SOME NOVEL APPROACHES IN BIOSEPARATION, BIOCATALYSIS AND PROTEIN REFOLDING STRATEGIES

By

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Submitted

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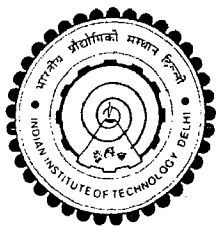

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"But I don't want to go among mad people," Alice remarked.
"Oh, you can't help that," said the Cat: "we are all mad here.
I'm mad. You're mad."
"How do you know I'm mad?" said Alice.
"You must be," said the Cat, "or you wouldn't have come here."

-Lewis Carroll
Alice's Adventure in Wonderland

To

Ma and Baba

CERTIFICATE

This is to certify that the thesis entitled **SOME NOVEL APPROACHES IN BIOSEPARATION, BIOCATALYSIS AND PROTEIN REFOLDING STRATEGIES** being submitted by **Ms Kalyani Mondal** 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 Mondal has worked under my supervision, and has fulfilled the requirements for the submission of the thesis which, to my 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.

March, 2006

Prof. M.N. Gupta
Department of Chemistry
IIT Delhi

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Kalyani Mondal
(Kalyani Mondal)

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. Downstream processing often is the more challenging aspect of protein production. While packed bed chromatography continues to be important in downstream processing, some nonchromatographic unit processes are becoming increasingly relevant. This is the first focus of this thesis.

Enhancing enzyme efficiency with microwave assistance, or novel biocatalyst designs is the second one. The experience of working with smart polymers in development of nonchromatographic bioseparation procedures led to the finding that such approaches infact can lead to not merely purification but simultaneous refolding of protein molecules if the latter is in the unfolded/ misfolded form. This outgrowth of separation work, development of refolding strategies is the third major component of the thesis.

Chapter 1 is an introductory chapter and gives a brief literature survey of the current status of the above mentioned three sub-areas of applied biocatalysis.

Three phase partitioning (TPP) is a nonchromatographic separation method which has been used in the past for purification of several proteins. It consists of addition of appropriate amounts of ammonium sulfate and *tert*-butanol to an aqueous solution of protein and incubationg it for some time. The proteins float as an

interfacial layer (between the aqueous phase and the upper *tert*-butanol phase) and is separated out.

Chapter 2 deals with TPP of water soluble polymers. TPP of starch (and its derivatives) and chitosan was carried out by adding *tert*-butanol to the aqueous solution of the polysaccharides in presence of ammonium sulfate. Starch and its modified forms could be recovered in more than 90 % yield in the form of an interfacial layer between aqueous and organic phases. Similarly, chitosan could be obtained as an interfacial precipitate with 88 % yield by subjecting 0.2 % (w v^{-1}) chitosan solution to TPP with 45 % (w v^{-1}) ammonium sulfate and an equal volume of *tert*-butanol at 40 °C. Quantitative FT-IR spectral analysis of TPP-treated potato starch revealed a clear change in the IR absorption band of the hydroxyl groups indicating a decrease in hydrogen bonding. TPP resulted in structural changes in chitosan which could be seen in its UV spectra, FTIR spectra and solubility characteristics. TPP-treated starch showed lower biodegradability with wheat germ α- amylase. TPP-treated chitosan also showed decreased susceptibility towards hydrolysis by chitinase.

In Chapter 3, TPP of alginates was used to purify α-amylases from porcine pancreas (animal), wheat germ (plant) and *Bacillus amyloliquefaciens* (bacterial source). The process is called 'macro-affinity ligand facilitated three phase partitioning' (MLFTPP). This technique was also used to purify other starch-degrading enzymes: glucoamylase (from *Aspergillus niger*) and pullulanase (from

Bacillus acidopullulyticus). In this process, the crude extract of the enzyme was mixed with alginate. On addition of equal volume of *tert*- butanol and appropriate amount of ammonium sulfate, alginate bound enzyme appeared as an interfacial precipitate between lower aqueous phase and upper *tert*-butanol phase. The precipitate could be dissolved in aqueous solution and α-amylase activity recovered with 1 M maltose. The process did not require any preclarification of the crude extract. The recovery of α-amylases were 74, 77 and 92 % in the case of *B. amyloliquefaciens*, wheat germ and porcine pancreas respectively. Glucoamylase and pullulanase were purified 20- and 38-fold with 83 and 89 % activity recovery, respectively. All purified preparations showed a single band on SDS-PAGE.

