SOME APPROACHES TO ENHANCE CATALYTIC PERFORMANCE OF ENZYMES IN NEARLY ANHYDROUS ORGANIC SOLVENTS

BY

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Dedicated to my family

CERTIFICATE

This is to certify that the thesis entitled "**Some approaches to enhance catalytic performance of enzymes in nearly anhydrous organic solvents**" being submitted by **Ms. Kusum Solanki** 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. Solanki 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.

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Abstract

The application of hydrolases (mainly lipases and proteases) in organic solvents for synthetic purpose has been attracting attention for the last two decades. The simplest way of using enzymes in non-aqueous media is to use them as dry lyophilized powders. But the activity of enzymes in organic media is inherently slow as enzymes by nature are made to work in aqueous media only. Biocatalyst development is one of the key towards the development of new synthetic transformations in industrial biotechnology. The present thesis, describes the optimization of different approaches/protocols used to design the biocatalyst in order to enhance their stability and activity in organic media. Broadly, the thesis aims at improving catalytic rates of enzymes in nearly anhydrous organic solvents.

The thesis is divided into six chapters.

Chapter 1 is the introductory chapter which focuses on the literature on improving performance of enzymes in non-aqueous media. The chapter essentially provides the background for the current work.

Chapter 2 deals with the study of effect of trehalose on alpha-chymotrypsin precipitation. Two enzyme preparations, EPRP (enzyme precipitated and rinsed with n-propanol) and PCMC (protein coated microcrystals) were prepared by precipitating alpha-chymotrypsin with *n*-propanol in the presence of trehalose. It was found that EPRPs of α-chymotrypsin prepared by precipitation with *n*-propanol in the presence of trehalose contained substantial amount of trehalose (even though trehalose alone at these lower concentrations was not precipitated by *n*-

propanol). The presence of trehalose in these EPRPs resulted in much higher transesterification rates (45 nmoles mg-1min-1) as compared with EPRPs prepared in the absence of trehalose (17 nmoles mg-1min-1) in *n*-octane. Both kinds of EPRPs gave similar initial transesterification rates in acetonitrile. Use of higher concentrations of trehalose (when trehalose alone also precipitates out), resulted in the formation of PCMCs, which showed higher transesterification rates in both octane and acetonitrile. SEM analysis showed the relative sizes of various preparations. Presence of trehalose resulted in EPRPs of smaller sizes. The two different forms of enzymes (EPRP and PCMC) known to show higher activity in organic solvents were found to be different only in the concentration of the low molecular weight additive which was present along with the protein during precipitation. Therefore, the enhancement in the transesterification activity in EPRPs prepared in the presence of trehalose was due to: (a) better retention of essential water layer for catalysis due to the presence of the sugar. This effect disappeared where the reaction media was polar as the polar solvent (acetonitrile) is more effective in stripping off the water from the enzyme; (b) reduction in particle size as revealed by SEM. In the case of PCMC, the enhancement in the initial rates presumably was due to an increase in the surface area of the biocatalyst since protein is coated over the core material (trehalose or salt).

Chapter 3 describes chemical modification of proteases (alpha-chymotrypsin and subtilisin Carlsberg) and *Pseudomonas cepacia* lipase with Pyromellitic dianhydride (PMDA) to obtain high active biocatalysts for use in non-aqueous media. Alpha-chymotrypsin was made more hydrophilic by modifying 10 (out of 16) o-amino groups with pyromellitic dianhydride. The modified preparation was precipitated with *n*-propanol to form EPRP (enzyme precipitated and rinsed with *n*-propanol). This preparation gave significantly higher initial rates at the optimum a_w

(127 nmol mg^{-1} min^{-1} in n-octane and 21 nmol mg^{-1} min^{-1} in acetonitrile at a_w 0.33) compared with the lyophilized preparation (53 nmol mg^{-1} min^{-1} in n-octane and 0.26 nmol mg^{-1} min^{-1} in acetonitrile at a_w 0.97). FT-IR showed that the precipitate of modified alpha-chymotrypsin has a higher content of alpha-helices and beta sheets compared to the lyophilized powder. PCMC (protein coated microcrystal) of PMDA modified alpha-chymotrypsin with potassium sulphate as core material was prepared. This preparation also gave significantly higher initial rates at the optimum a_w (960 nmol mg^{-1} min^{-1} in n-octane and 172 nmol mg^{-1} min^{-1} in acetonitrile at a_w 0.33) compared with the lyophilized preparation at its optimum a_w (53 nmol mg^{-1} min^{-1} in n-octane and 0.26 nmol mg^{-1} min^{-1} in acetonitrile at a_w 0.97). When solid state buffers were used as core material instead of potassium sulphate for the preparation of PCMC of PMDA modified alpha-chymotrypsin, the initial rate of 7420 nmol $min^{-1}mg^{-1}$ was obtained at a_w 0.33 with *n*-octane as a reaction medium.

