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Three phase partitioning leads to subtle structural changes in proteins

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ABSTRACT

Three phase partitioning consists of precipitation of proteins due to simultaneous presence of ammonium sulphate and *t*-butanol. The technique has been successfully used for purification and refolding of proteins. There are however indications that the structures of proteins subjected to three phase partitioning are different from native structure of proteins. Taking several proteins, the present work examines the structural changes in proteins by comparing their thermal stabilities, secondary structure contents, surface hydrophobicities, hydrodynamic radii and solubilities in the presence of ammonium sulphate. The results show 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.

1. Introduction

Three phase partitioning (TPP) was originally developed as an upstream technique for isolation of proteins [1,2]. It essentially consists of adding a salt together with an organic solvent to the crude extract of protein. The result is formation of three phases: upper organic solvent rich phase, lower water rich phase and an interfacial layer of precipitated protein. In a successful TPP protocol, the desired protein is recovered in the concentrated form as the interfacial layer and some contaminating material (like pigments) is recovered in the organic solvent phase. Initial work had found that generally ammonium sulphate and *t*-butanol are the best salt and organic solvent, respectively for carrying out TPP [2–4]. Subsequently, TPP was found to be a good strategy for protein purification in several cases [5–9] leading it to be recognized as a potential non-chromatographic method for protein purification in industrial enzymology [10]. Some discussion of mechanistic aspects of TPP is available [1,2,11]. While Dennison and Lovrein [2] view TPP as a result of various consequences of sulphate anion and *t*-butanol binding to the proteins, Borbas et al. [12] view TPP as an adsorption of protein at the fluid interface. TPP has also found to be a useful strategy for protein refolding [13,14]. In many cases, TPP leads to enhanced in vitro biological activity both in conventional aqueous buffers as well as in low organic media [15]. This observation as well as its use in protein refolding makes the issue of structural consequences of TPP for proteins a very important one. Very limited information is available on this. It has been shown by X-ray diffraction study that TPP treated proteinase-K has a very high B factor. The protein molecule is more flexible especially around active site region [16].

There are well defined states for the protein structure: native state, denatured state and molten globule [17]. There is obviously a need to understand how a precipitation technique invariably produces a biologically more active protein. So the present work is aimed at investigating the effect of TPP on protein structure by examining several biophysical parameters in case of several TPP treated proteins.

2. Materials and methods

2.1. Materials

Alpha-chymotrypsin (Bovine Pancreas), bovine serum albumin (BSA), beta-galactosidase (*Aspergillus oryzae*), porcine pancreatic alpha-amylase, proteinase-K and Subtilisin Carlsberg (*Bacillus licheniformis*) were obtained from Sigma Chemical Company, St. Louis, USA. Alpha-amylase (I and II) were purified from the fungus, *Penicillium polonicum*. Both purified preparations showed a single band on SDS-PAGE (G. M. Rather and M. N. Gupta, unpublished results). Alpha-amylase from *B. licheniformis* is sold as a commercial product, Termamyl 120L from Novozyme, Denmark and was obtained from Arun and Co., Mumbai, India. The enzyme was purified to obtain a preparation which showed a single band on SDS-PAGE (G. M. Rather and M. N. Gupta, unpublished results). Ammonium sulphate and *t*-butanol were purchased from the

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Table 1
Three phase partitioning (TPP) of different enzymes/proteins at their optimum conditions. The TPP of all the samples were carried out at 25 °C for 1 h. All the experiments were carried out in triplicates and the percentage error in each set of readings was less than 3%.

Enzymes	Ammonium sulphate (%)	Aqueous to *t*-butanol ratio	Recovery of enzyme in interfacial precipitate	
			Activity (%)	Protein (%)
Porcine alpha-amylase	10	1:2	86	78
Alpha-amylase (Termamyl 120L)	50	1:1	85	80
Alpha-amylase I[a]	50	1:1	92	87
Alpha-amylase II[a]	50	1:1	90	85
Proteinase-K (*Tritirachium album*)	30	1:2	186	90
β-Galactosidase (*Aspergillus oryzae*)	60	1:1	55	30
Alpha-chymotrypsin	50	1:2	182	63
Subtilisin Carlsberg	20	1:2	348	87
Bovine serum albumin	60	1:1	–	91

[a] Alpha-amylases from psychrotolerant fungus, *Penicillium polonicum* were purified before use (G. M. Rather and M. N. Gupta, unpublished results).

Merck Limited (Mumbai, India). All other reagents were of analytical grade.

