Three Phase Partitioning - A Novel Protein Purification Method

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Abstract: Investigation of food protein composition becomes more and more significant now a days, that is motivated by the claim for verification of food origin and / or identification, growing cultivation of genetically modified plants, commercialization of functional food, stress on plants etc. Numerous methods are used for investigation of food proteins, however, the need for rapid data collection and for better recognition requires a steady development and use of novel methods. Three phase partitioning (TPP) is a relatively new, three stage, batch method for rapidly and effectively purifying protein. TPP uses t- butanol and ammonium sulfate to precipitate enzymes and proteins from aqueous solution. The simplicity of the method combined with its rapid result make this a popular choice in large scale protein purification.
Key words: Three phase partitioning, protein, ammonium sulfate, purification

Introduction

Traditionally protein purification involves a complex array of techniques. But, process development constantly demands methods that increase purity and yields, and reduce the process time cost. The analysis of enzyme behaviour in anhydrous media has important implications in biotechnology and bioorganic chemistry. At present, the interactions between protein and organic solvent are inadequately understood[1]. A clear understanding will facilitate the use of a non-aqueous milieu for many applications such as organic synthesis, biosensors, bioseparation and perhaps enhancement of the enzyme activity and stability. Three phase partitioning (TPP), a novel bioseparation strategy, is useful as both an upstream and a downstream method in the production of enzyme proteins. Kosmotropy, electrostatic forces, conformation tightening and protein hydration shifts have been suggested as the physico-chemical basis for the protein coming out as an insoluble phase[2]. One of the main assumptions has been that the tert-butanol (the solvent, which has been used most frequently) binds to the protein interior[3].

Three phase partitioning (TPP) uses t-butanol and ammonium sulfate to precipitate enzymes and proteins from aqueous solutions. In TPP, proteins are excluded from two immiscible liquid phases into a middle zone that becomes concentrated by low speed centrifugation into a thin disk. The simplicity of the method combined with its rapid results should make this a popular choice in large-scale protein purification and in small-scale diagnostics, as well.

TPP was first developed as an upstream technique for the litre scale precipitation of crude cellulases[4] and other enzymes. However, TPP is frequently also useful down stream for isolation on a semi-micro, milliliter volume scale. TPP development has one root in 1972 work[5], which showed that numbers of enzymes maintain their activities in t-butanol water mixtures. A few enzymes and protein lose activity or function in such

cosolvent water mixtures. On the other hand, that can also be useful if unwanted protein can shunt aside, such as hemoglobin, in isolating erythrocyte enzymes via the TPP technique[6].

Nearly all development of TPP so far depended on t-butanol, which is a differentiating solvent. It might not necessary to always use t-butanol. Other C_4 and C_5 co solvents may also serve because they act as differentiating co solvents. TPP is related to conventional salting out, not simply because TPP uses the same salt, ammonium sulfate, but because TPP and conventional salting out has a common origins[7].

Principle of TPP

Three phase partitioning uses t-butanol and ammonium sulfate to precipitate enzymes and proteins from aqueous solution. Tertiary butanol is normally completely miscible with water, but upon the addition of enough salt, such as ammonium sulfate, the solution separates into two phases, a lower aqueous phase and an upper t-butanol phase. If protein is present in the original aqueous phase, it may depending on the concentration of ammonium sulfate added, separate into a third phase, intermediate between the lower aqueous and upper t-butabol phase. This is the basis of the technique called it as three phase partitioning[8]. The schematic representation of TPP is shown in Figure 1.
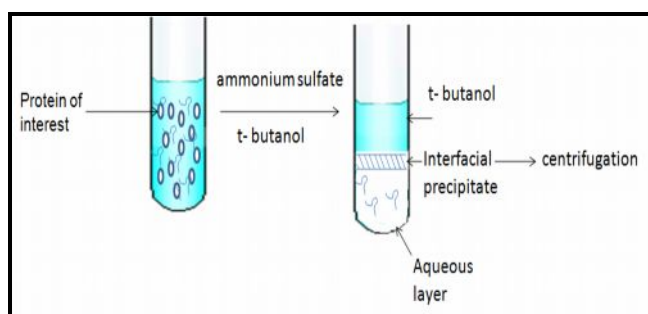
Figure 1. Schematic representation of TPP System

Some t-butanol appears bound to TPP precipitated proteins, which are actually protein – t-butanol coprecipitates. They float above denser aqueous salts because bound t-butanol increases the buoyancy[2].

