1.

Table 6.1 : Synthesis and Characterization of imprinted and non-imprinted gels.

No.	Polymer[a]	Acrylamide mg	Methylenebis acrylamide mg	Swelling ratio[b]	Mean pore radius[c]	Trypsin uptake[d]	Uptake ratio[e]
1	I(10)	800	100	3.578	1.012	0.984	
2	NI(10)	850	100	3.350	N.D.	1.272	0.77
3	I(30)	600	300	1.976	0.938	1.044	
4	NI(30)	650	300	1.981	N.D.	0.680	1.53
5	I(50)	400	500	1.878	0.816	0.620	
6	NI(50)	450	500	1.806	N.D.	0.212	2.92

[a] I - imprinted polymer, NI - nonimprinted polymer, ( ) - figures in parenthesis denotes the percentage ratio of crosslinker. Ac. PABA was 50 mg in all experiments. In imprinted polymers trypsin (50 mg each) was incorporated as the template.

[b] Swelling ratio = (weight of swollen polymer – weight of dry polymer) / (weight of dry polymer).

[c] Mean pore radius is expressed in Angstrom * 10^4.

[d] Trypsin uptake expressed in mg of trypsin/g of polymer. The values expressed are the average of three experiments.

[e] Uptake Ratio = trypsin $uptake_{imprinted}$ / trypsin $uptake_{nonimprinted}$

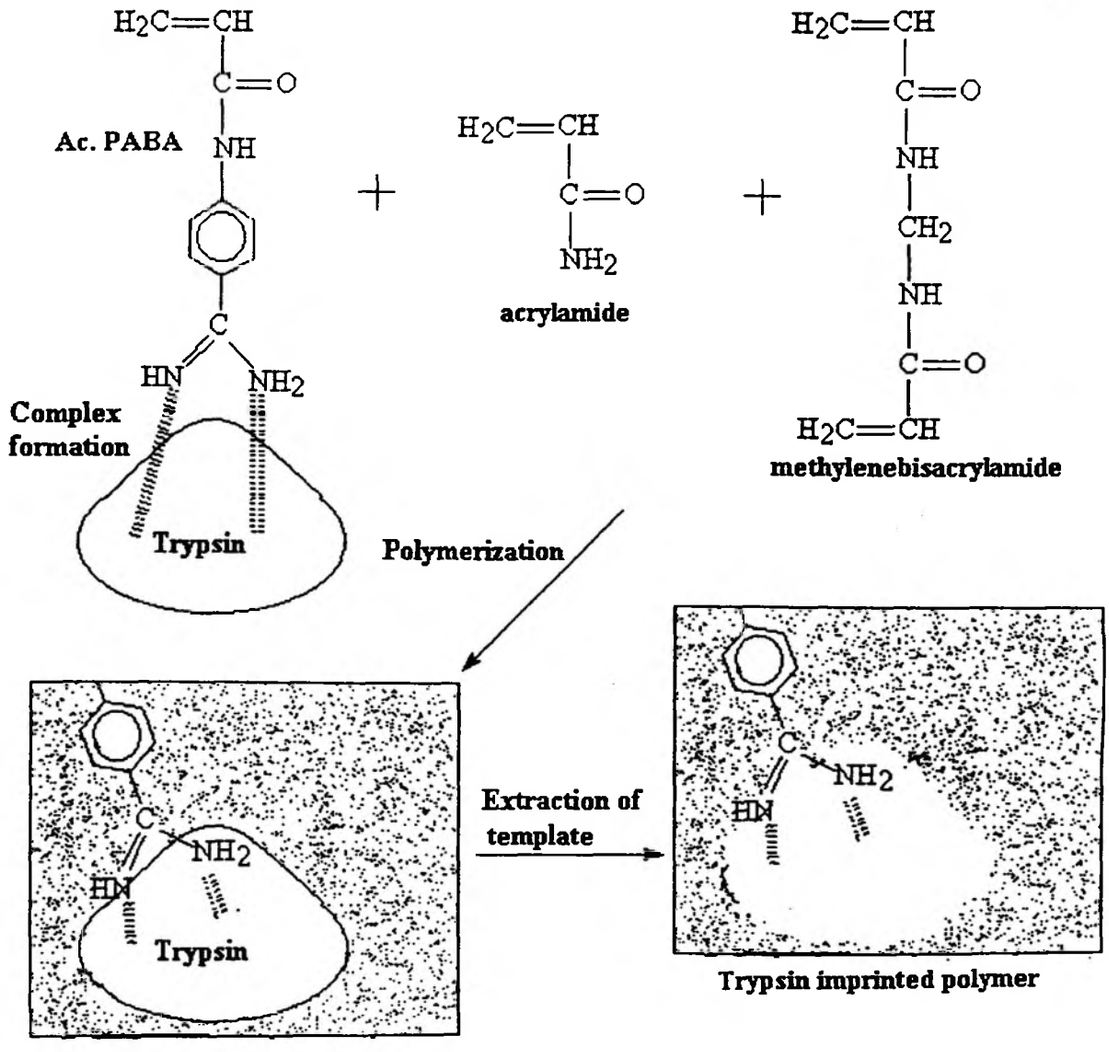


Figure 6.1: Affinity-imprinting methodology.