In Chapter 4, a two step sequence of MLFTPP is described to recover an enzyme in each step. The order in which this sequence is used is important since the first smart polymer should not bind the second target enzyme in a non specific fashion. Sequential MLFTPP was used to purify pectinase and cellulase from a commercial enzyme preparation called Pectinex™ Ultra SP-L, and wheat germ agglutin (WGA) and lipase from a crude wheat germ extract. In the first step, pectinase was purified (with alginate as the polymer) 13 fold with 96 % activity recovery. In the second MLFTPP step, using chitosan, cellulase was purified 16 fold with 92 % activity recovery. Both preparations showed a single band on SDS-PAGE. It was found that WGA does not bind to alginate. Hence in the case of this system

(crude wheat germ extract), MLFTPP was first carried out with alginate to affinity capture lipase. The dissociation and recovery of lipase from the complex was carried out by using 60 mM $CaCl_2$. The aqueous phase could in turn be subjected to MLFTPP using chitosan as the affinity macroligand to affinity capture WGA. The complex of chitosan with WGA was obtained as an interfacial precipitate and WGA was recovered from it using saturated $MgCl_2$ solution.

Wheat germ lipase was recovered in 94 % yield and 27 fold purification The purified preparation was found to have a single band on SDS-PAGE. Recovery of WGA activity was 99 % and 40 fold purification. This purified preparation also gave a single band on SDS-PAGE.

In Chapter 5, κ-carrageenan has been evaluated as a general purpose carrier of affinity ligands for use in affinity precipitation of proteins. Affinity precipitation is a simple, single plate separation process in which the complex of a smart affinity macroligand with the target protein (from a crude broth) can be selectively precipitated by application of a suitable stimulus.

The conjugate of κ-carrageenan with the dye Cibacron Blue was used for purification of yeast alcohol dehydrogenase (ADH) using the known affinity of the dye for the enzyme. κ-Carrageenan is a non-toxic polymer from seaweeds and consists of sulfated or non-sulfated galactose and 3,6-anhydro galactose units. A useful feature of κ-carrageenan as a smart polymer is that it becomes insoluble merely upon addition of K^+ . Response surface methodology was used to optimize

conditions for affinity precipitation of the enzyme with the polymer-dye conjugate. Recovery of 58 % of the enzyme activity with 13.6-fold purification was obtained.

The purification of pectinase by affinity precipitation was also tried using microwave-treated alginate as the affinity macroligand. It was found that the performance of alginate (in the purification of pectinase by affinity precipitation) as a result of its microwave pretreatment at a constant temperature changed. Microwave treatment of alginate at 75 °C for 30 min resulted in reduction in availability of guluronic acid residues for interaction with Ca^{2+} ions. Thus, whereas alginate required only 20 mM $CaCl_2$, microwave-treated alginate required 0.1 M $CaCl_2$ for complete precipitation. Microwave pretreatment of alginate at 75 °C was found to enhance the selectivity of the affinity precipitation of pectinase from a commercial preparation of *Aspergillus niger*. Using microwave-treated alginate, 83 % of the enzyme activity with 20-fold purification could be recovered. In comparison, affinity precipitation with untreated alginate led to 10-fold purification with 81 % recovery of enzyme activity. SDS-PAGE upon silver staining confirmed the enhanced selectivity of affinity precipitation when microwave-treated alginate was used.

Chapter 6 describes a pre-treatment process for chitin by microwave irradiation which resulted in increase of its enzymatic hydrolysis by cabbage chitinase. The microwave irradiation was carried out using a microwave equipped with non-contact infrared feedback controller which allowed fixing up a constant

temperature during microwave irradiation. Response surface analysis was used to determine optimum conditions [2 % (w v^{-1}) chitin, 57.5 °C, 38 min] for microwave-irradiation of chitin so as to improve its enzymatic hydrolysis. V_{max}/K_m of cabbage chitinase towards untreated and microwave-irradiated chitin was found to be 21.1 nmol h^{-1} mg^{-2} ml and 31.7 nmol h^{-1} mg^{-2} ml, respectively. Similar improvement was observed in the case of pectinase in its unusual catalytic activity of chitin degradation. It was found that greater extent of chitin hydrolysis by chitinase was possible after the substrate chitin was irradiated with microwaves. The effect of microwave pretreatment on chitin was seen by scanning electron microscopy (SEM).