Tertiary-butanol has wide application in the isolation of proteins from animal, plant or microbial sources. Unusually, for an organic solvent, t-butanol stabilizes protein structure. It also inhibits enzyme activities and protein / protein interactions. By inhibiting proteolysis and obviating unwanted protein / protein interactions, the presence of approximately 30 percent t-butanol in a homogenization buffer generally minimizes the formation of artifacts and gives a higher yield. The presence of t-butanol in the homogenate leads naturally onto concentration and fractionation of proteins by three phase partitioning, simply by the addition of increments of ammonium sulfate[7]

The basis of TPP's capacity to precipitate proteins originates from the abilities of t-butanol and sulfate anion to push on the left and pull from the right in two principal reactions.

Protein molecule in free solution conformationally coprecipitated motile penetrated by water, fully soluble	Reaction1 →	Protein molecule in solution, conformationally tighten, less penetrated by water	Reaction 2 →	Precipitated protein (or with bound sulfate), least penetrated water

Pushing and pulling is meant in both the thermodynamic sense and in measurable physical sense. Pushing and pulling both involve macromolecule hydrate water. In order to squeeze a macromolecule shut and forces conformation tightening, water molecules have to displace, moved out of the macromolecule's domain. How agents push or pull depends on how much, and even whether, such agents interact or bind directly with the macromolecule.

Pushing by large concentration of salts such as ammonium sulfate is a thermodynamic consequence of exclusion crowding mechanism[9][10]. Pushing produces conformation tightening together with protein hydration changes, seen by viscosity changes and related physical tools. Pulling frequently also results in protein molecule

conformation tightening, but for different reason and in much lower concentrations than SO_4^{2-} needs to push. In "pulling", SO_4^{2-} binds to protein molecule cationic groups : an electrostatic interaction, which draws protein conformation inward, also decreasing protein net hydration. Protein in this case emerges as sulfate salts. They frequently are markedly more stable in bound sulfate salt form, then when lacking salt, especially for proteins, which are rather fragile, easily denatured. The conformation changes fostered by sulfate anion and related agents, while protein are still in solution, are observed by several physico-chemical tools such as large decreases in protein intrinsic viscosities[11] and by spectroscopic means[12].

Ammonium Sulfate Precipitation

The solubility of protein depends on, among other things, the salt concentration in the solution. At low concentrations, the presence of salt stabilizes the various charged groups on a protein molecule, thus attracting protein into the solution and enhancing the solubility of protein. This is commonly known as "salting-in". However, as the salt concentration is increased, a point of maximum protein solubility is usually reached. Further increase in the salt concentration implies that there is less and less water available to solubilize protein. Finally, protein starts to precipitate when there are not sufficient water molecules to interact with protein molecules. This phenomenon of protein precipitation in the presence of excess salt is known as "salting-out"[13].

Many type of salts have been employed to effect protein separation and purification through salting-out. Of these salts, ammonium sulfate [$(NH_4)_2SO_4$} has been the most widely used chemical, because of its high solubility (about 3.6 M) and high ionic strength [which is proportional to the square of the charge on the ion, so that the ionic strength of 1 M $(NH_4)_2SO_4$ is 3 times that of 1 M NaCl]. Neither ion associates much with proteins, which is good since such association usually destabilizes proteins. Its solubility changes little with temperature, it is cheap, and the density of even a concentrated solution is less than that of protein, so that protein can be centrifuged down from concentrated solutions.

One generally uses "enzyme-grade" $(NH_4)_2SO_4$ crystallized from EDTA to minimize effect of contaminating heavy metals. The volume of the solution increases as $(NH_4)_2SO_4$ is added; the solubility is 533 g/L solution, but 761 g/L original solution. The one thing one must remember is that because ammonia is a weaker base than sulfuric is an acid, the pH tends toward about 5.3, base (usually ammonium hydroxide) must be added to hold the pH at 7.0. Alternatively, if your protein doesn't mind pH 5.3, it may be least soluble at that pH, which may be near its isoelectric point.
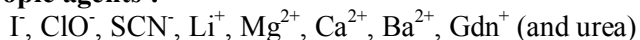
<p align="center">**Order of effectiveness in salting-out**</p>