6.1.3.3 Synthesis of non-imprinted gels

Non-imprinted gels containing Ac. PABA were synthesized following the same procedure described above for imprinted gels except that the template trypsin was not used during synthesis. Details are listed in Table 6.1.

6.1.3.4 Trypsin uptake studies

A typical procedure for trypsin uptake studies was as follows. In a 50 ml capacity conical flask, 250 mg dry gel particles (250 μ-500 μ size) were suspended in 25 ml of 10 mM Ca^{+2} aqueous solution containing 2 mg/ml trypsin. The flask was allowed to shake at a speed of 50 rpm at room temperature on a rotary shaker for 1 hr. An independent experiment was conducted, which showed that at this Ca^{+2} concentration there was no loss of trypsin activity due to self proteolysis. The swollen gel particles were then filtered and the trypsin content in the filtrate was estimated according to the procedure laid down by Lowery et.al (1951). Uptake of trypsin by the gel was determined from the difference between the initial amount and that present in the filtrate. Results of trypsin uptake by imprinted and non-imprinted gels are summarized in Table 6.1.

6.1.4 Characterization

6.1.4.1 Swelling ratios for receptor gels

Exactly weighed 200 mg of dry gel particles were placed in a stoppered conical flask. To this, 25 ml distilled water was added and the flask was kept at room temperature for 72 hrs. The swollen gel was then filtered, wiped with tissue paper and weighed. This procedure was repeated till constant weight was obtained. The swelling ratio was determined by dividing the difference between the weight of the swollen gel and the weight of the dry gel by the weight of the dry gel. The results are summarized in Table 6.1.

6.1.4.2 Estimation of K_d and number of binding sites

The ratio of trypsin uptake in the case of imprinted vs. non-imprinted gels was maximum at 50 % crosslinker concentration. Hence, trypsin imprinted gel i.e. I (50) and non imprinted gel i.e. NI (50) were evaluated for the determination of K_d (dissociation constant) and total number of binding sites as follows-

200 mg of I (50) or NI (50) dry gel was allowed to equilibrate with 5 ml trypsin solution of varying concentration ranging from 100 to 700 μg / ml containing 10 mM Ca^{+2} for 18 hrs. Then, trypsin present in the filtrate (S_f) was estimated and bound trypsin (S_b) was calculated from the difference. The Scatchard plot (S_b / S_f) vs. (S_b) was constructed. From the slope of the graph the dissociation constant K_d (-1/slope) was obtained and from the intercept on the X axis, total number of binding sites were calculated.

6.1.5 Synthesis of trypsin and chymotrypsin receptor gels

Trypsin imprinted receptor gel T (50) and chymotrypsin imprinted receptor gel C (50) were synthesized using 50 % crosslinker following the same procedure as described above in the experimental section. The details of synthesis are listed in Table 6.2.

6.1.5.1 Trypsin vs. chymotrypsin uptake experiments

Gel particles [T (50) and C (50)] were equilibrated with trypsin (concentration - 2 mg / ml) and chymotrypsin (concentration - 2 mg / ml) solutions separately on a shaker bath for 1 hr. at room temperature. The gel particles were filtered off, and the amount of trypsin and chymotrypsin present in the filtrate was determined by Lowery method [Lowery et al (1951)]. The uptake of respective enzyme by receptors was estimated from the difference between the initial amounts and those present in the filtrates. Relevant data are listed in Table 6.2.

To demonstrate selective uptake of trypsin in the presence of chymotrypsin, a mixture of trypsin and chymotrypsin containing identical initial activities was shaken with trypsin imprinted gel T (50) as described above. The filtrate was assayed for individual enzyme using

Table 6. 2 : Synthesis and uptake characteristics of trypsin and chymotrypsin imprinted gels.

No.	Polymer[a]	Acrylamide mg	Methylenebis acrylamide mg	Uptake of trypsin[b]	Uptake of chymotrypsin[c]	uptake ratio[d]
1	T(50)	400	500	0.600	No uptake	Exclusive trypsin uptake
2	C(50)	400	500	0.186	0.096	1.94
3	NI(50)	450	500	0.212	0.090	-----

[a] Ac. PABA was 50 mg in all the experiments. The percent crosslinker was 50 % for all the polymers. Also, 50 mg of trypsin and chymotrypsin were incorporated as template for T(50) and C(50) respectively.

[b] mg of trypsin / g of polymer.

[c] mg of chymotrypsin / g of polymer.

