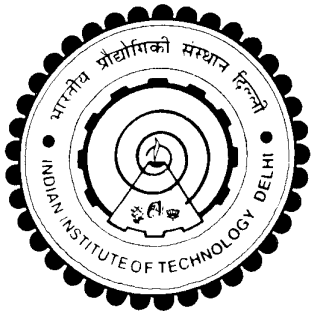
# STUDIES ON THREE PHASE PARTITIONING OF PROTEINS

**GULAM MOHMAD RATHER**




**DEPARTMENT OF CHEMISTRY**

**INDIAN INSTITUTE OF TECHNOLOGY DELHI**

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# STUDIES ON THREE PHASE PARTITIONING OF PROTEINS

**by**

**GULAM MOHMAD RATHER**

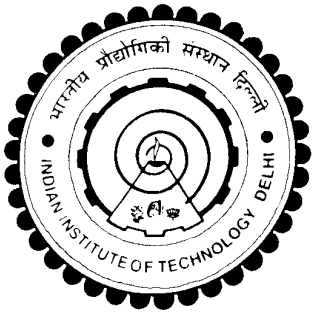
**Department of Chemistry**

**Submitted**

**In fulfillment of the requirement of the degree of**

**DOCTOR OF PHILOSOPHY**

**to the**




**INDIAN INSTITUTE OF TECHNOLOGY DELHI**

**AUGUST 2012**

*Dedicated*

*To*

*My Dear Family*

# CERTIFICATE

This is to certify that the thesis entitled "**Studies on Three Phase Partitioning of Proteins"** being submitted by **Mr. Gulam Mohmad Rather** 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. Rather 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 thesis 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

Emeritus Professor

Department of Biochemical Engineering and Biotechnology

Indian Institute of Technology Delhi.

(Supervisor)

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*(Gulam Mohmad Rather)*

# Abstract

Three phase partitioning (TPP) consists of precipitation of proteins due to simultaneous presence of ammonium sulphate and *t*-butanol. The technique has been successfully used for various purposes including protein purification and protein refolding.

The present thesis is an attempt to further investigate the changes in structural and biological activity of proteins as a result of TPP.

**Chapter 1** is the introductory chapter and reviews the literature relevant to three phase partitioning of proteins.

**Chapter 2 (Part A)** describes the results on the structural changes in several proteins (as a result of TPP) by comparing their thermal stabilities, secondary structure contents, surface hydrophobicities, hydrodynamic radii and solubilities in the presence of ammonium sulphate. The proteins studied for three phase partitioning are: Porcine alpha amylase, Termamyl 120 L (alpha amylase from thermophile, *Bacillus licheniformis*), Proteinase K (from *Tritirachium album*), Beta galactosidase (from *Aspergillus oryzae*), Alpha chymotrypsin (Bovine Pancreas), Subtilsin Carlsberg and Bovine serum albumin (BSA). However, in the present work (carried out with a larger set of proteins) TPP treated enzymes had much higher specific activity values. All possible effects on optimum temperature were observed as a result of TPP treatment. Beta galactosidase, Alpha chymotrypsin and Subtilisin Carlsberg showed no change. Porcine alpha amylase showed increase in optimum temperature. Alpha amylase (Termamyl 120L) and Proteinase K showed decrease in optimum temperature. While 'no change' may be understandable in terms of absence of drastic changes in flexibility, increase in $T_m$ in case of porcine alpha amylase was unexpected and difficult to understand. $T_m$ remained

unchanged except drastic decrease in Beta galactosidase. Thermal stability determination as indicated by half lives showed that TPP uniformly made all enzymes more thermolabile. The most significant decrease was observed in the case of Proteinase K (which is not surprising as X-ray diffraction had indicated a high increase in flexibility of the molecule) followed by Beta galactosidase. The TPP treatment resulted in increase in α-helical contents of Proteinase K. In this case, this increase in α-helical content was mostly at the cost of β-sheet content. In case of Beta galactosidase, Subtilisin Carlsberg and Bovine serum albumin, α-helical content decreased. In case of Beta galactosidase, β-sheet content decreased as well; the random structure content increased. In Subtilisin Carlsberg, β-sheet content increased considerably with some decrease in randomness contributing as well. Apart from porcine alpha amylase, $\lambda_{max}$ of emission (by fluorescence spectroscopy measurements) did not change. Hence changes in the microenvironments of tryptophan residues were generally not drastic. The changes in the intensities do however reflect some subtle changes in the microenvironments of tryptophan residues. The most interesting is the case of subtilisin Carlsberg whose tryptophan fluorescence is known to be quenched as a result of neighboring serine residues. Any significant unfolding generally should be reflected in increase in surface hydrophobicity. Changes in fluorescence emission of 8-anilino-1-naphthalene sulfonate (ANS) as a consequence of binding to the protein surface are the most frequently used method to estimate surface hydrophobicity of proteins. Except in the case of Subtilisin Carlsberg, TPP treatment led to decrease in surface hydrophobicity. The most drastic change was observed in the case of alpha chymotrypsin. Proteinase K, on the other hand showed only a marginal decrease. Any significant unfolding should also get reflected in changes in hydrodynamic radii of the various enzyme molecules after TPP treatment. No

