DEVELOPMENT OF SOME EFFICIENT STRATEGIES FOR BIOSEPARATION, BIOCATALYSIS AND BIOANALYSIS

By

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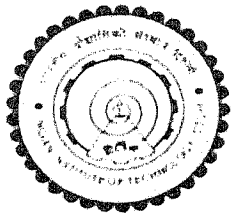
Submitted
In fulfillment of the requirement of the degree of Doctor of Philosophy
to the



INDIAN INSTITUTE OF TECHNOLOGY, DELHI
JANUARY, 2005

Dedicated to my Parents

CERTIFICATE

This is to certify that the thesis entitled **DEVELOPMENT OF SOME EFFICIENT STRATEGIES FOR BIOSEPARATION, BIOCATALYSIS AND BIOANALYSIS** being submitted by **Ms. SULAKSHANA JAIN** 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 Jain has worked under my supervision, and has fulfilled the requirements for the submission of the 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.

January, 2005

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ACKNOWLEDGEMENTS

I would like to express my deep sense of gratitude and respect to my teachers, past and present, colleagues, friends and parents who have contributed immensely at various stages while working on this thesis. It is not possible to mention all of them but I will still like to name a few people.

Prof. M. N. Gupta, for his guidance, untiring efforts, constructive criticism, precious advises and many insightful conversations during the development of the ideas for this thesis. With his enthusiasm, his inspiration, patience and his great efforts to explain things clearly and simply, he helped me in understanding this new world of enzymes/proteins. He has always been a driving force. I would have been lost without him. His sayings will be source of inspiration throughout of my life.

Head, Department of Chemistry Prof. U. K. Nadir for providing me all the necessary facilities.

Prof. A. K. Singh and Prof. Mathillisharan (IIT Delhi) for constant encouragement.

Dr. S. K. Khare (IIT Delhi) for his encouragement, useful discussions and help whenever needed.

My all teachers of Banasthali Vidyapith (Chemistry Department) for their encouragement and being so good and affectionate towards me. I feel myself lucky for having such caring teachers.

My labmates: Dr. Sunita Teotia, Dr. Meryam Sardar, Dr. Aparna Sharma, Dr. Sandhya Shenoy, Dr. Rajni Singh, Dr. Ipsita Roy, Dr. Shweta Sharma for their moral support, well wishes, uninhibited help and teaching different aspects of research. Anshu, Kalyani, Ruchi, Shweta, Shalini, Smita, Veena, Parul, Abir and Sohel for providing friendly environment in the lab, being ready to help whenever needed, for good discussions over hostel problems and bearing with my short temper at times.

I would like to extend my heartful thanks to Dr. Srinivas, Anamika, Rashmi, Varsha, Bharti, Renuka, Shweta and all other friends whose names are not listed here, for their best wishes and kind help during my stay at IIT, Delhi.

All help at various stages by Mr. Nautiyal, Mr. Anjani, Kuldeep ji and Gangaji was indispensable.

It is a futile attempt on my part to express my deep love and respect to my parents, to whom I owe everything I have achieved today. This thesis is dedicated to them. I express appreciation to my sister Rachana and

already known to bind to IMAC column. It is shown that in case of papain optimization of level of Cu^{2+} charging has significant effect on the performance of the IMAC columns. One disadvantage of using commercially available media for immobilized metal ion affinity chromatography is their high cost. In this chapter, It is shown that alginate beads (wherein metal ions bind directly with the matrix) can be used directly for IMAC. Alginate is a non-toxic polysaccharide consisting of guluronic acid and mannuronic acid residues and complexes with metal ions via its carboxyl groups. These beads are evaluated as a media for immobilized metal affinity chromatography and used successfully for purification of papain from crude papaya latex (with 68% recovery and 5 fold purification) and soybean trypsin inhibitor (STI) from crude aqueous extract of soybean flour (with 90 % recovery and 18 fold purification). In this design, the step of covalent coupling of the affinity ligand is totally eliminated. Apart from elegance in design and making the process more economical, this approach circumvent the problems of traces of affinity ligand and coupling reagents ending up in the product by slow leaching. This may be extremely critical in bioseparation of pharmaceutical proteins.

In order to create more stable beads which do not require the presence of the metal ions in working buffers, the efforts were made to extend the previous design of earlier chapter to crosslinked alginate beads. This is described in chapter III. Metal ion charged crosslinked alginate beads are evaluated as media for IMAC. The copper ion charged beads were used for purification of goat immunoglobulin G (IgG) as an illustrative and biochemically useful application. The extent of binding of IgG as well as elution was dependent upon the density of the metal ion charged carboxyl groups on the beads. In the batch mode, beads with 188 Cu^{2+} μmole ml^{-1} were found to bind IgG most efficiently and

maximum elution (62.6% IgG) was possible with these beads upon elution with 0.1 M imidazole in equilibration buffer. However, the use of same beads in the packed bed mode gives no elution with imidazole in equilibration buffer. The best results in the packed bed mode were obtained by using beads charged 95.4 Cu^{2+} μmole ml^{-1}, 97.4 % IgG could be recovered with 8 fold purification by using 0.1 M imidazole as eluent.

