PURIFICATION AND IMMOBILIZATION OF SOME BIOCHEMICALLY USEFUL PROTEINS

by

SOHEL DALAL

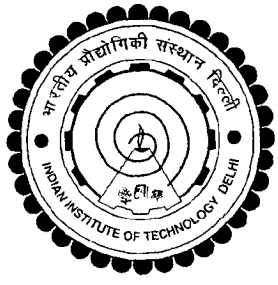
Department of Chemistry

Submitted

In fulfillment of the requirement of the degree of

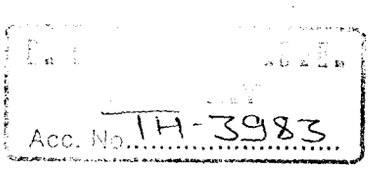
DOCTOR OF PHILOSOPHY

to the



Indian Institute of Technology Delhi

January 2010



Dedicated to my dear parents

CERTIFICATE

This is to certify that the thesis entitled "**Purification and immobilization of some biochemically useful proteins**" being submitted by **Mr. Sohel Dalal** 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 him. Mr. Dalal has worked under our supervision, and has fulfilled the requirements for the submission of the thesis which, to our knowledge, has reached 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.

Prof. M.N. Gupta

(Supervisor)

Department of Chemistry

IIT Delhi

Dr. S. K. Khare

(Co-Supervisor)

Department of Chemistry

IIT Delhi

Acknowledgements

It is indeed a great pleasure and a feeling of achievement to write the acknowledgement for my research work.

First of all, I would like to thank Prof. M. N. Gupta for his supervision, invaluable discussions, advice and guidance during my research work. I am deeply indebted to him for being available anytime for discussions and get me out of problems whenever I found myself in trouble. He was always easily accessible and willing to help. I also thank him for providing all the facilities which helped me to accomplish my task with such an ease.

I gratefully acknowledge Dr. S. K. Khare for his advice and guidance as a co-supervisor.

I am indebted to Prof. A. K Singh, Head, Department of Chemistry, for providing the necessary facilities during the work.

I would like to acknowledge All India Council for Technical Education (AICTE) for providing me National Doctoral Fellowship and funds to carry out my research work.

Prof. A. K. Singh and Dr. Shashank Deep, Department of Chemistry, IIT Delhi, and Dr. Lata, Department of Microbiology, Indian Agricultural Research Institute (IARI), New Delhi, deserve special thanks as my research committee members.

I gratefully thank Prof. T.P. Singh, Department of Biophysics, All India Institute of Medical Sciences (AIIMS), New Delhi, for helping me with N-terminal sequence analysis.

I would like to express my sincere and big thanks to my beloved parents (abba-ammi) without whom I couldn't have reached here. I simply cannot describe their love and support in words. I would like to thank my wife Nafisa for her support, confidence in me, patience and love. I would also like to thank my brother (bhai) who is also very supportive during these years. I also thank my sister (aapa), brother-in-law (Shahidbhai) and nephew (Suhaan).

My sincere thanks to Dr. Aparna Sharma, Dr. Sunita Teotia and Dr. Meryam Sardar for their help to me in initial days in the lab and for their valuable suggestions.

I would also acknowledge my seniors Dr. Sulakshana Jain, Dr. Kalyani Mondal, Dr. Shweta Shah and Dr. Smita Raghava for their advice and their willingness to share their bright thoughts with me, which were very fruitful for shaping up my ideas and research. I thank Dr. Ruchi for extending her helping hand whenever needed.

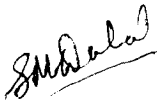
I am fortunate to have colleagues like Dr. Abir, Kusum and Veena who always helped me in one or another way. My special thanks to Dr. Abir for his valuable discussions and suggestions during a coffee break. I would like to thank my juniors Manali, Ghulam Mohmad, Benu, Sarah and Mahendra for creating a pleasant working atmosphere in the lab.

I would also like to thank Anjani for his technical assistance.

To Piar Chand (PC) for making the lab a cleaner place to work.