2.2. Three phase partitioning (TPP) of enzymes

Enzyme solution was mixed with varying concentrations of ammonium sulphate (w/v). The varying amount of *t*-butanol was added to this solution, vortexed, and incubated at the 25 °C. After 1 h the mixture was centrifuged at 2000 × *g* for 10 min for three-phase formation. The upper *t*-butanol layer was pipetted out. Thereafter, the interfacial precipitate (of the enzyme) was pierced to collect the lower aqueous layer. The precipitated enzyme was dissolved in their respective buffers and thus the TPP treated enzyme was extensively dialyzed at 4 °C before any further study. The amount of precipitated enzyme was calculated by measuring the amount in aqueous phase and interfacial precipitate (by taking absorbance at 280 nm) with the starting amount as 100%. The optimized conditions of TPP for various enzymes for maximum recovery in the interfacial precipitates are given in Table 1.

2.3. Enzyme and protein assay

Alpha-chymotrypsin activity was measured using BTEE as a substrate [15]. Subtilisin Carlsberg activity was measured using casein as a substrate [18]. Amylase activity was measured using starch as a substrate [19]. Proteinase-K activity was measured as described by Singh et al. [16]. Beta-galactosidase activity was measured using *o*-nitrophenyl-β-D-galactopyranoside (ONGP) as substrate [20].

Protein estimation was carried out by dye binding method using bovine serum albumin as the standard protein [21].

2.4. Optimum temperature and thermal stability

The optimum temperature of all the native enzymes and their respective TPP treated enzymes were determined by assaying the enzymes at various temperatures in the range of 20–100 °C. The solutions of native enzymes and their respective TPP-treated preparations were incubated at different temperatures (45 °C, 50 °C, 60 °C and 65 °C). The aliquot were taken at different time intervals, immediately cooled and assayed. The activity at 0 min was taken as 100%. Half-life values were calculated by plotting In (residual activity) vs time for each enzyme [22].

2.5. Circular dichroism measurements

The circular dichroism (CD) spectrum of various TPP treated enzymes were recorded with a spectropolarimeter (JASCO 815) with homogeneous protein samples in 20 mM sodium phosphate, pH 7.0 at 25 °C. Spectra were recorded using a 2 nm band width, 8 s response rate, scan rate of 50 nm min^{-1} and a 0.1 nm pitch. Five accumulations were recorded and averaged for spectrum using a 0.1 cm path length cell. The blank spectrum of an aqueous buffer was used to correct the observed spectrum of the sample. The CD data were expressed as mean residual ellipticity in deg cm^2 $dmol^{-1}$. The spectrum was subjected to secondary structure analysis using k2d2 online program.

The melting temperature of native and TPP treated enzymes were also determined by recording the spectra over different temperatures. The CD data were expressed as mean residual ellipticity in deg cm^2 $dmol^{-1}$. The T_m values were also confirmed from the first derivative [$d\theta/dT$ vs T] plot.

2.6. Fluorescence measurements

Fluorescence spectra were recorded at 25 °C with a Varian Cary Eclipse Fluorescence spectrophotometer using a 1 cm cuvette. All protein solutions were made in 20 mM sodium phosphate, pH 7.0. All fluorescence spectra were normalized and corrected for buffer contributions. The identical protein concentrations for each native enzyme and its TPP treated counterpart were used by matching the absorbance at 280 nm.

Surface hydrophobicity parameter (S_o) of various enzymes was measured by a fluorimetric approach and applying the slope method of Kato and Nakai [23]. An excess of fluorescent probe ANS at 100 μM was titrated with a typical protein in the concentration range of 0–7.0 μM in 20 mM sodium phosphate buffer, pH 7.0. Excitation and emission wavelengths were 390 and 470 nm respectively. Relative fluorescent intensity (F_R) calculated as $[(F - F_o)/F_o] \times 10$ was plotted as a function of protein concentration [24] and the slope of the regression line was taken as the S_o value.

2.7. Ammonium sulphate precipitation profile measurement

Native enzymes (proteinase-K, alpha-chymotrypsin and bovine serum albumin) and their TPP treated preparations were precipitated (both native and TPP-treated proteins had the same protein concentration which was ensured by matching absorbance at 280 nm) with different ammonium sulphate concentrations at 4 °C (overnight) with continuous stirring. The protein estimation was done in the supernatant by taking the absorbance at 280 nm after extensive dialysis of each sample in order to remove the ammonium sulphate.

2.8. Dynamic light scattering measurements

Dynamic light scattering (DLS) measurements were performed at 25 °C using a RiNA GmbH laser spectroscatter 201 (Berlin