[d] uptake ratio = uptake of trypsin by imprinted polymer / uptake of chymotrypsin by imprinted polymer.

standard substrates [Erlanger et al (1961)]. For trypsin, DL-BAPNA was used. For chymotrypsin, L-BTPNA was used. From the percentage of substrate hydrolyzed, the residual enzyme activity was estimated. The data are shown in Table 6.2.

6.2.0 Results and Discussion

The objective of the present investigation was to synthesize a polymer based on the affinity monomer, which is molecularly imprinted for its corresponding enzyme so that the resulting polymer will have strong affinity, as well as imprinted cavity for very selective uptake of the enzyme. In the present work trypsin was selected as a model enzyme to demonstrate affinity-imprinting concept. Guanidine group present in PABA exhibits strong affinity for aspartate group in the active site of trypsin. We therefore selected N-acryloyl PABA (Ac. PABA; $K_i = 67 * 10^{-6}$ M) as trypsin specific affinity monomer for the use in the polymers [Luong et al (1988a)]. In the following sections, the synthesis of trypsin imprinted Poly (acrylamide) gels containing PABA and optimization efforts for maximum uptake and selectivity are discussed.

6.2.1 Choice of acrylamide based gels

Trypsin-Ac. PABA complex formation takes place in aqueous medium. Therefore water soluble acrylamide and methylenebisacrylamide were selected as comonomer and crosslinker respectively, for synthesizing the gels. Here it may also be noted that Poly (acrylamide) gels, which are biocompatible, are routinely used in gel electrophoresis technique for the separation of enzymes and various other proteins.

6.2.2 Affinity-imprinting methodology

In brief, our methodology is a combination of affinity based separation and molecular imprinting which is shown schematically in Figure 6.1. The steps involved include the formation of affinity complex between Ac. PABA and trypsin, polymerization of the complex

with acrylamide and methylenebisacrylamide and the removal of the template- trypsin from the gel to create an imprint polymer selective for trypsin.

6.2.3 Trypsin-Ac. PABA complex

Conventionally, stoichiometric amounts of functional monomers and small template molecules have been used to form the monomer-template assembly. But in case of macromolecular templates like enzymes, the molar ratio required for the formation of such monomer-template assembly is best determined by the active site titration of the enzyme with its inhibitor. The active site titration of trypsin with Ac. PABA was carried out by monitoring the inhibition of trypsin activity against the standard substrate DL-BAPNA. Data listed in the Table 6.3 show that trypsin is completely inhibited at trypsin : Ac. PABA molar ratio of 1:200. White and Chen (1995) reported 1:236 molar ratio of trypsin : benzamidine for the complete inhibition of trypsin. Thus it can be concluded that in the present case the ideal molar ratio for trypsin-Ac. PABA complex formation would be 1:200. Yet, any inadvertent excess of Ac. PABA in the complex would result in this fraction of Ac. PABA acting as merely an affinity chromatography ligand. To eliminate any such possibility we selected slightly lower ratio of trypsin : Ac. PABA, which consumes all the affinity monomer for complex formation and only 6 % residual activity of trypsin remains. For this to happen, the molar ratio of trypsin : Ac. PABA is 1:127. On the weight basis this ratio turns out to be 1:1. Thus 1:1 w/w amounts of trypsin and Ac. PABA were used to form the complex between the two.

Table 6.3 : Active site titration of trypsin in the presence of Ac. PABA.

No.	Mole ratio of trypsin : Ac. PABA	% residual activity
1	1:0	100
2	1:1	98
3	1:25	32
4	1:60	17
5	1:127	6
6	1:200	0

6.2.4 Synthesis of imprinted gels and removal of template

Trypsin : Ac. PABA complex (1:1 w/w) was polymerized with acrylamide and methylenebisacrylamide, as described in the experimental section. After crosslinked gels were synthesized, an attempt was made to extract the template trypsin out of the gels by the conventional treatment of gel particles with dilute HCl, which dissociates the complex into free trypsin and inhibitor [Senstad and Mattiasson (1989)]. But dilute HCl treatment could not extract out trypsin completely. Therefore, organic solvents were resorted to. Chloroform / acetone treatment degrades trypsin into smaller fragments and elutes them out of the gel particles [Fahien and Kmiotek (1979)]. This method of template removal resulted in 94 % removal of trypsin from the imprinted gels. Since trypsin so extracted is in degraded form, it does not show any activity. Here it may be noted that once the cavity specific for trypsin is formed and frozen by crosslinking, subsequent removal of trypsin by its degradation and drying the gel particles does not affect the imprinted cavity. Further, trypsin bound to the gel is also not expected to show any activity as it is inhibited by Ac. PABA. This work aims at demonstrating the receptor concept i.e. selective uptake (recognition) of imprinted enzyme and not the recovery.

