APPLICATIONS OF REVERSIBLY SOLUBLE - INSOLUBLE POLYMERS IN BIOSEPARATION AND ENZYME IMMOBILIZATION

By

MERYAM SARDAR

PARTMENT OF CHEMISTRY

Submitted in fulfilment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

to the

INDIAN INSTITUTE OF TECHNOLOGY, DELHI

INDIA

July 1998

For my daughter

Sahar Rizvi

CERTIFICATE

This is to certify that the thesis entitled "***APPLICATIONS OF REVERSIBLY SOLUBLE-INSOLUBLE POLYMERS IN BIOSEPARATION AND ENZYME IMMOBILIZATION*** " being submitted by **Ms. MERYAM SARDAR** to the Indian Institute of Technology, Delhi for the Award of the degree of **Doctor of Philosophy** in Chemistry is a record of bonafide research work carried out by her. Ms. Meryam Sardar has worked under my guidance and supervision, and has fulfilled the requirements for the submission of this thesis which, to my knowledge, has reached the requisite standard.

The result contained in this dissertation have not been submitted in part or in full to any other University or Institute for the award of any degree or Diploma.

July , 1998

M.N. Gupta
Professor
Department of Chemistry
IIT, Delhi

ACKNOWLEDGEMENT

I would like to express my deep gratitude to Prof. M.N. Gupta, for his invaluable ideas, guidance and encouragement throughout my research work. I have gained in experience and learnt more, than I would have in any other place of work.

I am grateful to the Head, Department of Chemistry, for providing all the necessary facilities.

My sincere thanks are due to all my colleagues Dr. Renu Tyagi, Ritu, Renu, Aparna and Ipsita for suggestions and help during various stages of this work.

I am also thankful to Indian Institute of Technology, Delhi for the financial support.

Lastly, I owe a debt of gratitude beyond repayment to my parents and specially my husband for never ending love and support.

Meryam Sardar

MERYAM SARDAR

ABSTRACT

Today, two key operations in biotechnology are protein bioseparation and enzyme stabilization. The former is important since as high as 70-90% of production cost of a protein involves its downstream processing. The latter assumes importance in the context of actual use of the enzyme. A variety of approach for protein stabilization are well known, one which is frequently employed is immobilization of the protein.

The present thesis describes the efforts to exploit interactions between protein and polymeric surfaces for the dual applications of separation and noncovalent immobilization.

The first chapter is an introductory chapter and briefly surveys the literature in order to provide the necessary perspective for the work described in this thesis. After reviewing reversibly soluble insoluble polymers, the area of bioseparation of enzymes with a focus on precipitation is discussed. This is followed by a description of recent trends in protein immobilization with an emphasis on enzymes dealing with macromolecular and insoluble substrates.

The second chapter of the thesis focuses on the interaction between some biotechnologically important enzymes / proteins and some reversibly soluble - insoluble polymers.

Three water soluble polymers have been used in this work. Two are naturally occuring polymers (viz. alginate and chitosan) the third is a synthetic

polymer (viz. Eudragit). The binding of several enzymes (viz. wheat germ acid phosphatase, trypsin, chymotrypsin, alkaline phosphatase, alpha amylase, xylanase, cellulase, pectinase and beta-glucosidase) with these polymers was studied. The results indicated that these polymers show pronounced nonspecific adsorption with the large number of enzymes and the exact extent of binding and its strength depends upon the individual pair (consisting of polymer and an enzyme). For example, none of the enzymes bound to alginate could be eluted to a significant extent by using salt solutions.

The result with chitosan shows that 100% of lysozyme, wheat germ lectin and cellulase binds to it, thus it can be used as an affinity matrix, this is further investigated in chapter four.

The result with Eudragit S-100 shows that alpha amylase and alkaline phosphatase bound rather firmly to it, it should be worth exploiting this for the immobilization of the enzymes.

The third chapter described the noncovalent immobilization of alpha amylase and alkaline phosphatase on Eudragit S-100 and xylanase on Eudragit L-100. Immobilization of alkaline phosphatase and alpha amylase on Eudragit S-100 was carried out at different polymer concentrations. In the polymer concentration range of 0.1 to 4%, the enzymes did not leach from the polymer when washed with 1M NaCl, 0.2% triton and 50% ethylene glycol. The immobilized alpha amylase was characterized in terms of catalytic activity, kinetic parameters and thermal stability. At 50^0C, the immobilized enzyme was found to completely lose its activity after 80 minutes whereas the free enzyme still retained 72% activity. In the case of alpha amylase, this reduced

thermostability is desirable since thermolabile alpha amylase is reported to be useful for improving both loaf volume and crumb softness of bread products without the danger of overdextrination.