significant changes in hydrodynamic radii were observed (An exception was alpha chymotrypsin, this was studied more extensively as described in **chapter 3**). In fact, porcine alpha amylase, Beta galactosidase, Subtilisin Carlsberg and Bovine serum albumin showed marginal decrease in the hydrodynamic radii after TPP treatment. Significantly, proteinase K which is known to became highly flexible, did not show any change in its hydrodynamic radius after TPP treatment. This indicated that whatever structural changes occurred as a result of TPP treatment were too subtle to affect hydrodynamic radius of the protein molecules. TPP treated enzymes also showed different precipitation behavior in the presence of ammonium sulphate. With Proteinase K, both untreated and TPP treated preparations showed higher solubility at 30% saturation of ammonium sulphate. On the other hand, the differences in the solubility curves were much larger in the case of untreated and TPP treated alpha chymotrypsin. While untreated alpha chymotrypsin had more solubility at ~ 20% saturation of ammonium sulphate, TPP treated alpha chymotrypsin had higher solubility at ~ 10% saturation of ammonium sulphate. With bovine serum albumin, the precipitation curve was flat over a large range of ammonium sulphate concentration. TPP treated BSA was more soluble than untreated BSA, noticeably so at the "salt-out" component of the precipitation curve. In general, TPP treated enzymes were more soluble in the presence of ammonium sulphate as compared to their untreated counterparts. The results showed that while the nature or extent of structural changes may vary, in all the cases the changes are rather subtle and not drastic in nature. Hence, the technique can be safely used for protein purification and refolding.

**Chapter 2 (Part B)** describes the work on purification and characterization of alpha amylases (alpha amylase I and II) from psychrotolerant fungus, *Penicillium polonicum.*

The present work was undertaken to investigate the structural consequence of TPP on psychrophilic enzymes (which are known to be more flexible and less stable at moderate temperatures) in order to understand the role of three phase partitioning on flexibility of enzymes and their structural comparison with the thermophilic enzymes (which are known to be more rigid). Interesting enough, TPP treatment seem to make these enzymes even more thermolabile. In that respect, the consequences of TPP treatment of these enzymes from psychrotolerant organisms were no different than what was observed with enzymes from mesophiles and thermophiles.

**Chapter 3** describes the results on subtle structural changes in alpha chymotrypsin leads to aggregation accompanied by increase in biological activity. Three phase partitioning resulted in alpha chymotrypsin preparations wherein the molecule showed greater tendency for self aggregation. Precipitation with increasing amount of ammonium sulphate led to increase in size of the aggregates. While light scattering method estimated the hydrodynamic diameter of these aggregates in the range of 242-1124 nm, Nanoparticle tracking analysis gave the value as 130-462 nm. Scanning electron microscopy and Gel filtration on Sephadex G-200 showed extensive aggregation in these preparations. Transmission electron microscopy showed that the aggregates had irregular shapes. All the aggregates had about 3X higher catalytic activity than native enzyme. These aggregates did not differ in $\lambda_{max}$ of fluorescence emission which was around 340 nm. Far-UV and Near-UV circular dichroism also showed no significant structural changes as compared to the native molecule. Interestingly, HPLC gel filtration (on hydroxylated silica column) gave 14 nm as diameter for all TPP treated alpha chymotrypsin preparations. Light scattering of these TPP treated alpha chymotrypsin preparations in presence of 10 % ethylene glycol also dissociated these aggregates to

monomers of 14 nm. Both these results indicated that hydrophobic interactions were the driving force behind this aggregation. These results indicate: (1) Even without any major structural change, three phase partitioning led to alpha chymotrypsin molecules becoming highly prone to aggregation (2) Different methods gave widely different estimates of sizes of aggregates. It was however possible to reconcile the data obtained with various approaches (3) Nature of gel filtration column is crucial and use of this technique for refolding and studying aggregation needs careful rethink.

**Chapter 4** describes the results on improving the efficiency of enzymatic hydrolysis of β-casein by subjecting it to three phase partitioning. Casein is the main protein component of milk constituting about 80 % of the total milk protein fractions. Although, neither casein nor individual casein fractions have any established physiological role, peptides derived from casein have been shown to possess various bioactive properties. Protein hydrolysates have been used in the manufacture of special foods for several groups, including premature newborn, children with diarrhoea, gastroenteritis and low-absorption syndrome. The results showed that bovine β-casein when subjected to three phase partitioning (TPP) became a better substrate for alpha chymotrypsin and trypsin. The optimized conditions for TPP of β-casein consisted of mixing 40 % saturation of ammonium sulphate and 1:1 ratio ($v.v^{-1}$) of *t*-butanol to 0.5 % ($w.v^{-1}$) β-casein solution. Both fluorescence emission spectra and surface hydrophobicity data (as obtained by ANS fluorescence titration) showed significant changes in the tertiary structure of β-casein. The $V_{max}.K_m^{-1}$ values (in $min^{-1}$) for hydrolysis of TPP treated β-casein and β-casein by alpha chymotrypsin were 39.35 and 29.20, respectively. SDS-PAGE analysis of β-casein hydrolysates obtained by alpha chymotrypsin treatment showed that larger % of ~3 kDa peptides were produced when β-casein was subjected to TPP before the enzymatic

hydrolysis. There is nothing in TPP process or β-casein structure to make the strategy "case specific". Hence, possibly the results have general significance in the context of production of protein hydrolysates and proteolysis as the wider context.

**Chapter 5** describes the results on refolding of urea denatured ovalbumin with three phase partitioning generates many conformational variants. Generally, the interfacial protein precipitate (formed between upper *t*-butanol rich and lower aqueous phase) obtained by TPP can be easily dissolved back in aqueous buffers. In case of ovalbumin, this led to a precipitate which was insoluble in aqueous buffers. This precipitate when solubilized with 8 M urea and again subjected to three phase partitioning under various conditions led to many conformational variants. One of these had trypsin inhibitory activity, showed higher β-sheet content and had higher surface hydrophobicity (both with respect to native ovalbumin). Scanning electron microscopy and Atomic force microscopy of this preparation showed a thread like structure characteristic of amyloid fibrils. The behavior of ovalbumin during three phase partitioning makes it a valuable system for further understanding of protein aggregation.

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