6.2.5 Optimization of percentage crosslinker in affinity-imprinted gels

Poly (acrylamide) gels swell substantially in water. This swelling disturbs the imprinted cavity and results in poor imprinting effect. This has been overcome by using high percentage (>90 %) of crosslinker which reduces the swelling and thus improves the specificity of the imprinted cavity. But this holds good only for small substrate molecules e.g. rhodaniline blue, safranine [Ekberg and Mosbach (1989)]. For macromolecular substrates, like trypsin in the present case, the amount of crosslinker has to be optimized

because small pore sizes of highly crosslinked gels limit the access of high molecular weight proteins towards the imprinted cavities [Venton and Gudipati (1995)]. A detailed study of the effect of percent crosslinker on the imprinting efficacy was undertaken. The imprinting efficacy was quantified in terms of the uptake ratio of trypsin by imprinted and nonimprinted gels prepared with same percentage of crosslinker ($uptake_{imprinted}$ / $uptake_{nonimprinted}$).

Trypsin imprinted and nonimprinted gels (both containing affinity monomer Ac. PABA) were synthesized using 10 to 90 % crosslinker by weight of the monomers. The feed compositions and other relevant data are summarized in Table 6.1. The gels were evaluated for the uptake of trypsin. It was observed that at the lowest percentage of crosslinker (10 %), imprinted gel I (10) exhibited lower uptake of trypsin (0.984 mg/g) than that of the nonimprinted gel NI (10) (1.272 mg/g). Swelling ratios of I (10) and NI (10) were very high (3.3 to 3.5). Lower trypsin uptake by I (10) suggested that the high swelling caused the loss of imprinted shape of the cavity. Thus, I (10) did not exhibit the imprinting effect ($uptake_{imprinted}$ / $uptake_{nonimprinted}$ = 0.77).

With further increase in the percentage of crosslinker, swelling ratios of both the gels decreased. This helped maintaining the shape of the imprinted cavity and the imprinting effect was then evident. See data listed in Table 6.1. Uptake ratios ($uptake_{imprinted}$ / $uptake_{nonimprinted}$) increased from 0.77 for I (10) to 1.53 for I (30). At 50 % crosslinker the maximum imprinting effect was observed (uptake ratio 2.92). Still, higher amounts of crosslinker in the gels (>50 %) decreased their pore radii and the net uptake of trypsin by either imprinted or non-imprinted gels. This is consistent with the trend

reported by Venton and Gudipati (1995) for urease imprinted silica gels of decreasing pore radii that restricted the free access of urease to the imprinted cavities.

Further, when percentage of crosslinker was increased above 50 %, uptake ratios again decreased to less than one, indicating the loss of imprinting effect. As mentioned above, this can be attributed to the poor access of trypsin to highly crosslinked gels. In summary, at 50 % crosslinker, maximum selectivity was exhibited by imprinted gels when compared with nonimprinted ones. I (50) exhibited trypsin uptake of 0.62 mg /g. This uptake capacity of the gel is indeed low, when compared with the large molar excess of Ac. PABA over that of trypsin used in the gels (trypsin : Ac.PABA = 1:127). But this is a typical drawback of affinity based gels. Since the binding takes place in heterogeneous mode, a large fraction of ligand in the dense gel matrix is unavailable for the enzyme. However, this uptake (mg/g) is still hundred folds higher than the results reported for imprinted silica gels [Burow and Minoura (1996)]. Here we would like to emphasize that I (50) exhibited three times higher trypsin uptake (0.62 mg/g) than that of NI (50) (0.21 mg/g) due to the imprinting effect. In order to discern between the affinity and imprinting and validate our methodology, we performed additional control experiments using the optimized 50 % crosslinker and 1:1 w/w trypsin : Ac. PABA complex. These are detailed below.

6.2.6 Affinity-imprinting: validation

Trypsin imprinted gel was synthesized using 450 mg acrylamide as the functional monomer and 500 mg methylenebisacrylamide (50 % of the total feed) as the crosslinker and 50 mg trypsin as the template molecule. This gel did not exhibit any uptake of trypsin because there are no sites (affinity groups) within the gel to which the enzyme could

bind. From this it is clear that voidage created by the mere presence of trypsin during polymerization is not adequate for the uptake of trypsin. This also indicates the limitations of hydrogen bonding in synthesizing MIPs for enzymes.

A gel containing blocked guanidine group using 400 mg acrylamide, 500 mg methylenebisacrylamide, 50 mg trypsin and 50 mg Ac. PABA. HCl. was also synthesized. This gel also did not exhibit any trypsin uptake, since the guanidine group, which binds to the active site of trypsin has been blocked by hydrochloride.

Non-imprinted gel NI (50) containing Ac. PABA was synthesized according to the composition shown in Table 6.1. The data show that trypsin uptake by this gel was 0.212 mg/g, despite the fact that it was not imprinted for trypsin. This is because of the inherent ability of Ac. PABA to bind to trypsin. This gel behaves as an affinity chromatography gel.