Microwave pre-treatment of polygalactouronic acid, xylan and carboxymethyl cellulose was also found to improve the catalytic efficiencies of pectinase, xylanase and cellulase by 1.5, 2.3 and 1.6 times, respectively. The temperature and time of pre-treatment and substrate concentration during the pre-treatment were optimized by response surface methodology. Scanning electron microscopy revealed significant morphological changes in the substrate as a result of microwave pre-treatment. Time course of enzymatic hydrolysis in each case showed that the use of microwave pre-treated substrates gave higher catalytic rates. Also, higher extent of bioconversion was observed in the case of polygalactouronic acid when microwave pre-treated substrate was used.

Apart from pretreatment, it was found that microwave can enhance enzyme performance in aqueous media as well.

Subtlisin Carlsberg and Protizyme™ (a commercial preparation of *Aspergillus flavus*) were used for removal of blood stains from cloth under microwave irradiation at 37- 40 °C. The stains were completely removed within 2 minutes when 0.6 U of proteolytic activity was used along with 0.1 % (w v^{-1}) sodium dodecyl sulfate or 0.1 % (v v^{-1}) Triton X-100. A control in which conventional heating was used for maintaining 37-40 °C temperature did not remove the stains under similar conditions.

Chapter 7 evaluates three novel biocatalyst designs. The first is the immobilization of an enzyme on a reversibly soluble-insoluble polymer. Alginate was used to prepare a bioconjugate of *Pseudomonas cepacia* lipase by simple adsorption. The immobilized enzyme preparation exhibited increased esterase activity in terms of high effectiveness factor (effectiveness factor was 3 for the immobilized preparation) and greater V_{max}/ K_m value (V_{max}/ K_m increased 25 times upon immobilization). The bioconjugate was also more stable at 55 °C as compared to the free enzyme and could be reused for olive oil hydrolysis upto 4 cycles without any loss in activity. Fluorescence emission spectroscopy indicated that enzyme as a part of the bioconjugate had undergone definite conformational changes. Atomic force microscope (AFM) images provide visual information about the way enzyme molecules are interacting with the matrix.

Three phase partitioning was found to improve the catalytic efficiency of the commercially available aldolase antibody 38C2 for aldol condensation between

p-nitrobenzaldehyde and acetone to give 4-(4'-Nitrophenyl)-4-hydroxy-2-butanone. This is the second biocatalyst design tried. While untreated-38C2 gave 24 % conversion, TPP-treated 38C2 gave 65 % conversion during the same period of time. TPP-treated antibody also gave an additional product. The latter resulted in overall lower product formation for the desired product. Hence, an alternative biocatalyst design called crosslinked aggregates (CLEA) (the third design) was attempted to enhance the utility of 38C2. CLEA of 38C2 were prepared using bovine serum albumin as the protic feeder. The biological activity of CLEA as compared to equivalent amount of free 38C2 was 120 %. CLEA of 38C2 were found to be more stable at 50 and 60 °C as compared to the free aldolase antibody and could be reused upto 10 cycles with a residual activity of 80 % activity.

In Chapter 8, affinity precipitation was evaluated as a strategy for protein refolding. Urea denatured lipase from *Chromobacterium viscosum* lipase could be refolded by addition of alginate with high guluronic acid content. The refolded molecule could be recovered by affinity precipitation. Dynamic light scattering showed that binding required about 45 minutes and activity data obtained from affinity precipitation experiments indicated that refolding was almost instantaneous after binding. Circular Dichroism (CD) and fluorescence data showed that refolded molecule was identical to the native molecule. It also showed that refolding takes place at the binding stage and not at the precipitation stage.

In similar experiments, affinity precipitation with another smart polymer, Eudragit™ S-100 was exploited for simultaneous refolding and purification of

xylanase. Affinity precipitation consisted of this reversibly soluble-insoluble polymer binding xylanase selectively. The complex was precipitated by lowering the pH and xylanase was eluted off the polymer using 1 M NaCl. For refolding experiments, the commercial preparation of *Aspergillus niger* xylanase was denatured with 8 M urea. Addition of microwave-treated Eudragit™ S- 100 and affinity precipitation led to recovery of 96 % enzyme activity by refolding. Simultaneously, the enzyme was purified 45 times. Thermally inactivated preparation, when subjected to similar steps, led to 95 % recovery of enzyme activity with 42- fold purification. This strategy has the potential for recovering pure proteins in active forms from overexpressed proteins which generally form inclusion bodies in *E. coli* due to incorrect folding.

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