As the enzymes immobilized on smart polymers are considered specially useful with macromolecular or insoluble substrates, the work was initiated with preparation and evaluation of xylanase bound to reversibly soluble insoluble polymers (viz. chitosan, alginate, esterified alginate, Eudragit S-100 and L-100). It was found that in all the cases, the binding of the enzymes increases as the polymer concentration increases. Eudragit S -100 and L-100 in the concentration range of 1-2% seemed to be the best choice, since the enzymes quantitatively bound to the polymers.

One major application of xylanase at the industrial level is in prebleaching of paper pulp. For this application, xylanase should be free from any cellulase activity. Hence, it was desirable to investigate the binding of cellulase activity present in the xylanase preparation to these polymers. It was found that Eudragit L-100 did not bind any cellulase activity. Thus xylanase immobilized on Eudragit L-100 by adsorption constitutes a cellulase free xylanase preparation. The immobilized xylanase retained 60% of its activity towards xylan as the substrate. No change was observed in pH optimum (5.5) of the enzyme upon immobilization. The immobilized xylanase retained all its activity even after 90 minutes at 60°C whereas the free enzyme showed 20% decrease in activity. The only marginal increase in the K_m of the free enzyme (0.36gm/100ml to 0.50gm/100ml) upon immobilization to the soluble polymer reflected that the enzyme substrate binding continues to be efficient inspite of the macromolecular

nature of the substrate. The UV, fluoroscence and CD data indicate that about 40% loss of enzyme activity is likely to be largely due to the drastic change in the secondary structure of the enzyme.

In the fouth chapter chitosan was used as an affinity material for the purification of lysozyme, wheat germ and potato lectin and cellulase. The binding of hen egg white lysozyme to chitosan was a straightforward procedure, elution of the lysozyme from the precipitated polymer enzyme complex had to be optimized. Out of the numerous eluting agents, viz. 1M NaCl, 10-50% ethylene glycol, 1M N-acetyl-D-glucosamine, 3.0M KSCN and 2.5M $MgCl_2$, the last one worked best and 70% of the enzyme activity could be eluted.

It was observed that chitosan also bound to *A.niger* cellulase. 2.9 activity units of cellulase were added to 4 mg of chitosan and the enzyme bound quantitatively. The 65% enzyme activity could be eluted with phosphate buffer (1.0M, pH 7.0). However, the attempts to develop purification protocols using chitosan for wheat germ lectin and potato lectin did not succeed. While these lectins did bind almost quantitatively, most of these could not be eluted from the matrix. These results indicated the difficulties one is likely to encounter in developing a successful affinity precipitation/purification protocol for purifying a protein.

The following issues related to the process optimization in case of affinity precipitation were examined.

How does the percent binding at a fixed enzyme load change as the polymer concentration is varied.

- Is the enzyme recovery dependent upon the enzyme load.

- Is fold purification obtained dependent upon the enzyme load.

Fifth chapter explores the possibility of preparing or designing some useful insoluble materials made from reversibly soluble-insoluble polymers.

It has been reported that extensive crosslinking of chitosan with gluteraldehyde resulted in an insoluble material which showed selectivity in binding to proteins. It was also reported that salt promoted binding to the matrix made it easier to elute protein from this matrix. As further application of this novel media (for hydrophobic interaction chromatography) two target enzymes chosen were : alkaline phosphatase from bacteria (*Baccillus licheniformis*) and a commercial preparation of cellulase (from *Aspergillus niger*). It was found that 60% of alkaline phosphatase bound to the modified chitosan at pH7 in the presence of 2.4 M ammonium sulphate. The elution of the enzyme was tried with 80 % ethylene glycol and 58% of the enzyme could be recovered.

The binding of cellulase with modified chitosan in presence of 2.4 M ammonium sulphate was studied at different temperatures (5, 10, 15 and 25^{0}C). The elution of the enzyme was tried with 50% ethylene glycol. The data shows that at lower temperature (5^{0}C) binding of cellulase to modified chitosan decreases (from 100% at 25^{0}C to 65%), but 50% of the cellulase was eluted at lower temperature while there was no elution at 25^{0}C.