Subtilisin Carlsberg was also modified with PMDA and TNBS analysis showed that 5 out of total 9 free lysine amino acids were modified by reaction with PMDA. PCMC of modified subtilisin was then evaluated for transesterification activity with polar solvents like DMF and DMSO as a reaction medium. In anhydrous DMF, pH tuned subtilsin (both modified and unmodified) gave no product. PCMC of modified and unmodified subtilisin gave 0.7 nmoles min^{-1} mg^{-1} and 0.8 nmoles min^{-1} mg^{-1} respectively. This showed that in anhydrous DMF, PCMC worked better than lyophilized powders but modification of subtilsin did not help in increasing its activity. With 1 % (v/v) water content in DMF, the PCMC of modified subtilsin was found to be 110 and 4 fold more efficient than lyophilized enzyme and PCMC of unmodified subtilisin respectively. Increasing the water content to 5 % (v/v) from 1 % (v/v) had no effect on transesterification activity of all the enzyme preparations. In DMSO (containing 1 % (v/v) water),

PCMC of modified subtilisin was found to be 2.3 fold more active in catalyzing transesterification reaction than unmodified pH tuned enzyme.

Modification of *Pseudomonas cepacia* lipase with PMDA resulted in acylation of 5 out of 7 free lysine amino acids. Different high performance biocatalyst (EPRD, CLEA, PCMC and CLPCMC) of modified lipase were prepared and its transesterication activity (1-hexanol with tributyrin) was studied in *n*-octane and DMF. The initial rates obtained during the assay showed that CLPCMC was the best catalyst followed by PCMC>EPRD>pH tuned in *n*-octane. For all the preparations, biocatalyst formulation of modified lipase were better than corresponding formulation of unmodified lipase. These preparations were also studied for transesterification reaction in DMF with two different water contents (1 % (v/v) and 5 % (v/v)). Again, the performance of all the four preparations (EPRD, CLEA, PCMC and CLPCMC) exceeded well beyond the pH tuned lyophilized powders of lipase (which showed no activity at all).

In **Chapter 4** the effect of drying conditions on the catalytic performance of enzymes in organic transformations in low water media is investigated. These two proteases (alpha-chymotrypsin and subtilisin carlsberg) were used in the present work to gain further understanding in the context of best way of using the enzymes in low water media for organic synthesis. In addition, FT-IR was again employed, CD spectroscopy was also simultaneously used to probe secondary and tertiary structures of the two enzymes after different pretreatment (way of drying). The latter was possible by use of an innovative accessory (and corresponding theoretical framework) which spins the CD cuvette to allow CD measurements of suspensions of small particles. An agreement between FT-IR and CD data was observed. So, in a way, these two independent tools validated each other. The FT-IR spectrum of alpha-chymotrypsin in aqueous solution showed a maximum

at 1637 cm^{-1} (which corresponds to high content of anti parallel beta sheets). FT-IR spectra of preparations suspended in propanol showed that the band at 1637 cm^{-1} has decreased and shifted to lower frequencies (perhaps due to formation of beta sheet aggregates). A new peak also appeared at 1657-1659 cm^{-1} which corresponds to alpha-helices. With subtilisin also the spectra of the more active preparations resembled aqueous solution to a larger extent, particularly the intensity around 1650 cm^{-1}.

The CD spectrum of alpha-chymotrysin consists of a negative band at 230 nm which is reported to be closely associated with catalytically active conformation of alpha-chymotrypsin. There was a clear correlation between larger shifts in this wavelength minimum and lower catalytic activity of different chymotrypsin formulations. The far UV CD spectra of subtilisin in solution shows negative bands at 222 nm and 208 nm and a positive band at 190 nm. CD spectra of all the preparations shows decrease in molar ellipticity in the following order – Solution>PCMC>EPRP>RPCMC>CLEA. In case of REPRP and RCLEA the shape of far UV CD is also changed which indicates a major change in secondary structure. This order correlates with order of catalytic activity of these formulations in n-octane i.e. PCMC>EPRP>RPCMC>CLEA>RCLEA>REPRP.

