BIOCATALYSTS: BIOSEPARATION STRATEGIES, SMART DESIGNS AND MEDIUM ENGINEERING

By
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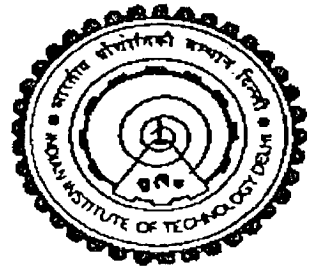
Submitted
in fulfillment of the requirement of the degree of Doctor of Philosophy
to the



INDIAN INSTITUTE OF TECHNOLOGY, DELHI
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TO
MY PARENTS

"Education is not the filling of a pail, but the lighting of a fire."
-William Butler Yeats

CERTIFICATE

This is to certify that the thesis entitled **BIOCATALYSTS: BIOSEPARATION STRATEGIES, SMART DESIGNS AND MEDIUM ENGINEERING** being submitted by **Ms. SHWETA SHARMA** 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. Sharma 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.

21st November, 2002

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Shweta

(SHWETA SHARMA)

Gratitude unlocks the fullness of life. It turns what we have into enough, and more. It turns denial into acceptance, chaos to order, confusion to clarity. It can turn a meal into a feast, a house into a home, a stranger into a friend. Gratitude makes sense of our past, brings peace for today, and creates a vision for tomorrow.

- *Melody Beattie*

ABSTRACT

Enzymes are biocatalysts with proven importance in both *in vivo* as well as *in vitro* situations. This thesis is concerned with three important aspects of these biocatalysts: bioseparation, designing reusable and stable bioderivatives and medium engineering.

The first introductory chapter reviews the literature on enzymes concerning the aspects relevant to the present thesis: various bioseparation strategies (viz., affinity precipitation, three-phase partitioning and expanded bed chromatography), bioconjugates with smart polymers, bioconversion in two phase systems and nonaqueous enzymology.

Chapter II deals with two techniques, viz. affinity precipitation and expanded bed chromatography which are used for the purification of enzymes/proteins. Alginate has been used as a macroaffinity ligand. This "stimuli sensitive" or "smart polymer" has been used for the purification of α-amylases, lipases and phospholipase D using the two above mentioned techniques.

Purification of α-amylases: α-Amylase from various sources was found to bind to alginate in free solution. The alginate-enzyme complex could be precipitated with Ca^{2+}. The enzyme activity could be recovered by dissolving the precipitate in 1M maltose and precipitating alginate alone by addition of Ca^{2+}. This led to

purification of wheat germ α-amylase by 68 fold with 74 % activity recovery. The molecular weight estimated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was 18 kDa. α-Amylase from whole wheat seed could also be purified 54 fold with 70 % activity recovery and gave the estimated molecular weight as 45 kDa. SDS-PAGE showed single band in both the cases.

Purification of Lipases: Lipases are being increasing employed for synthesis of drug intermediates and pharmaceutically important molecules as well as for the resolution of racemic mixtures for obtaining physiologically active enantiomers. Alginate was used as a macroaffinity ligand to purify lipases from *Chromobacterium*, porcine pancreas and wheat germ by employing the technique of affinity precipitation. The process gave adequate yields of 87, 75 and 62 % in the cases of *Chromobacterium*, porcine and wheat germ with 0.5 M NaCl, 1.0 M NaCl and 0.06 M $CaCl_2$ as the respective eluants. Alginate was also found to activate the enzymes, this effect was most dramatic (4 to 5 times) in the case of wheat germ enzyme. Addition of alginate enhanced the thermal stability of lipases. The half lives of lipases calculated with and without alginate were 160 and 45 min for *Chromobacterium* (at 55 °C), 61 and 27 min for porcine pancreas (at 45 °C) and 95 and 50 min for wheat germ lipase (at 60 °C) respectively.

Purification of phospholipase D: A simple titrimetric assay with soybean lecithin has been used for screening phospholipase D activity. The enzyme from peanut has been purified by binding to alginate. The purification consisted of co-

precipitation of enzyme with alginate upon addition of 0.06 M Ca^{2+}. The enzyme was eluted from the polymer using 0.2 M sodium chloride. The activity recovery was 55 % with 34 fold purification.

Another attractive option of expanded bed affinity chromatography was used for the purification of phospholipase D. Alginate was crosslinked with epichlorohydrin to obtain beads. The crosslinked alginate beads were used in a fluidized bed to purify phospholipase D from peanut. The binding followed Langmuir model. The maximum binding capacity (q_m) for the enzyme was around 416 U mL^{-1} and the dissociation constant (K_d) was 217 U mL^{-1}. The enzyme could be purified 317 fold directly from crude extract with 78 % activity recovery.

The somewhat underexploited technique of three-phase partitioning is dealt with in chapter III. TPP uses a combination of ammonium sulphate and *t*-butanol to precipitate proteins from crude aqueous extracts. The precipitated proteins form an interface between lower aqueous phase and upper organic solvent phase. The physicochemical basis of the method is poorly understood and hence it is difficult to design a TPP protocol in a predictable way. Purification of chicken intestine alkaline phosphatase and *Dacus carota* phospholipase D was explored by using TPP.

