Three-Phase Partitioning for Protein Purification

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Three-phase partitioning (TPP) represents an incredibly simple, rapid method for extracting, purifying and concentrating proteins for use as either a protein mini-prep kit or scaled up to large manufacturing operations.

Three-phase partitioning (TPP) is a relatively new, three-stage, batch method for rapidly and effectively purifying proteins (1,2). The TPP method may be used as a protein mini-prep kit (3) or scaled up to large manufacturing operations (2). The method employs high concentrations of well-buffered aqueous ammonium sulphate (0.8 to 2.4M) together with an equal volume of water-miscible aliphatic alcohol (most generally tertiary butanol). While methanol, ethanol, 1-propanol, 2-propanol and t-butanol are all miscible with pure water, none is miscible with aqueous solutions of the anti-chaotropic salt, ammonium sulphate – so two liquid phases form (alcohol on top and salty water below). The method may be used with crude homogenates of animal or plant tissues or with whole, unlysed microbial cells collected in pellets after fermentation (see Figure 1). There is no requirement for the protein of interest to carry a genetically inserted affinity tag, although affinity tags are not precluded. In this article, I will first describe the mechanical procedures of TPP and then, later, explain the theoretical basis of the method.

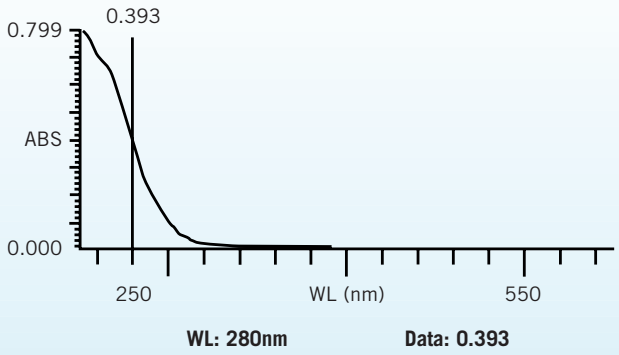

Figure 1: Absorption spectrum of whole *E coli* cell suspension, clarified by heating in 5M NaOH. Total absorbance at 280nm = 38,200

At Brighter Ideas, Inc, we have studied the TPP method as a key, initial step in purifying recombinant wild-type Aequorea Green-Fluorescent Protein (GFP) from appropriately transformed *E coli* cells (see Figure 1). A-GFP is a stable, water-soluble, globular protein of molecular weight 27 Kdal and isoelectric point near pH 5.3. At high protein concentrations, and especially in high concentrations of ammonium sulphate, A-GFP forms dimers, stabilised primarily by hydrophobic, intermolecular interactions (4).

STAGE I OF TPP

Using our TPP procedure, we suspend GFP-expressing, whole, unlysed *E coli* cells in 1.6M aqueous ammonium sulphate solution, buffered at pH 8.0 with 50mM Tris buffer (starting buffer). Sufficient 4 M ammonium sulphate stock solution is added to account for the water content of the cells. At this point, a droplet of the suspension is placed into a small tube of starting buffer as a simple way to check sample density (that is, salt concentration). If the droplet floats, salt is too low; if it sinks, salt is too high. Adjustments are made so that the droplet remains suspended in the starting buffer, neither sinking nor floating. Less than one per cent of the total GFP leaches from the whole cells, so high-speed centrifugation (10,000rpm, 10 minutes) is an appropriate next step. The cellular pellet is resuspended, as before, in fresh starting buffer. A significant amount of 280nm-absorbing material is removed in this simple step. In the case shown in Figure 1, the suspended bacterial pellet, corrected for dilution factors, has registered 38,200 total absorbance units at 280nm. If we wish to process 50ml of total suspension, (much smaller quantities than that shown in Figure 1), we split the sample equally into two Falcon tubes, topping off the tubes – if necessary – to the 25ml mark, with more starting buffer.

The cellular suspension is then treated with an equal volume of t-butanol (25ml of t-butanol per 25ml aqueous cell suspension in starting buffer). After one minute of vigorous shaking, this suspension is subjected to low speed centrifugation at room temperature (4,000rpm, 10 minutes). Two liquid phases appear, but now – with biological material present – there appears a semi-solid, spongy disk at the interface of the two liquid layers. This disk contains most of the cellular proteins (up to 98 per cent), virtually all of the cell wall polysaccharide and the chromosomal DNA. The upper organic layer contains the dissolved membrane lipids and low molecular weight pigments; this upper layer is then aspirated away, effectively removing all lipids and pigments. If the ammonium sulphate concentration was properly adjusted at the outset of TPP (this adjustment having been determined empirically for the protein of interest), up to 95 per cent of the protein of interest will now be dissolved in the lower aqueous layer. The bottom phase will have lost about 20 per cent of its volume, as some water will have partitioned into the organic phase.