This media was also used for fractionation of serum proteins. Judicious combination of IMAC (with this media) and various other purification techniques led to recovery and purification of some major proteins like albumin, IgG and some other minor proteins like α_1-proteinase inhibitor, α_2-macroglobulin and transferrin. Use of EDTA gradient (0-0.3 M) for elution of bound proteins from goat serum on 188 μmole ml^{-1} Cu^{2+} charged crosslinked alginate beads fractionate serum proteins into three major fractions, a) minor proteins b) purified IgG c) albumin with minor contaminant proteins. By using gel filtration, lectin affinity chromatography and dye affinity chromatography; α_2-macroglobulin, α_1-proteinase inhibitor and transferrin were recovered from fraction containing minor proteins. Albumin is purified using dye affinity chromatography from albumin rich fraction. All purified proteins were found to be homogenous on SDS-PAGE.

The chapter also contains evaluation of microbeads of calcium alginate as a fluidized bed medium for affinity chromatography of *Aspergillus niger* pectinase. Calcium alginate microbeads (212-425 μm) were prepared by spraying 2 % (wv^{-1}) alginate solution into 1 M $CaCl_2$ solution. The fluidization behavior of these beads was studied and the bed expansion index and terminal velocity were found to be 4.3 and 1808 cm h^{-1}, respectively. Residence time distribution curves showed that the dispersion was much less than the conventionally prepared calcium alginate beads. The fluidized bed of these

beads was used for the purification of pectinase from a commercial preparation. The media performed well even with diluted feedstock. 90 % activity recovery with 211 fold purification was observed.

The work described in chapter IV focuses on the development of an efficient protocol for purification of green fluorescent protein (GFP). The technique of three phase partitioning (TPP) was used to purify the green fluorescent protein (GFP) from recombinant *E. coli* in single step. TPP uses a combination of ammonium sulphate and t-butanol to precipitate proteins from their crude extracts. In the first round of TPP with 20% (wv^{-1}) ammonium sulphate at the ratio (vv^{-1}) of crude extract to t-butanol as 1:1, most of the GFP remains in lower aqueous phase. This phase when subjected to second round of TPP with 60% (wv^{-1}) ammonium sulphate at the ratio of crude to t-butanol as 1:2 (vv^{-1}) gives 78 % recovery of GFP with 20 fold purification. The SDS-PAGE analysis of purified preparation shows single band. The fluorescence excitation and emission spectra agreed with what is reported in the literature.

The above purification was carried out with a clear supernatant as obtained after centrifugation of sonicated cells. Aqueous two-phase extraction is a well-established bioseparation strategy which can directly handle crude suspensions without preclarification. This technique was used for purification of GFP without any preclarification step. Aqueous two phase partioning of GFP with PEG-phosphate phase system in presence of 14 % sodium chloride leads to 78.5 % recovery and 6.2 fold purification.

Simple precipitation with metal ions was also attempted. Use of 0.002 M Zn^{2+} at pH 6.5 is found to be the best condition resulting in 83 % recovery with 7.6 fold purification.

These results showed that each of the two nonchromatographic steps (Aqueous two-phase extraction and precipitation with metal ions) could lead to partial purification of GFP. As two-phase aqueous extraction can be carried out directly without any centrifugation or preclarification step, it was decided to use this as a first step in exploring the purification strategy based upon combination of two-phase extraction and metal precipitation.

Using above approach green fluorescent protein was purified from sonicated recombinant *E. coli* and its mutant obtained after exposure to UV light. The latter overexpresses green fluorescent protein. The recoveries of green fluorescent protein were 73 % and 83 % in the cases of recombinant *E. coli* and its mutant, respectively. The corresponding fold purifications were 24 and 9, respectively. In both cases, the purified protein showed a single band on SDS-PAGE corresponding to 28 kDa.

Chapter V deals with the preparation of smart bioconjugates of trypsin and penicillin acylase. Immobilization of penicillin acylase on eudragit S-100 (a methacrylate polymer) was studied using different modes of precipitation. Precipitation by lowering the pH (4.7 in case of buffer and 5.2 in presence of 1 M ammonium sulphate) results in 56 % retention of penicillin acylase activity. Both preparations showed higher thermal stability than native enzyme at 50 °C but could not be reused. Use of copper ion for precipitation of penicillin eudragit conjugate results in 86 % retention of penicillin acylase activity.

Trypsin was immobilized noncovalently on alginate with 100 % retention of activity. The enzyme did not leach off the polymer even in the presence of 0.01 M HCl and Triton X-100 (0.2 % vv^{-1}). The V_{max}/ K_m values did not change significantly on immobilization. There was 22% loss of activity in first cycle of pH change and after that the conjugate could be reused upto 4 precipitation cycles without any further loss of activity. This smart

bioconjugate was also found to have better operational stability in the presence of casein than free enzyme. Fluorescence studies were carried out to probe structural changes upon immobilization.

Recent developments in biochemistry demand faster screening methods which can work with small volumes. In chapter VI three fast and sensitive protocols which can work with small volumes have been described. The protocols are based upon using microwave assistance to estimations carried out in microtitre plate. The protocols relate Protein estimation by Lowry and BCA method and an assay for lipases.

TABLE OF CONTENTS