Alkaline phosphatase from chicken intestine was purified from the crude preparation by sequential use of three - phase partitioning and Phenyl Sepharose - 6B in the batch mode. The enzyme after TPP was still heterogeneous as indicated

by SDS-PAGE analysis. The homogenous preparation was obtained after using phenyl sepharose beads in the batch mode. The 100 % enzyme bound in 0.05 M Tris-HCl, pH 7.0 containing 1M ammonium sulphate. Elution was carried out with 50 % ethylene glycol in 0.05 M Tris-HCl buffer, pH 7.0. The fold purification of the finally purified enzyme was 81 and the activity recovery was 61 %. The sodium dodecyl sulphate - polyacrylamide gel electrophoresis analysis of this enzyme showed a single band and its molecular weight was found to be around 67 kDa.

Purification of phospholipase D from *Dacus carota* was also carried out by TPP. The single step of three phase partitioning led to 13 fold purification with an activity recovery of 72 %. SDS-PAGE analysis showed a single band with a minimum molecular weight corresponding to nearly 60 kDa. The purified enzyme had a pH optimum in the range of 6.0 - 6.5 and was unstable above 30 °C. Kinetic studies showed a K_m value of 9.5 mM and a V_{max} of 0.35 mL min^{-1}. The enzyme purified by three phase partitioning was found to resolve into two isoenzymes on a DEAE - cellulose column.

Chapter IV details the studies carried out using a smart derivative of α-chymotrypsin. α - Chymotrypsin was immobilized on Eudragit S-100 via covalent coupling with 94 % retention of proteolytic activity. The bioconjugate behaved as a smart biocatalyst and functioned as a pH dependent reversibly soluble - insoluble biocatalyst. Various spectroscopic techniques, viz. UV, fluorescence and CD were

used to probe structural changes in the enzyme upon immobilization. As per UV spectra, insignificant changes around 280 nm indicated absence of drastic changes in the microenvironments of tryptophan/tyrosine residues. However, drastic changes around 250 nm reflected that the enzyme had undergone some conformational changes upon conjugation.

The fluorescence data confirmed the results of UV absorbance that gross changes in the microenvironment of tryptophan and tyrosine have not occurred upon immobilization. On thermal denaturation (at 90 °C) of α-chymotrypsin, the λ_{max} shifted from 330 to 347 nm and intensity decreased by ten fold. Similarly, in the case of immobilized enzyme, λ_{max} shifted towards red by 17 nm (335 to 352 nm). However, the fluorescence intensity decreased only by 18 %. The latter perhaps reflects that the "residual structure" at 90 °C in the case of immobilized enzyme is greater. The CD spectra showed that some definite changes have occurred in the secondary structure upon immobilization. The small α-helical content was totally gone and there was decrease in β-sheet/β-turn structure. The randomness in the structure had increased by about 17 %.

The same conjugate of α-chymotrypsin and Eudragit S -100 was used for carrying out casein hydrolysis in the PEG/dextran two phase system. This biocatalyst along with the substrate casein was found to partition predominantly into the upper PEG phase. Under the optimized conditions, the product partitioned (84 %) into the lower dextran phase. Removal of dextran phase at an appropriate time interval and

replacing it with fresh dextran phase led to considerable enhancement of casein hydrolysis. The biocatalyst could be separated from the PEG phase by lowering the pH to 3.8 and again dissolved in PEG phase by increasing the pH to 7.6. Thus, this smart biocatalyst could be reused for casein hydrolysis in PEG-dextran two phase aqueous system. To reduce the process cost, casein hydrolysis was also carried out in another two phase system based on PEG/salt. Removal of the lower phase at an appropriate time interval (as in the case of PEG/dextran phase) and replacing it with fresh salt phase also led to considerable enhancement of casein hydrolysis.

Yet another underexploited immobilization technique is that of bioaffinity immobilization. Another hydrolase, tannase from *Aspergillus niger* van Teighem, was immobilized on Con A - sepharose and used for bioconversion of methyl gallate to gallic acid. Conjugate of tannase from *Aspergillus niger* van Teighem and Concanavalin A - sepharose was prepared via bioaffinity interaction. The immobilized enzyme showed pH optima similar to that of the free enzyme. K_m values for free and immobilized enzymes were 0.3 and 0.6 mM respectively. V_{max} changed from 0.013 to 0.02 μmoL min^{-1} upon immobilization. The immobilized preparation was quite stable to reuse, there was no loss of enzyme activity after three cycles and it retained 81 % activity even after sixth cycle. Ester hydrolysis using the immobilized enzyme led to 40 % conversion of methyl gallate to gallic acid as compared to 30 % obtained with the free enzyme.

Enzyme catalysis in anhydrous organic solvents has become an important approach in the area of applied biocatalysis. The enzymatic synthesis of an antioxidant, propyl gallate from tannic acid in non aqueous media is described in chapter V. Propyl gallate was produced by enzymatic transesterification of tannic acid with *n*-propanol using *Aspergillus niger* van Teighem tannase tuned at pH 6.0. Immobilization of enzymes on supports or carriers is desirable in anhydrous organic solvents in order to minimize mass transfer limitations or protect the enzyme molecule from inactivation by the organic solvent. So, the enzyme was immobilized on celite, which doubled the percent conversion of tannic acid to propyl gallate. The interaction of support with the water present is important in determining the availability of the water required by the biocatalyst to maintain its conformation, and hence the catalytic properties. The addition of optimum amount of water to the free and immobilized enzyme preparations led to a further increase in the yield of propyl gallate.

Effect of pH tuning on mushroom tyrosinase and duranta polyphenol oxidase was also studied in non aqueous media. The enzymes displayed 'pH memory' in acetonitrile. Addition of 1.0 – 1.5 % water to both tyrosinases led to optimum reaction rates.

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