Yet another way of using water soluble polymers is to convert these materials into insoluble beads. It has been mentioned earlier that the presence of alginate was found to completely inhibit alpha amylase activity. The addition of alpha amylase from porcine pancreas to alginate beads showed that the 83% of

the enzyme activity bound to the beads. The 84% of the bound enzyme could be eluted by 1.0 M maltose. Similarly, a commercial preparation of *Baccillus subtilis* alpha amylase could also be purified on alginate beads. In this case 71% of the enzyme could be recovered. Thus, alginate beads constitute an alternative affinity medium for purification of alpha amylases (both from animal as well as microbial sources). To further explore the usefulness of alginate beads for purification of alpha amylases from different sources, a crude extract of wheat amylase was added to alginate beads. 83% of the enzyme activity bound to the beads, and 70% could be eluted with maltose. The eluted enzyme was 45 fold purified.

The thesis also contains references quoted during various chapters; this section is given in the end of the thesis.

CONTENTS

Lately, reversibly soluble - insoluble polymers are finding increasing applications in bioseparation and design of reusable biocatalysts[1,2]. In both approaches; naturally occuring polymers as well as synthetic polymers have been used successfully. However, these approaches constitute underexploited strategies and it is worthwhile to explore further possibilities of applications of such polymers in these twin areas. The present work is an attempt in this direction.

The bioseparation component of this work exploits interactions with the target enzyme and a polymeric surface. An enzyme selectively bound to a smart polymer can be taken out of a crude mixture by precipitating out the polymer along with the bound protein. In many cases, the interaction between the enzyme and the polymer is adequately strong so as to constitute an immobilized enzyme preparation. Because of the nature of the polymer, it also constitutes a reusable enzyme form.

This introductory chapter briefly describes such polymers and then reviews the relevant aspects of bioseparation and immobilization which provide the necessary perspective for the work described in the thesis.

REVERSIBLY SOLUBLE - INSOLUBLE POLYMERS

Smart materials respond to changes in their environment in predictable and pronounced ways[1-4]. This ability can be finely tuned for a wide variety of applications (Table 1.1) including the downstream processing of biological macromolecules. The water soluble polymers undergo fast and reversible changes in the micro structure triggered by small changes in the characterstics (e.g. pH, temperature, ionic strength, presence of specific chemicals, light,

electric or magnetic field) of the medium in which they are used. These changes are reversible.

Table 1.1: Applications of reversibly soluble - insoluble polymers

Field	Specific Use	Ref.
Biocatalysis	Immobilization of living cells	5
	Reversibly soluble biocatalysis	6,7
	Feedback control of biocatalyst	8,9
	Enhanced mass	10
Energy transducer	Artificial muscle	11,12
Medicine	Controlled drug release	13,14
	Site directed delivery	15,16
Analysis	Light, Thermo, pH & ions selective	17
	Immunoanalysis	18,19
	Biosensors	20,21
Size selective	Dewatering of slurries	22
Separation	Concentrating macromolecular solutions	23,24
	Controlled permeation of membranes	25,26
Downstream	Affinity precipitation	1
Processing	Partitioning in two phase systems	27,28
	Modulated chromatography	29,30
	Detachment of cells	31

In general, the soluble-insoluble polymers can be divided into three groups (Fig 1.1). The first group consist of polymers for which poor solubility conditions are created by decreasing the net charges on their surface e.g by changing the pH. This reduces the repulsion between the polymer segments. For instance, methylmethacrylate-methacrylic acid copolymers (Eudragit S and L) precipitate from aqueous solutions on acidification to pH below ~ 5, whereas chitosan, partially deacetylated chitin is soluble below pH 6 and insoluble at higher pH[1-3].

The charges on the macromolecule can also be neutralised by adding an efficient counterion, for example, a low molecular weight counterion or a polymer molecule with the opposite charges. In the latter case a polycomplex is formed. The cooperative nature of interaction between two polymers with opposite charges makes polycomplexes very sensitive to changes in pH or ionic strength[32]. For instance, the complex of poly (N-ethyl-4-vinyl pyridinium bromide) and poly (methacrylic acid) quantitatively precipitates and dissolves with a pH changes of only 0.3 units[33].

The second group of soluble-insoluble polymers consists of uncharged polymers soluble in water because of hydrogen bonding with water molecules. The changes in the hydrophobic-hydrophilic balance induced by increased temperature or ionic strength cause the polymers to aggregate and eventually precipitate. Hydrogen bonding efficiency is reduced when the temperature is raised.

Fig.1.1 Types of reversibly soluble insoluble polymers and their mechanisms of phase separation.