ENZYME FUNCTION AND STABILIZATION IN AQUEOUS AND NON-AQUEOUS MEDIA

by

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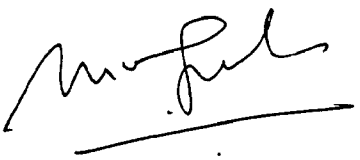
CERTIFICATE

This is to certify that the thesis entitled "**Enzyme Function and Stabilization in Aqueous and Non-Aqueous Media**" being submitted by **Ms. Renu Batra** 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. Renu Batra has worked under my guidance and supervision and has fulfilled the requirements for the submission of this 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.



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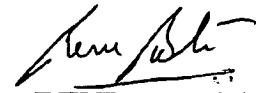

RENU BATRA

ABSTRACT

The work described in the present thesis is an attempt to look at the enzyme function in aqueous-organic cosolvents and in some cases compare their behaviour in purely aqueous and nearly anhydrous media. As most of the work with organic cosolvents in the literature has been restricted to a single enzyme chymotrypsin, in this work a variety of enzymes have been chosen to get the general picture. Mostly, the focus has been on enzyme stability using enzyme activity measurement as the monitoring parameter.

The first chapter is an introductory chapter and briefly surveys the literature on enzyme function and its stability in aqueous, aqueous-organic cosolvent mixtures and nearly anhydrous media. As considerable portion of this thesis also deals with the effect of adsorption of enzymes on polymeric surfaces and the effect of aggregation by chemical crosslinking on enzyme activity and stability, these two approaches of immobilization have also been reviewed.

For obtaining a general picture of effect of water-miscible organic solvents on enzyme activity, four different enzymes viz. polyphenol oxidase, trypsin, acid phosphatase and peroxidase were assayed in the presence of aqueous-organic cosolvent mixture (acetonitrile/tetrahydrofuran/N,N dimethyl formamide/dioxane) where the % of organic cosolvent varied from 0-60% (v/v). In all the cases, stimulation of the enzyme activity was observed at a specific concentration range (10-20%, v/v) of the cosolvent present in the reaction media. The extent of increase in activity varied with the solvent and the enzyme. Beyond this concentration, the activity drops rapidly with all the enzymes and solvents except peroxidase, which shows 90% activity retention even in the presence of 60% (v/v) acetonitrile. No significant structural changes were detected by fluorescence spectroscopy at the cosolvent concentrations where activity increases were significant. Properties such as K_m, V_{max} and catalytic efficiency at cosolvent concentrations (showing maximum enzyme activity) were also examined.

In order to compare the enzyme activity in different milieu (viz. aqueous, aqueous-organic cosolvent mixtures and anhydrous organic solvents), polyphenol oxidase was selected as this enzyme could be assayed with an identical protocol in all media. *Solanum tuberosum* (potato tubers) was chosen as the readily available source for this enzyme. The enzyme was isolated, purified and characterized for its function in aqueous and non-aqueous media. The partially purified (seven fold) enzyme preparation obtained after ammonium sulphate precipitation was immobilized on chitin by simple adsorption at pH 6.8. As it was observed that the enzyme was bound to chitin with an adequate strength, (as no leaching was observed with 1 M NaCl and pH 5.0 buffer), effect of water-miscible organic solvent viz. acetonitrile as a cosolvent was examined and compared with that of the free enzyme. The enhancement was found to be much more in the case of immobilized enzyme, as high as 200% whereas the maximum activity observed with the free enzyme was only 145% (where 100% is taken as the enzyme activity in aqueous buffer).

The work described in chapter III examines the effect of simple adsorption of enzymes to the polymeric surfaces upon their activity and stability in organic cosolvents. Lately, multipoint attachment to polymeric matrices have been shown to protect the enzyme from denaturation in organic cosolvents but simple adsorption has not yet been tried. The data with PPO / chitin in acetonitrile (as a cosolvent) indicated that similar systems may be worth investigating.

Four enzymes viz. polyphenol oxidase, trypsin, acid phosphatase and peroxidase were adsorbed on eudragit S-100, chitin and chitosan. The enzyme activities of immobilized enzymes were assayed in the presence of varying concentrations of some organic cosolvents viz. acetonitrile, THF, DMF and dioxane. Again, the enhancement in activity was observed in the presence of low concentrations (upto 20%) of organic cosolvents. It was observed that the increases were greater in the case of immobilized enzymes as compared to the free enzymes.

Another feature observed with immobilized enzyme systems was the greater stabilization towards the presence of higher concentration (50%) of an organic cosolvent. In the case of trypsin immobilized on eudragit it retained 100% of its original activity in the presence of 50% acetonitrile (v/v) and 75% of its activity in 50% DMF (v/v) whereas complete deactivation of the free enzyme occured under same conditions. Similar results were obtained with other enzymes as well. From these observations it was also concluded that influence of matrix upon enzyme activity varies with the solvent (medium effect) and did not correlate well with a solvent parameter such as log P.

This work also examines the phenomenon of enzyme inactivation and its reversibility in the same four different water miscible solvents. Reversibility of enzyme activity was found to be clearly dependent upon the choice of the organic cosolvents e.g. acetonitrile and DMF proved to be better cosolvents as compared to THF and dioxane. In case of all the four enzymes, the actvity retained (measured by standard assay in aqueous system) was above 90% after 30 minutes incubation in 50% (v/v) acetonitrile and DMF whereas the loss in activity was quite rapid when the enzymes were exposed to 50% (v/v) THF and dioxane. A good correlation of the phenomenon of irreversibile enzyme inactivation and a simple solvent parameter - Polarity Index has been proposed. The results with all the above four enzyme systems show that the solvents with polarity index of 5.8 and above are 'good' solvents as they donot denature the enzymes irreversibly. Enzyme(s) exposed to high concentrations (50%,v/v) of these solvents reatin most of its activity even after 48 hr exposure whereas that in the case of solvents with a polarity index of < 5.1 denature the enzyme completely within 0-4 hr in most of the cases studied. This correlation was found valid for free as well as the immobilized (by adsorption) enzymes.

