

**DEVELOPMENT OF SOME STRATEGIES FOR  
SEPARATION AND IMMOBILIZATION OF  
PROTEINS/ENZYMES**

*by*

**RITU AGARWAL  
Department of Chemistry**

***SUBMITTED  
IN FULFILMENT OF THE REQUIREMENTS  
OF THE DEGREE OF  
DOCTOR OF PHILOSOPHY***

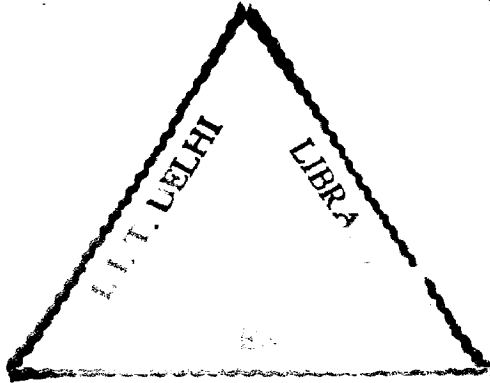


**to the  
INDIAN INSTITUTE OF TECHNOLOGY, DELHI  
INDIA**

**February, 1996**

TH  
543.87  
AGA-D

I. T. DELHI.  
LIBRARY  
Acc. No. TH-2398



**FOR MY SON,  
ANIRUDH**

## CERTIFICATE

This is to certify that the thesis entitled "**DEVELOPMENT OF SOME STRATEGIES FOR SEPARATION AND IMMOBILIZATION OF PROTEINS/ ENZYMES**" being submitted by **Ms. RITU AGARWAL** 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. Ritu Agarwal 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 results 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.

2nd February, 1996



**M.N.GUPTA**  
Professor  
Department of Chemistry  
IIT, Delhi

## ACKNOWLEDGEMENT

*I feel very much honoured to express my deep sense of gratitude and respect to Prof. M.N.Gupta for his brilliant guidance, constructive criticism and constant encouragement during the tenure of this work.*

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

*I am thankful to all my laboratory colleagues Dr. Renu Tyagi, Dr. Ashok Kaul, Ms. Renu Batra and especially Ms. Maryam Sardar and Ms. Zeba for their help at various stages of this work.*

*I am also thankful to Council of Scientific and Industrial Research, New Delhi, for the award of Junior and Senior Research Fellowship.*

*Lastly, I owe a debt of gratitude beyond repayment to my family members specially my husband and dear son, for providing a cordial atmosphere which helped me to reach this level.*

*Ritu Agarwal*  
RITU AGARWAL

## ABSTRACT

This work exploits interactions between proteins and reversibly soluble-insoluble polymers for dual applications of protein separation and immobilization. Protein separation is important because as high as 60-80% of production cost of a protein involves its downstream processing. The protein stabilization assumes importance in the context of actual use of the enzyme. An enzyme during transport, storage or reaction may be subject to relatively harsh conditions. Variety of approaches for protein stabilization are well known, one which is frequently employed is the immobilization of the protein on a support.

Chapter 1 consists of literature survey which provides the backdrop for separation processes which have been used in the present thesis. This survey also briefly touches upon the area of protein immobilization in general and emphasizes noncovalent methods of immobilization in particular.

The second chapter of the thesis focuses on metal affinity interactions in free solution. To start with, the extent of precipitation of various proteins (containing varying number of histidine residues/mol of protein) by metal ions ( $\text{Cu}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Zn}^{2+}$ ) in free solution was studied. Precipitation by  $\text{Cu}^{2+}$  was investigated in detail and was used for purification of Concanavalin A, soybean agglutinin, and soybean

nitz trypsin inhibitor. In the last case it was conformed that data obtained in free solution agrees rather well with what is obtained in immobilized metal affinity chromatography. The result with soybean trypsin inhibitor also prompted us to look at behaviour of evolutionary related inhibitors from other plant sources.

Two thermoprecipitable polymers, copolymer of 1-vinyl imidazole with vinyl pyrrolactam (poly VI-VCl) or N-isopropylacrylamide (poly VI-NIPAM) were used to carry out metal affinity precipitation in free solution.  $\text{Cu}^{2+}$  was immobilized on these polymers. Both the polymers were found to bind Kunitz trypsin inhibitor from soybean (STI). Maximum precipitation with  $\text{Cu}^{2+}$  loaded poly VI-VCl was 83% and recovery was 65% at 0.83% polymer concentration. STI could be precipitated quantitatively using  $\text{Cu}^{2+}$  loaded poly VI-NIPAM with complete recovery at 0.67% polymer concentration.  $\text{Cu}^{2+}$ -poly VI-VCl did not precipitate quantitatively from the solution. On the other hand, precipitation efficiency of poly VI-NIPAM was 100%. Therefore, poly VI-NIPAM was chosen and Kunitz trypsin inhibitor was separated from crude soybean extract.