Trypsin imprinted gel I (50) containing Ac. PABA was synthesized. The composition is shown in Table 6.1. Trypsin uptake by this gel was 0.620 mg/g, which is almost three times higher than that of NI (50). This is because this gel contains affinity monomer Ac. PABA, which has its inherent trypsin binding capacity as well as an imprinted cavity specific for trypsin, which results in synergistic "affinity-imprinting" effect.

Finally, I (50) was treated with hydrochloric acid (35 %) to block the active guanidine groups. The gel did not exhibit any uptake of trypsin. This further lends support to the hypothesis that the uptake of trypsin by affinity imprinted gel is indeed due to synergistic effect.

6.2.7 Affinity-imprinted gels as receptors for trypsin

In order to highlight the difference between affinity chromatography gels (non imprinted gels) and receptors (trypsin - imprinted gels), Scatchard plots were constructed for I (50) and NI (50) (Figure 6.2). The number of binding sites in I (50) were 0.165 μM/g and K_d = 0.0375 μM. Scatchard plot for NI (50) was curvilinear indicating thereby that this gel did not exhibit the characteristics of a selective receptor. In fact this curvilinear nature of the plot shows that it indeed functions as an affinity chromatography gel. Thus although both the gels were synthesized using same amounts of Ac. PABA, only I (50) exhibited trypsin binding like a true receptor [Segel (1973)].

6.2.8 Recognition of trypsin and chymotrypsin by receptors

Trypsin imprinted T (50) and chymotrypsin imprinted C (50) gels comprising Ac. PABA were synthesized using 50 % crosslinker. The composition of these gels is shown in Table 6.2. The ability of these receptors to discriminate between trypsin and chymotrypsin, a closely related enzyme was evaluated in independent batch experiments and also in the mixture of trypsin and chymotrypsin. An independent experiment was conducted to confirm that in the presence of 10 mM Ca^{+2}, the trypsin activity was not lost due to proteolysis during the time span of the experiment. Data in Table 6.2 show that trypsin uptake for T (50) was 0.68 mg/g of gel. However, T (50) exhibited no uptake of chymotrypsin. We confirmed this result by estimating chymotrypsin in the filtrate, which remains after separating the receptor gel particles. The estimation by Lowery method and activity measurement confirmed that the amount of protein and the activity of chymotrypsin in the filtrate was the same as in the initial solution. Thus trypsin imprinted receptor gel exhibited exclusive recognition of trypsin. We believe that this exclusive