Anions :
$$SO_4^{2-} > H_2PO_4^- > CH_3COO^- > Br^- > I^- > ClO_4^- > SCN^-$$
Cations :
$$NH_4^+, Cs^+, K^+, Na^+ > Li^+ > Mg^{2+} > Ca^{2+} > Ba^{2+}$$
Chaotropic agents :
$$I^-, ClO^-, SCN^-, Li^+, Mg^{2+}, Ca^{2+}, Ba^{2+}, Gdn^+ \text{ (and urea)}$$

The sulfate ion has been viewed in five principal ways, concerning how it salts out protein :

- Ionic strength
- Kosmotrophy
- Osmotic stressor
- Cavity surface tension
- Exclusion crowding agent

Possible all five such factors or mixtures of them operate in various proportions depending on sulfate concentration and the protein molecule charge in response to pH.

There is a sixth matter, the divalent sulfate anion SO_4^{2-} tends to bind into few cationic sites of many proteins, which proteins have a net positive charge Z_H^+.

Sulfate's function not only in TPP, but also in other protein separation – isolation uses. Sulfate ion binding to proteins tends to tighten protein conformation[14,15]. The capacity of sulfate to precipitate and coprecipitate protein depends on the protein molecule binding salt anions and on protein conformation tightening, if the starting point is water penetrated protein molecule. The abundance of protein cationic sites for

salt anion binding depends on the protein molecule net charge. Z_H^+, and therefore, depends on pH and PI. The pH dependence of sulfate promoted precipitation and coprecipitation of proteins in relation to each protein's isoionic point, i.e., to the positive charge borne on proteins, indicates that sulfate binding is largely electrostatic in nature. Below salt concentration 0.2 M, solvent ionic strength may be the dominant factor governing protein conformation motility. Above 0.2 M concentration, especially in the case of sulfate, kosmotropy and osmotic stress become strong contributors. Sulfate binding to the discrete site of proteins, when it occurs usually is completed when about 0.1 to 0.2 M sulfate concentration is reached. If the protein molecular tightening in coprecipitation also orders protein molecule conformation, protein crystallization may occur with salt anions placed in discrete place[16]. Sulfate has an enhanced ability to bind to protein cationic sites, but it is divalent. TPP uses t-butanol and ammonium sulfate in which some sulfate bound to protein and other acts as a kosmotrope when its concentration is increased. The same likely to pertains to t-butanol, some t-butanol becomes bound and remaining in bulk solvent either acts as a kosmotrope or act in a synergism with sulfate kosmotropy[17].
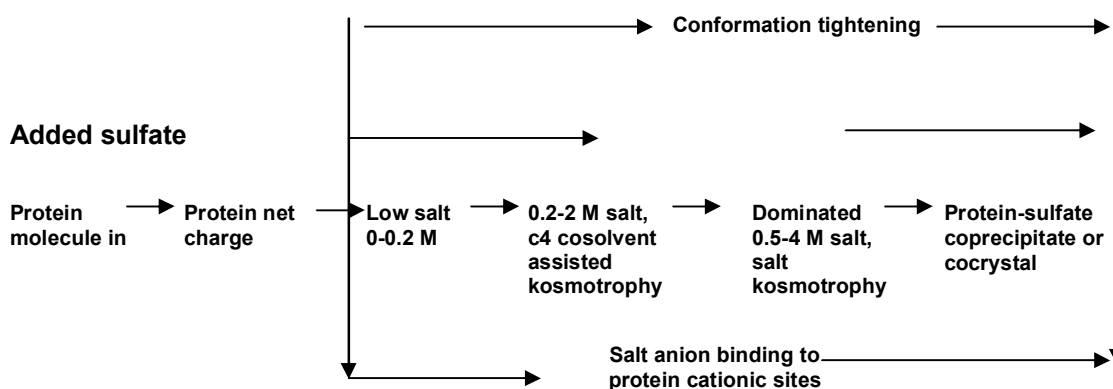
Advantages of TPP

Three phase partitioning is a simple but elegant non chromatographic process used for purification and concentration of protein. TPP seems to have evolved out of these early results and at first glance appears to be a hybrid of salting out and alcohol precipitation. However, there are definite indications that TPP is different from simple combinations of these steps a) concentration of ammonium sulphate in TPP is much lower than that required for salting out of proteins. b) In salt precipitation the precipitate is a function of the precipitating salt concentration, while in TPP precipitation also depends upon the initial protein concentration. c) Organic solvent precipitations must be carried out at low temperatures, whereas TPP can be carried out at room temperature[18]. Thus, process environments in TPP are more conductive to the maintenance of native structure. Several advantage of TPP are