I wish to thank my friends, Maroof Ali (Maroofbhai) and Mohammed Afzal (Afzalbhai) for providing me a good company and for religious talks. I am fortunate to have friends like Kamalakantha, Mohammed Imran, Zubair and Raja Mohammed.

Last but not the least, I thank IIT-Delhi for providing accommodation (Satpura House) and good environment (lots of trees) in the campus.



Sohel Dalal

Abstract

A major part of biotechnology is concerned with producing proteins in a reasonably purified form for using their biological activity in an efficient fashion. A detailed study and characterization of novel proteins from new sources require purified protein in substantial amount. Therefore, protein purification and characterization continues to be a challenging area in protein biotechnology.

Downstream processing often is a more challenging aspect of protein production. Therefore, a purification protocol with less number of steps which can ultimately reduce the cost of production is desirable.

For application of enzymes, creating efficient biocatalyst designs involving greater catalytic efficiency, higher stability and reusability for various industrially important biotransformations is the next important step in protein biotechnology.

These twin aspects form the basis of the work described in the current thesis. The thesis is divided into eight chapters. The chapters 2-6 relate to development of efficient protein purification protocols for proteins/enzymes of diverse kinds. The chapters 7 & 8 describe the somewhat novel strategies of protein immobilization.

Chapter 1 is the introductory chapter and reviews the literature on various topics covered in the rest of the thesis.

Chapter 2 describes production, purification and characterization of an alkaline lipase from *Burkholderia cepacia* ATCC 25609. A *Burkholderia cepacia* strain ATCC 25609, which had been isolated from the bronchial washings of a cystic fibrosis patient was used to produce lipase. The presence of sodium alginate at an optimum concentration of 8 mg/ml in the growth medium nearly doubled the production of extracellular lipase activity. The enzyme could be purified with 38 fold purification and 96% activity recovery using a two-step purification protocol. The

minimum molecular weight of the purified lipase determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was shown to be 28000 Da. Lipases from *Burkholderia cepacia* are known to be monomeric proteins with a molecular weight in the range of 25000-36500 Da. The purified enzyme showed pH optimum at pH 9 and it was stable upto 12 h at pH 9 and 10. The enzyme has temperature optimum at 40 °C and its half-lives were 54 min and 46 min at 50 °C and 60 °C respectively. The lipase was found to be stable in the presence of detergents Tween-20 and Triton X-100. Hence, the lipase could be useful in detergents. The Secondary structure analysis of lipase by circular dichroism spectroscopy showed 52% α-helix, 7.7% β-sheet, 12.6% β-turn and 27.8% random structure.

Chapter 3 describes extraction, purification and characterization of a novel trypsin inhibitor (JCTI) from *Jatropha curcas* seeds have been described. JCTI was purified to homogeneity using three chromatography steps: i) Cu^{2+} Streamline™; ii) DEAE-cellulose; iii) Trypsin-sepharose CL 4B. Purity of >99% was achieved with 70% yield and 13 fold purification. JCTI is found to be heterodimeric protein with a minimum molecular weight of 10500 Da as determined by HPLC and SDS-PAGE under non-reducing conditions. SDS-PAGE under reducing conditions showed two protein bands of 6000 Da and 4500 Da molecular weights. The N-terminal amino acid sequence of JCTI (both the peptides) showed similarity (29-36%) with Bowman-Birk trypsin inhibitors from *Zea maiz*, *Oliva sativa japonica* and *Oliva sativa indica*. The N-terminal sequence for the higher molecular weight peptide (6000 Da) was Gly-Pro-Pro-Glu-Ala-Ala-Leu-Glu-Thr-Glu-Asn-Gly-Ileu-Phe. The N-terminal sequence for the lower molecular weight peptide (4500 Da) was Val-Arg-Asp-Ileu-Ala-Lys-Lys-Glu-Ala-Ala-Arg-Arg-Asp-Asp. JCTI has a high affinity for trypsin with K_i of 6.5×10^{-9} M. JCTI does not inhibit α-chymotrypsin. JCTI is completely stable in the pH

range of 2-8 for 24 h at room temperature. JCTI has a remarkable thermal stability retaining 93% inhibitory activity at 70 °C for 30 min. The inhibitory activity measurements and CD studies at different temperatures showed that transition midpoint for JCTI lies close to 85 °C. CD analysis of JCTI revealed that it is an α, β protein rather than a predominantly β-sheet protein (26% α-helix, 15% β-sheet and 59% random structure).