TPP-BASED PROTEIN MINI-PREP

For a protein mini-prep application (3), we start with a much smaller volume (0.5ml). All TPP steps will be the same, except that volumes are reduced by a factor of 100. After Stage I, the aqueous layer will now be quite clear and free of viscosity and turbidity, so spectrally-based assay methods (spectrophotometry, fluorometry or colorimetry) can be performed quite accurately. Removed at Stage I, the complicating factor of light scatter that might otherwise give falsely high or falsely low assay values is no longer an issue. The unwanted organic layer is easily aspirated away and the spongy disk, having the consistency of an almost fully cooked pancake, is easily removed with a spatula. 'Flipping' the spongy disk away with a spatula removes most proteins, nearly all nucleic acids, and nearly all polysaccharides. This leaves the highly purified protein of interest in a clear aqueous layer as shown in Figure 2. In parallel experiments of this sort, clear aqueous layers from TPP may be sampled and then optically quantified to determine specific protein expression levels.

If the goal is to determine the time course of protein expression in a given fermentation run, Stage I of TPP may be highly useful. Others may use TPP to facilitate measurement of the levels of a protein of interest derived from a variety of different biological source materials. In each case, quantifying the level of the protein of interest is made more accurate by application of TPP. As the protein mini-prep produces, in just a few minutes, a clear aqueous layer at Stage I, this may be as far as one needs to take three-phase partitioning for a variety of protein screening projects such as these.

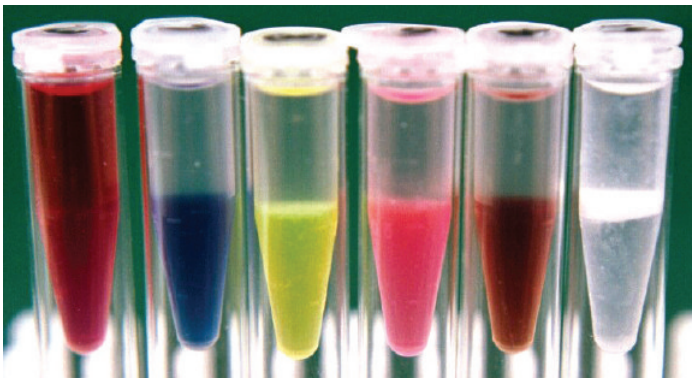

Figure 2: These tubes illustrate the first stage of three phase partitioning. Tubes contain, from left to right, vitamin B-12 (only case of partitioning into two liquid phases), blue dextran, wild-type recombinant GFP, recombinant DsRed, cytochrome c and ovalbumin (premature movement to interface due to inappropriate salt concentration)

STAGE II OF TPP

Whether for mini-preps or for larger scale operations, when the highest level of purity is desired, one can take TPP two stages further. Stage II is initiated by adding fresh t-butanol (the same volume as used earlier) to the aqueous ammonium sulphate layer saved from Stage I. When we process samples in two 50ml Falcon tubes, we add 25ml of fresh t-butanol to the now diminished volume of aqueous solution (~20ml) that remains in each tube saved from Stage I. This mixture is again shaken vigorously for one minute and centrifuged, as before (4,000rpm for 10 minutes at room temperature). Two liquid layers again separate, but now a paper-thin zone of precipitate

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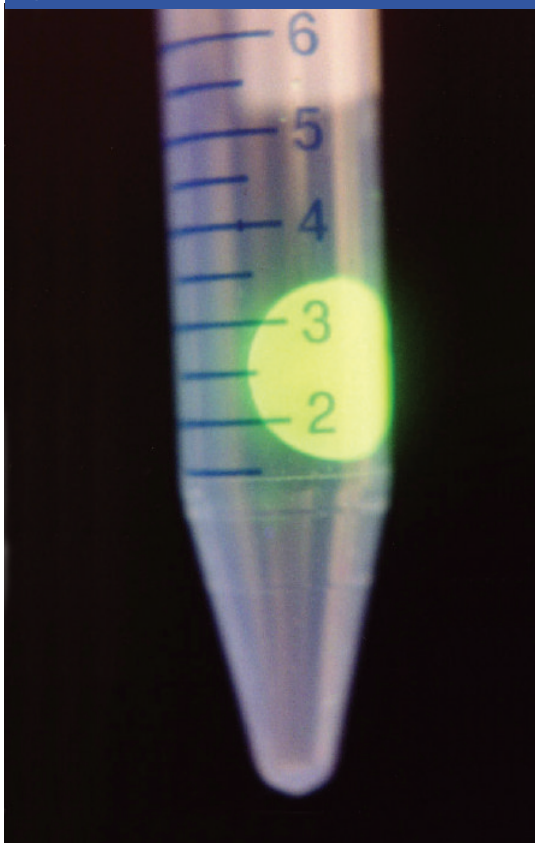
Figure 3: Isolated disk from Stage II of TPP