> - It is a simple procedure with short processing time
> - Ammonium sulphate and tert- butanol are inexpensive chemicals, thus making TPP an economical protocol
> - TPP conditions are mild and do not denature proteins
> - It is usually carried out at room temperature
> - TPP can be scaled down to semi- microlevels or scaled up to litre scale
> - TPP purifies as well as concentrates the protein, unlike chromatography, which dilutes the purified protein
> - Purification fold achieved in TPP is much higher than that achieved in simple salting out procedures
> - TPP can be used with direct crude culture containing cell debris: no pre-clarification steps such as centrifugation are required

Applications of TPP

Cellulases

Cellulases are mixtures of three excreted enzymes from fungi such as exo-, endo- cellulases and β-glucosidase. TPP in the pH range 4.5 to 5.5 precipitates all the three enzymes simultaneously from *T. reesei* crude fermentation mixture. Total TPP activity yields are 90 to 95 percent of original cellulase activity assayed on crystalline cellulose substrate. Crude cellulases from most fungi are often quite coloured, pigmented. During TPP, the majority of such pigments are extracted into the t-butanol (upper) layer[4].

Cathepsins D, L, S

Cathepsins are intracellular, proteolytic enzymes occurring mostly in animal cell lysosomes. They are generally monomeric proteins and commonly have molecular weights of 25 to 35 kDa. TPP denatures hemoglobin [6] suggested that it might be useful in the isolation of Cathepsin D from spleen, where removal of contaminating hemoglobin is the major challenge.

Erythrocyte cytosol proteins

TPP has proved useful in the rapid isolation of soluble proteins from erythrocyte cytosol such as carbonic anhydrases, catalases and superoxide dismutase, but requires the removal of hemoglobin [6].

Recently isolated procalpain I and calpastatin, an inhibitor of the cytosol calcium-dependent cysteine proteinase calpain, from porcine erythrocyte cytosol using a procedure called as five phase partitioning.

Lipases

In 1987 Lovrein and his coworkers[19] found t-butanol based TPP is useful for the microbial lipase from *Candida cylindracea*. The large changes around the isoionic point (pH 4.0) for this lipase, in its TPP behaviour dependent on pH, is another example of governance by electrostatic forces in TPP. The sign of protein net charge, Z_H^+, abruptly changes at the isoionic point, sharply changes the protein's ability to bind or to reject sulfate anions, with consequence very similar to other proteins in their pH dependent TPP behaviour.

Cholesterol esterase

In 1987 Rudd [20] have isolated cholesterol esterase from pancreas using t-butanol TPP and obtained a 2.8 fold increase in specific activity.

Catalase

Three-phase partitioning (TPP) was used to purify and recover catalase from potato crude extract. The method consists of ammonium sulfate saturation, t-butanol addition, and adjustment of pH, respectively. The best catalase recovery (262 %) and 14.1-fold purification were seen in the interfacial phase in the presence of 40 % (w/v) ammonium sulfate saturation with 1.0:1.0 crude extract/t-butanol ratio (v/v) at pH 7 in a single step. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the enzyme showed comparatively purification and protein molecular weight was nearly found to be 56 kDa. This study shows that TPP is a simple, economical, and quick method for the recovering of catalase and can be used for the purification process[21]

Modification of TPP

Two Step TPP

It has been observed that under certain circumstances, upon subjecting the crude protein solution to TPP, three phases are formed; however, the desired protein remains predominantly in the aqueous layer. In such cases, the three protein remains predominantly in the aqueous layer. In such cases, the three layers are separated and the aqueous layer is subjected to a second TPP by adding more ammonium sulphate and tert-butanol. Three phases are formed again and this time most of the desired protein partitions into the interfacial precipitate. Such two- step TPP has been used for purification of several enzymes, such as α- amylase[19], GFP[22], phospholipase D from carrots[23], alkaline phosphatise from chicken intestine[24] and pectinase[25]

Metal – affinity based TPP

TPP was interfaced with metal affinity based step to selectively purify proteins with surface histidine residues present at the appropriate distance[26].

Affinity macroligand facilitated three phase portioning

Water soluble polymers also underwent TPP in the presence of ammonium sulphate and tert-butanol. This led to the development of a purification protocol called macroaffinity ligand facilitated three phase partitioning, which may be considered as an extension of TPP[27].

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