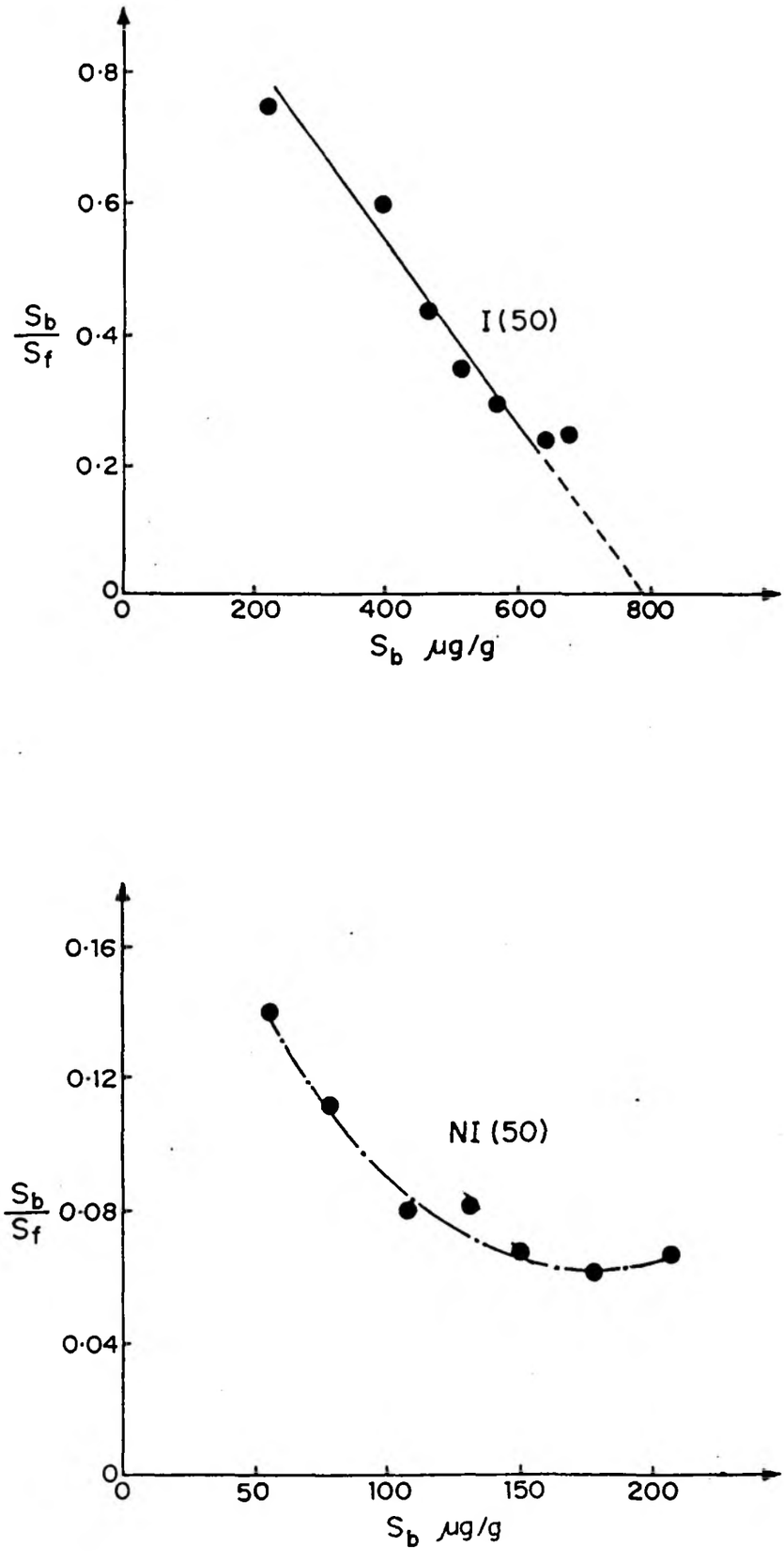


Figure 6.2: Scatchard plots of I (50) and NI (50).

uptake arises from imprinted cavity created around inhibitor-enzyme complex, very specific for that enzyme.

Non-imprinted gel NI (50) exhibited nonspecific chymotrypsin uptake of 0.090 mg/g. This is consistent with non-specific adsorption of chymotrypsin by Ac. PABA observed in affinity precipitation [Luong et al (1988a, 1988b); Schneider et al (1981)]. Thus affinity-imprinted gels reported here have an advantage over conventional affinity gels in that they can eliminate the nonspecific adsorption of proteins other than the imprinted one. C (50) gel exhibited chymotrypsin uptake of 0.096 mg/g, a trend consistent with the above described in NI (50). C (50) also exhibited trypsin uptake of 0.186 mg/g due to inherent affinity of Ac. PABA for trypsin.

The ability of T (50) to discriminate between trypsin and chymotrypsin from a mixture containing identical initial activities of the two was also evaluated. It was found that activity wise, 40 % trypsin and only 2.6 % chymotrypsin was taken up by T (50). This uptake of chymotrypsin by trypsin imprinted gel T (50) was surprising in view of the exclusivity exhibited in separate batch experiments. Here, it should be noted that trypsin hydrolyses chymotrypsin and *vice a versa* which can result in lowering chymotrypsin activity in the eluate. To confirm whether the loss in chymotrypsin activity was due to the presence of trypsin, we followed the loss in chymotrypsin activity in the presence of trypsin in the preparations used in above experiment. The loss in chymotrypsin activity over the time period of the experiment was exactly 2.6 %. Thus we concluded that 2.6 % loss in the activity of chymotrypsin is not due to its uptake by T (50) but it is because of decrease in its activity caused by trypsin. Thus, from the mixture as well, exclusive uptake of trypsin was exhibited by T (50) receptor gel.

6.3.0 Conclusions

Molecularly imprinted receptor for trypsin was synthesized by employing a novel affinity – imprinting technique. N-acryloyl para aminobenzamidine which contains active site inhibitor of trypsin viz. guanidine group was used as a functional monomer in these gels. The affinity-imprinting effect was demonstrated. For an optimum percentage of crosslinker (50 %) the imprinted gel exhibited linear Scatchard plot, which is a characteristic of a true receptor. But the non-imprinted gel exhibited curvilinear plot indicating that it functions as an affinity chromatography gel. Trypsin receptor gel T (50) exhibited exclusive uptake of trypsin and no uptake of chymotrypsin in a batch experiment. It also exhibited exclusive uptake of trypsin from a mixture of trypsin and chymotrypsin. The affinity - imprinting concept proposed in this work can be extended to the development of biosensors for detection of clinically important enzymes such as lactate dehydrogenase and glutamic-oxaloacetic transaminase.

Chapter 7

Conclusions and suggestions for future work

This work was undertaken with following objectives in mind.

1. To conceptualize and synthesize thermoprecipitating polymers containing enzyme specific ligands conjugated to spacers, which would enhance ligand-enzyme interactions in polymer, and lead to enhanced recovery of the desired enzyme.