There was a good correlation between the transesterification activities observed and the closeness to the known native structure of the enzyme molecule.

Chapters 5 deals with enhancement of activity and stability of enzymes by entrapment inside dextran beads. As mentioned earlier that drying by lyophilization affects the structure of the enzyme. This insight has led to removal of water by precipitation instead and some high activity preparations like Crosslinked enzyme crystals (CLEC), Propanol rinsed enzyme preparation

(PREP), Enzyme precipitated and rinsed with propanol (EPRP), Cross linked enzyme precipitate (CLEA), Protein coated micro crystals (PCMC) and cross linked protein microcrystals (CLPCMC) have been described. In most of these cases, morphology of the biocatalyst design does not lend itself to their use in packed bed and fluidized bed and some studies cast doubts over their operational stability. The CLEA of alpha-chymotrypsin was formed inside sephadex G 100 beads with retention of complete activity as measured by established assays in aqueous buffers. These preparations showed high activity in organic solvent (CLEA) and high thermal stability in aqueous media. The half life of entrapped CLEA was found to be 100 min at 50 °C (in 50 mM phosphate buffer, pH 7.8), far better than 25 min and 41 min for free alpha-chymotrypsin and CLEA. Specific activity (as determined by esterase assay) of CLEA entrapped inside beads is 62 U/mg of enzyme as compared to 63 U/mg given by 'straight out of bottle' enzyme i.e. on encapsulation of alpha-chymotrysin CLEA inside beads the specific activity does not change. Specific activity of free CLEA was 55 U/mg. The initial rates of transesterification of *N*-acetyl-L-phenylalanine ethyl ester with n-propanol was found to be 28 $nmolesmin^{-1}mg^{-1}$ for CLEA entrapped inside beads while it is 26 $nmolesmin^{-1}mg^{-1}$ for free CLEA with anhydrous octane as a reaction medium. Leaching of CLEA from beads was also studied using Green Fluorescent protein (GFP) as a model protein. It was found that GFP CLEA did not show any leaching even upto 20 cycles (1 cycle – 1h incubation in an aqueous buffer, centrifuged and resuspended). Use of CD (with a special accessory which allows us to work with suspensions) has enabled us to look at structural aspects of the enzyme precipitates/aggregates inside the beads. Far UV CD spectrum of alpha-chymotrypsin consists of two minima-one at 230 nm (due to antiparallel beta sheets) and another at 202 no parallel beta sheets). The spectrum of free CLEA shows that the characteristic negative peak at 230 nm is there but minima at 202 nm is

completely disappeared (could be due to absorption flattening). Far UV spectra of CLEA entrapped in sephadex beads consists of negative peaks at 230 nm and 205 nm. Though the intensity of these peaks is less than that of alpha- chymotrypsin solution but the appearance of negative peak at 205 nm shows that there is less absorption flattening in the case of CLEA entrapped in the beads as compared to free CLEA (not entrapped in the beads). This showed that there was less clumping in case of CLEA entrapped in beads than free CLEA.

Chapter 6 involves simultaneous purification and immobilization of *Candida rugosa* lipase on superparamagnetic nanoparticles for catalyzing transesterification reactions. In the present work, immobilization of *Candida rugosa* lipase on Fe_3O_4 superparamagnetic nanoparticles has been tried. It was found that these immobilized preparations show unusually high transesterification activity (11 nmoles min^{-1} mg^{-1} for lipase immobilized on PEI coated Fe_3O_4 nanoparticles as compared to 0.12 nmoles min^{-1} mg^{-1} for 'straight out of bottle' lipase). The racemic 1-phenylethanol, underwent a more efficient enantioselective transacetylation with this formulation than the 'straight out of bottle' enzyme. Thus, with the 'straight out of bottle' enzyme, after 24 h only 2 % conversion could be obtained while with the above mentioned formulation, 41 % conversion was obtained with a marginal loss of 1 % in ee value. Far-UV CD spectra of lipase shows that there is no change in secondary structure of lipase after immobilization on PEI coated iron oxide nanoparticles.

Simultaneous purification of lipase was also observed as specific activity of lipase immobilized on PEI coated iron oxide nanoparticles was 663 U/mg enzyme as compared to 233 U/mg enzyme for 'straight out of bottle' lipase. This could be due to selective adsorption of lipase to PEI coated nanoparticles.

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