Chapter 4 deals with purification of recombinant green fluorescent protein (r-GFP) overexpressed in *Escherichia coli* using immobilized metal ion chromatography (IMAC) in both packed bed as well as expanded bed. Performance of cross-linked Ni^{2+} alginate beads with that of commercial Ni^{2+} Streamline™ has been compared. Cross-linked alginate beads can constitute an attractive alternative matrix for expanded bed affinity chromatography since these beads are easy to make and very cheap as compared to Streamline™. The adsorption isotherms to both IMAC media followed the Langmuir model. The maximum binding capacity (q_{max}) of Ni^{2+} Streamline™ and Ni^{2+} cross-linked alginate for the GFP was 1,42,860 FU/ml and 18,000 FU/ml, respectively. The packed bed chromatography with Ni^{2+} Streamline™ gave 2.85 fold purity with 84% recovery, while Ni^{2+} alginate gave 3.1 fold purity with 88% recovery. The expanded bed chromatography using Ni^{2+} Streamline™ gave 2.7 fold purification with 89% of GFP recovery, while Ni^{2+} alginate gave 3.1 fold purification with 91% of GFP recovery. SDS-PAGE of purified GFP in both cases showed single band.

Chapter 5 describes purification of three isozymes of peroxidase from the roots of *Brassica rapa* (Indian variety). Furthermore, acidic isozyme (TP I, eluted from DEAE-cellulose) has been characterized in more detail. Three peroxidase isozymes have been purified to homogeneity using combination of ammonium sulphate

precipitation, Con A-sepharose 4B chromatography, DEAE-cellulose chromatography and CM-cellulose chromatography. Turnip peroxidase isozyme I (TPI) was purified to 28 fold purity with 53% yield using DEAE-cellulose. Turnip peroxidase isozyme II (TP II) and isozyme III (TP III) has been obtained from unbound and eluted fractions of CM-cellulose column, respectively. TP II constituted 19% while, TP III constituted 21% of total peroxidase activity. Molecular weight of TP I, TP II and TP III were found to be 45000 Da, 36000 Da and 34000 Da respectively. Most of the plant peroxidases are known to be monomeric enzymes with molecular weight in the range of 40000-50000 Da. The N-terminal amino acid sequence of TP I (Gln-Phe-Val-Ile-Pro-Thr-Tyr-Ala-Trp-Gln) showed similarity (40-60%) with that of *C. sativus, S. aureus, Cyanothecs* sp. PCC 8802, *L. xyli, T. vibrio and M. domestica*. Far-UV CD spectroscopy study revealed α-helix structure for TP I (44% α-helix, 16% β-sheet and 40% random structure). Melting temperature (T_m) calculated from the first-order derivatives of ellipticity-temperature plot was 50 °C for TP I. TP I showed highest specificity towards TMB (3,3`,5,5` tetramethylbenzidine) among the substrates studied. TP I has a pH optimum at pH 5.5 and temperature optimum of 25 °C. TP I was chemically modified with PMDA (pyromallitic dianhydride) with 0.75 degree of modification. PMDA modified TP I showed enhanced thermal stability (retains 80% activity at 60 °C) and pH stability (retains 80% activity at pH 7), enhanced *p*-chlorophenol removal efficiency and increased catalytic efficiency towards various substrates. PMDA modified TP I was examined for wastewater treatment using *p*-chlorophenol as a model system and compared with native TP I.