The extensive chemical crosslinking of enzymes to produce insoluble enzyme aggregates is known to lead to enzyme stabilization. Chapter IV deals with preparation and characterization of some enzyme aggregates in water and the

evaluation of their performance in the aqueous-organic cosolvent mixtures. Conditions for the preparation of aggregates of polyphenol oxidase, acid phosphatase, β-glucosidase and trypsin by crosslinking with glutaraldehyde were optimized. Except in the last case, BSA (bovine serum albumin) was used as a proteic feeder. The aggregates of polyphenol oxidase, acid phosphatase, β-glucosidase and trypsin showed the activity retention of 86%, 88%, 86%, and 30% respectively. The enzyme aggregates were examined for the properties such as pH optimum and thermal stability. All the enzyme aggregates showed considerable improvement in thermal stability. While the complete denaturation of free enzyme occurs after 3 hrs of incubation at 60°C, the aggregates retained more than 50% of the original activity in the cases of polyphenol oxidase and β-glucosidase. The acid phosphatase aggregate was found to retain 100% of the original activity whereas that of free enzyme decreases to 25% of the original activity when exposed to 50°C for 3 hrs. The effect of four different organic cosolvents (viz. acetonitrile, tetrahydrofuran, dimethyl formamide and dioxane) on the activity of the enzyme aggregates was investigated. With all the enzyme aggregates except acid phosphatase (no change in activity observed), the enzyme activity in the presence of organic cosolvents in the reaction media substantially improved as compared to the free enzyme. This work also investigates the inactivation of the enzymes after exposure to the organic cosolvents. In the case of acid phosphatase and trypsin, chemical aggregates retained better activity (as compared to the free enzyme) upon exposure to organic cosolvents such as acetonitrile, tetrahydrofuran, dimethyl formamide and dioxane (in the concentration range of 30-50%, v/v). On the other hand, aggregates of polyphenol oxidase and β-glucosidase showed no improved activity retention upon the organic cosolvent exposure. Finally, the effect of ultrasonication on the activity of aggregates was investigated. A four fold increase was observed in the case of polyphenol oxidase aggregates in buffer when sonicated for 90 seconds. The performance of sonicated polyphenol oxidase aggregates in aqueous-organic cosolvent mixtures was also

compared with the non-sonicated aggregates.

Its a well known fact that the enzymes/proteins show enhanced stability in anhydrous organic solvents. Chapter V describes the consequences of thermal exposure in anhydrous organic media and in the presence of other additives on the enzyme activity in water. Various enzymes viz. peroxidase, chymotrypsin, acid phosphatase, β-glucosidase, trypsin and α-amylase were exposed to 70°C in anhydrous acetonitrile for 0-7 hr. The same investigation was also carried out with an apolar organic solvent-toluene with the first three enzymes mentioned above. All the enzymes (except α-amylase) showed enhanced enzyme activity, when assayed in aqueous media with or without the removal of organic solvent. The enhanced activity was found to be as high as 200% with peroxidase, 150% with chymotrypsin and 130% with acid phosphatase when toluene was used as the solvent. In acetonitrile, it was 130% with peroxidase, 160% with β-glucosidase, 130% with acid phosphatase, 130% with chymotrypsin and 110% with trypsin. No inactivation was observed with any of the enzymes examined even after 7 hrs incubation in anhydrous organic media at 70°C, where complete denaturation of the enzymes occurs in water under similar conditions.

Also, it was observed that the preincubated enzyme - pectinase, at 50°C in glycerol (40%,v/v) showed about two fold increase in enzyme activity when assayed in aqueous medium. Also, marginal increase in enzyme activity was observed in the presence of trehalose (50 mM) and sucrose (50 mM). No conformational changes, however, could be detected by fluorescence spectroscopy.

Chapter VI, the last chapter of this thesis describes some exploratory work which seeks to exploit some unique possibilities which arise in aqueous-organic cosolvents/nearly anhydrous organic solvents. The first application, in the area of bioseparation, consisted of developing an alternative mode of precipitation of eudragit S-100 with a combination of Ca^{++} and water miscible organic solvents. The best conditions for this purpose turned out to be 0.2 M Ca^{++} with 20% acetonitrile/iso-propanol at 25°C and pH 7.0. The compact precipitate so obtained

could be redissolved easily and within 30 min by using 1 M NaCl solution. It was found that in the case of several enzymes viz. trypsin, polyphenol oxidase, xylanase, acid phosphatase, alkaline phosphatase and α-amylase, the extent of nonspecific binding to the polymer decreased considerably when precipitation using organic cosolvents was carried out instead of usual well established pH dependent precipitation. For example, only 35% of the trypsin acitvity bound to eudragit (when coprecipitated using 0.2 M Ca^{++}/20% acetonitrile) as compared to the 98% binding in case of pH dependent precipitation. This alternative mode of precipitation may be especially valuable for purification of acid labile enzymes/proteins.

In a second application, it was found that activity staining of polyphenol oxidase in organic cosolvents on polyacrylamide gels led to sharper activity bands as compared to the ones obtained by standard protocols. This chapter also takes a close look at the phenomenon of pH memory exhibited by enzymes in non-aqueous solvents.

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