2. To synthesize polymers, which would overcome the crowding effect.

3. To exploit the results generated, to design and synthesize new spacer containing ligands/polyligands which would exhibit not only enhanced binding but also increased recovery of an industrially important enzyme. Lysozyme was chosen as a typical enzyme and the efficiency of polymeric ligands synthesized in this work was compared with the natural ligand i.e. N-acetylglucosamine (NAG).

4. To evaluate a variety of acidic ligands for thermoprecipitation of lysozyme and compare their performance *vis a vis* affinity ligands both synthetic and natural.

5. To synthesize molecularly imprinted polymers using affinity ligand as the functional monomer. To evaluate specificity and capacity of such affinity-imprinted polymers against imprinted polymers, which are synthesized using non-affinity based functional monomers exhibiting hydrogen bonding interactions only.

The key conclusions arrived at from present investigations are as follows.

Chapter-3

- The inhibition constant (K_i) of the copolymers of N-isopropylacrylamide (NIPAM) and N-acryloyl amino acids conjugated with para aminobenzamidine (PABA) for trypsin decreased with increase in the spacer chain length. It demonstrates that the efficiency of PABA - trypsin binding increased with spacer chain length. (page 125)

- Further investigation showed that this enhancement was due to the spacer as well as microenvironmental effects. (page 125-126)
- The inhibition constants of these affinity polymers were not adversely affected by the crowding effect. (page 126-127)
- The recovery and specific activity of the trypsin recovered, increased with the spacer chain length as well as selectivity in the separation of trypsin from a mixture of trypsin and chymotrypsin. (page 128 to 130)

Chapter-4

- Synthetic ligands comprising spacers and terminally attached acetamido groups were conjugated with an acrylic monomer and copolymerized with NIPAM. These thermoprecipitating polymers bind to lysozyme far more efficiently than those containing NAG; a natural ligand for lysozyme. (page 163 to 165)
- The spectrophotometric analysis reveals that these synthetic ligands with pendant acetamido groups bind to tryptophan residue in lysozyme as does NAG. (page 160 to 162)
- The amount and activity of lysozyme recovered from aqueous solution as well as lysozyme-ovalbumin mixture increased with the length and the hydrophilicity of the spacer. Also, these synthetic ligands are more stable than their natural counterpart i.e. NAG. (page 165 to 169)
- This investigation can lead to a new method for the recovery of lysozyme. Also, the approach is generic and could be extended to other enzymes as well. (page 169)

Chapter-5

- Acidic monomers viz. acrylic acid, methacrylic acid, itaconic acid and 2-acryloylamido-2-methyl-propane-sulfonic acid were copolymerized with NIPAM. The resulting thermoprecipitating polymers exhibited lower critical solution temperatures (LCST) in the ambient range. These polymers bind to lysozyme far more efficiently than those containing NAG and synthetic N-acetamido ligands reported earlier. (page 189 to 191)
- For a given polymer, the total number of binding sites (q^{app}_{max}) was higher below LCST than above LCST. The dissociation constant (K_d) was marginally higher above LCST. (page 191 to 194)
- Lysozyme recovery from aqueous solution showed that a polymer comprising acrylic acid exhibited higher recovery in terms of protein as well as activity of lysozyme than the polymer containing NAG. More than 90 % of lysozyme activity could be recovered from egg white using this polymer. Also, acidic thermoprecipitating polymers are more stable than affinity polymers. Therefore, this approach could provide an alternative to ion-exchange method presently used for the lysozyme recovery. (page 194 to 196)

Chapter-6

- Molecularly imprinted polymeric receptor for trypsin was synthesized by employing a technique, which is a combination of affinity chromatography and molecular imprinting. Various control experiments demonstrated the synergistic affinity and imprinting effect. (page 214-215)

- Imprinting efficacy is governed by the percentage of crosslinker used when the percentage of crosslinker was optimized, trypsin imprinted gel exhibited exclusive recognition for trypsin from a mixture of trypsin and chymotrypsin. Moreover, the uptake capacity of trypsin imprinted gel was hundred folds higher than that of molecularly imprinted silica gels employing hydrogen bonding interactions. (page 216 to 218)

While this work has led to methodologies which enhance enzyme-ligand binding in affinity based separations; it has also opened up further possibilities which could be exploited to enhance the binding. These also depend upon the mechanism of binding between the ligand and the enzyme.

1) The investigation carried out in chapter 3 demonstrates that the binding strength of PABA for trypsin increased with the spacer chain length. Further, enhancement in binding was achieved by enhancing the hydrophobicity of the comonomer used. Thus, it would be logical to investigate if increasing the hydrophobicity of the guanidino group would enhance the binding further.