In the third chapter, chitosan (a polymer of N-acetyl glucosamine) and Eudragit S-100 (copolymer of methyl methacrylate and methyl methacrylic acid) were used for purification of  $\beta$ -glucosidase from two sources (almond meal and *Pododerma longibrachiatum*).  $\beta$ -Glucosidase from almond meal was purified 5.6 fold by simple pH precipitation step followed by precipitation with Eudragit S-100 with 73% recovery of activity. Recovery of activity was critically dependent on the polymer concentration and the recovery increased with decrease in polymer

concentration. Therefore, 0.1% Eudragit S-100 was used in all the experiments.  $\beta$ -Glucosidase from *Trichoderma longibrachiatum* could be purified 3.7 fold with 83% recovery by precipitation with Eudragit S-100 alone. By sequential precipitation with chitosan and Eudragit S-100, followed by a gel filtration step, enzyme could be purified 29 fold with 74% recovery of activity. This purified preparation was homogeneous as shown by SDS-PAGE.

It has been reported earlier that there might be significant amount of noncovalent adsorption in immobilized enzyme preparation obtained by covalent coupling procedures. This is an important issue which may, perhaps, account for the leaching off the enzyme from immobilized preparations obtained by covalent coupling procedures. The fourth chapter looks at this issue more extensively. Four enzymes viz trypsin,  $\beta$ -glucosidase,  $\beta$ -galactosidase and wheat germ acid phosphatase (WGAP) were covalently coupled by EDC on Eudragit S-100. Simultaneously, enzymes were bound noncovalently to Eudragit S-100. The coupling and adsorption were carried out at two pH (4.5, when the polymer was not soluble; 7.2 when the polymer was in soluble form). In the case of WGAP, it was found that while there were measurable differences between protein which came off the matrix with various solutions (1 M NaCl, 0.15% triton X-100 and ethylene glycol) in case of adsorbed and covalently conjugated enzymes, a significant amount of covalent conjugation in fact seems to involve binding of the enzyme through noncovalent interactions only. In case of trypsin, conjugation was carried out in presence of 1 M NaCl and two polymer concentrations were taken (0.1% and 1%). Even in presence of 1 M NaCl, the enzyme binds to 1% polymer.

both by adsorption and covalent coupling. The nonspecific binding was 45% at pH 7.2 and 5 while the covalent binding was 89%. At pH 4.5 nonspecific binding was 78% and covalent binding was 97%. When the polymer concentration was reduced to 1% the nonspecific binding was 18% at pH 7.2 and 53% at pH 4.5. Thus, at lower polymer concentration, nonspecific binding in presence of salt was much less as compared to the nonspecific binding at 1% polymer concentration. Higher polymer concentration was chosen to immobilize trypsin and  $\beta$ -glucosidase on Sepharose S-100. Various parameters like effect of pH, thermostability and  $K_m$  of the immobilized preparations were studied.

In the last chapter, alkaline phosphatase was purified on chitosan modified with glutaraldehyde. When alkaline phosphatase was bound (99%) to modified chitosan in presence of 2.4M  $(NH_4)_2SO_4$ , enzyme could be completely recovered with 50% ethylene glycol at 10°C. It appears that in presence of high salt concentration, binding was mostly due to hydrophobic interactions. This way, the enzyme could be purified 4.5 fold with 99% recovery of activity.

# CONTENTS

CERTIFICATE		i
ACKNOWLEDGEMENT		ii
ABSTRACT		iii
CHAPTER I	INTRODUCTION	1-35
CHAPTER II	SOME BIOSEPARATION STRATEGIES BASED UPON METAL AFFINITY TOWARDS PROTEINS	36-90
CHAPTER III	SEQUENTIAL PRECIPITATION BY POLYMERS AS A BIOSEPARATION STRATEGY	91-115
CHAPTER IV	IMMOBILIZATION OF ENZYMES ON EUDRAGIT S-100	116-149
CHAPTER V	HYDROPHOBIC CHITOSAN AS A MATRIX FOR ENZYME PURIFICATION AND IMMOBILIZATION	150-165
REFERENCES		166-181