Chapter 6 describes purification of horseradish peroxidase (HRP) and laccase by using affinity precipitation with crude Concanavalin A. Addition of a simple crude extract of a lectin when added to a protein mixture would lead to formation of lectin-

glycoprotein precipitates. This simple economical and nonchromatographic approach can be useful in search for biomarkers in proteomic research. The concept was tested with commercial horseradish peroxidase (HRP) and laccase from modified *Aspergillus oryzae* (Denilite IIS) which are well known Concanavalin A (Con A) specific glycoproteins. Crude extract of jack beans containing Con A was mixed with HRP and laccase solutions. The contaminating non-glycoproteins remained in the supernatant. The precipitates of lectin-glycoprotein affinity complex thus obtained were redissolved in 1 M α-D methyl mannoside. The HRP and laccase was then separated from Con A using Sephacryl S-100 gel filtration chromatography. HRP could be purified to 9.3 fold purification with 93% yield and Con A could be purified to 7.3 fold purification with 72% yield. Laccase was purified to 10 fold purification with 80% yield and at the same time Con A was purified to 8 fold purification with 73% yield. SDS-PAGE analysis revealed single band of each purified glycoprotein and Con A.

Chapter 7 describes the preparation and characterization of biocatalyst preparation in which horseradish peroxidase was immobilized by bioaffinity layering. Immobilized preparation was then used for the treatment of phenolic wastewater using *p*-chlorophenol as a model system. For this purpose, lectin Concanavalin A was bound to Sephadex beads. The glycoenzyme peroxidase was layered upon this Con A layer. Subsequently, alternate layers of the enzyme and Con A were applied. The most efficient design consisted of three layers of Con A and peroxidase each. This immobilized enzyme preparation retained 80% of the activity of the free peroxidase used for immobilization. Kinetic parameters for the immobilized and free enzyme were determined. The V_{max}/K_m value of the enzyme significantly increased on immobilization from 100 to 160. PEG at the concentration of 0.1 mg/ml was found to

prevent enzyme inactivation by the products, although it increased the process time. Thus 60 U/ml of enzyme completely converted the *p*-chlorophenol (into products) in 4 min in the absence of PEG. On the other hand, only 0.05 U/ml of enzyme was required for this purpose in the presence of PEG but the process required 60 min. Peroxidase converts phenol molecules into free radicals. These free radicals then polymerize and get precipitated. As a further means of minimizing exposure of the enzyme to free radicals and enhancing the reusability, it was decided to remove the enzyme from reaction medium after 10 min. With this strategy, the bioaffinity layered peroxidase preparation could be reused five times without any loss of activity.

Chapter 8 describes preparation and characterization of multipurpose cross-linked enzyme aggregates (multipurpose-CLEAs) of two commercial preparations, i.e. Porcine pancreatic extract and Pectinex™ Ultra SP-L. Porcine pancreatic extract is known to be rich in lipase, α-amylase and phospholipase A_2 activities. Complete precipitation of all the three enzyme activities were achieved with Dimethoxyethane (DME). When precipitates were cross-linked with 20 mM glutaraldehyde, all the three enzymes retained 100% activity in resulting CLEAs. The lipase present in CLEAs showed greater thermal stability at 50 °C as compared to the free enzyme. For lipase and phospholipase A_2, V_{max}/K_m showed no significant change upon multipurpose-CLEAs formation but decreased significantly for α-amylase activity from 190 to 114 min^{-1}. The lipase and α-amylase activity in CLEAs were completely retained upto three cycles of reuse. The scanning electron microscopy (SEM) studies showed that morphology of CLEAs changed upon inactivation by reuses. Pectinex™ Ultra SP-L contains pectinase, xylanase and cellulase activities. Complete precipitation of the three enzyme activities was obtained with n-propanol. When resulting precipitates were subjected to cross-linking with 5 mM glutaraldehyde, the three activities initially

present (pectinase, xylanase and cellulose) were completely retained after cross-linking. The V_{max}/K_m values were increased from 11, 75 and 16 to 14, 80 and 19 for pectinase, xylanase and cellulase respectively. The thermal stability was studied at 50, 60 and 70 °C for pectinase xylanase and cellulase, respectively. Half-lives were improved from 17, 22 and 32 minutes to 180, 82 and 91 minutes for pectinase, xylanase and cellulase actvities, respectively in CLEAs. All the three enzymes in multipurpose-CLEAs could be reused three times without any loss of activity.

TABLE OF CONTENTS