2) The extent of interaction between the enzyme and affinity ligand incorporated in the polymer depends upon the nature of active site. The active site of lysozyme is multimeric and hydrophilic whereas trypsin has a single active site containing a hydrophobic pocket. The PABA attached to a relatively hydrophobic spacer (5 methylene groups) exhibits highest binding with trypsin whereas, the acetamido group conjugated to hydrophilic spacer glycylglycine binds lysozyme most efficiently. Lysozyme, being a multimeric enzyme showed enhanced binding with the affinity polymer *vis a vis* affinity ligands as compared to trypsin. Chitin is a natural affinity polymer used routinely for lysozyme

separation. It has many folds higher binding capacity for lysozyme than the random copolymers synthesized in the present work. This could be due to the fact that the NAG residues in chitin are in juxtaposition. Investigation of the effect of block copolymer comprising synthetic affinity ligands for the binding of lysozyme *vis a vis* trypsin will elucidate the reasons for enhanced binding further.

3) The cationic exchangers are routinely used for the recovery of lysozyme on industrial scale. Acidic thermoprecipitating polymers discussed in the chapter 5 also demonstrate increased recovery of lysozyme as compared to the affinity polymers, both synthetic and natural. To enhance the recovery of lysozyme by thermoprecipitation process, binding strength of acidic groups with lysozyme should be increased. This could be achieved by introducing hydrophilic spacers in the side chain.

4) Recovery of both trypsin and lysozyme was carried out by thermoprecipitation process. But, similar to Eudragit other ways of NIPAM precipitation, e.g. change in ionic strength, use of organic solvents etc. could be investigated.

5) The affinity-imprinting concept developed in chapter 6 showed that at 50 % crosslinking the polymer exhibit maximum imprinting efficiency. At this percentage of crosslinker the polymer can only be used as a receptor. As reported earlier, NIPAM based imprinted polymers were used to selectively separate restriction enzymes. Similarly, we envisaged that replacing acrylamide in the present case with stimuli responsive monomer the resulting receptor can also be used as a separation tool.

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1. N.Kulkarni, **A.Vaidya** and M.Rao
   Extractive Cultivation of Recombinant *Escherichia coli* Using Aqueous Two Phase Systems for Production and Separation of Extracellular Xylanase. Biochemical and Biophysical Research Communications, **255**: 274-278 (1999).

2. **A.A.Vaidya**, B.S.Lele, M.G.Kulkarni, R.A.Mashelkar.
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3. **A.A.Vaidya**, B.S.Lele, M.V.Deshmukh, M.G.Kulkarni and R.A.Mashelkar.
   Synthetic Ligands Outperform N-acetylglucosamine in Lysozyme thermoprecipitation. Biotechnology and Bioengineering (*communicated*)

4. **A.A.Vaidya**, B.S.Lele, M.G.Kulkarni, R.A.Mashelkar.
   Affinity-Imprinting: A Technique for Creating Receptor with Enhanced Selectivity for Trypsin. Bio-macromolecules (*communicated*)

5. **A.A.Vaidya**, B.S.Lele, M.G.Kulkarni, R.A.Mashelkar.
   Acidic Thermoprecipitating Polymers Enhance Lysozyme Recovery. Biotechnology progress *(communicated)*

6. M.V.Deshmukh, **A.A.Vaidya**, M.G.Kulkarni, P.R.Rajmohanan, S. Ganpathy.
   LCST in poly (N-isopropylacrylamide) copolymers: High resolution proton NMR investigation. Polymer *(communicated)*

List of patents

1. **A.A.Vaidya**, B.S.Lele, M.G.Kulkarni, R.A.Mashelkar.
   A process for the preparation of molecularly imprinted polymers useful for separation of Enzymes. Indian Patent (Filed).

2. **A.A.Vaidya,** B.S.Lele, M.G.Kulkarni, R.A.Mashelkar.
   A process for the preparation of thermoprecipitating affinity polymers. Indian Patent (Filed).

3. **A.A.Vaidya**, B.S.Lele, M.G.Kulkarni, R.A.Mashelakar.
   A thermoprecipitating polymer containing enzyme specific ligands and a process for the preparation therefor and a process for the separation of enzymes. U.S.Patent (Under Filing).

Abstract Submitted in the Conference

1. N.Kulkarni, **A.Vaidya** and M.Rao.
   Extractive Cultivation of Recombinant *Escherichia Coli* for Production of Extracellular Xylanase Using Aqueous Two-Phase System - Submitted at Diamond Jubilee Symposia and 39th Annual Conference Association of Microbiologist of India; Mangalore, India. Held on December 5-7, 1998.
2. M.V. Deshmukh, P.R. Rajamohanan, **A.A.Vaidya**, M.G. Kulkarni, S. Ganapathy.
   Proton (^{1}H) NMR Study of LCST Phenomenon in Copolymers of poly (N-isopropylacrylamide) - Submitted at Workshop on Applications of NMR in Petroleum and Petrochemical Industries and 5th National Symposium on Magnetic Resonance. Indian Institute of Petroleum, Dehradun, India. Held on February 23-26, 1999.

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