(substantially enriched with the protein of interest) appears between the organic and aqueous layers. The organic layer is aspirated away, but the thin disk of precipitate is usually too fragile to remove physically. Fortunately, if a tube is very slowly decanted, the disk will almost always adhere to the side wall, allowing all liquid contents to drain (see Figure 3). This completes Stage II of TPP.

STAGE III OF TPP

To these thin disks, we add a tiny volume of starting buffer. If the protein of interest is highly soluble, no more than 2ml of starting buffer will be needed. Serial addition of starting buffer, in four small aliquots of 500µl, is sufficient for quantitative transfer from the two 50ml Falcon tubes into two microfuge tubes. Serial transfer maximises total recovery of the protein of interest, while minimising transfer volume. In some cases we have been able to effect a 100-fold concentration from the beginning of TPP to the final step.

This transferred suspension contains the soluble protein of interest plus various levels of insoluble contaminants. A five-minute spin at top speed in a microfuge (swinging bucket is preferable, but not essential) will generate up to four phases. Generally, there is a small amount of precipitate at the bottom of the tube, a clear solution of the protein of interest above (occupying most of the volume), a very thin disk of precipitated protein floating near the top and, finally, a tiny bit of clear t-butanol on the very top. The soluble protein of interest is easily removed with a micropipette (see Figure 4, page 32). In the example shown in Figure 4, the starting absorbance at 280nm of 38,200 absorbance units has been reduced to just 168 absorbance units by TPP alone – a purification factor of 227-fold.

OVERALL PURITY AND CONCENTRATION FACTORS

In many cases, TPP generates greater than 100-fold purification, 70-85 per cent of final purity and at least a 50-fold decrease in volume. The GFP sample shown in Figure 4 has reached just 22 per cent of final purity, as we started with a 17-litre fermentation batch of very poorly expressing *E coli* cells. However, a simple run of the TPP-purified material through a 6ml hydrophobic interaction column of Phenyl Sepharose achieves a purity of 94 per cent in less than two hours (see Figure 5, page 34). GFP recovery, as in the case illustrated here, is usually 70 per cent or greater. Prior to discovering this method, we needed a separate lysis step and sometimes four traditional purification steps to get from whole *E coli* cells to 70 per cent pure product, and many of these steps were time-consuming – overnight

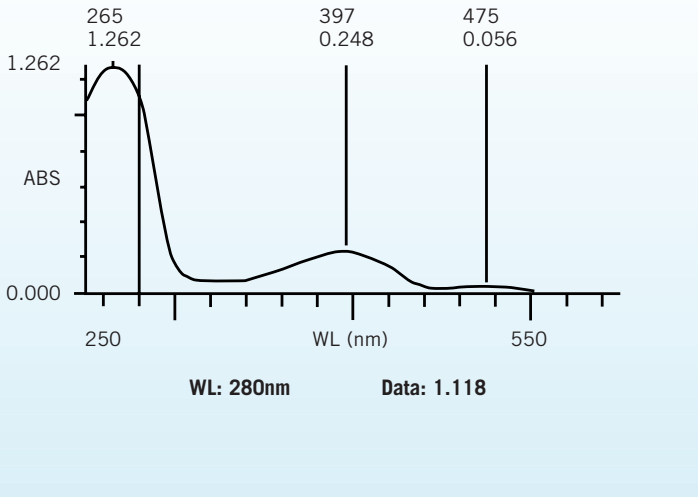


Figure 4: Absorption spectrum of whole *E coli* cell suspension after TPP (GFP chromophore signature at 397nm and 475nm now apparent). Total absorbance at 280nm = 168

or longer. Assuming each of these traditional steps yields 70 per cent recovery, four such steps would yield just 24 per cent overall recovery. A fifth step drops the recovery to just 17 per cent.

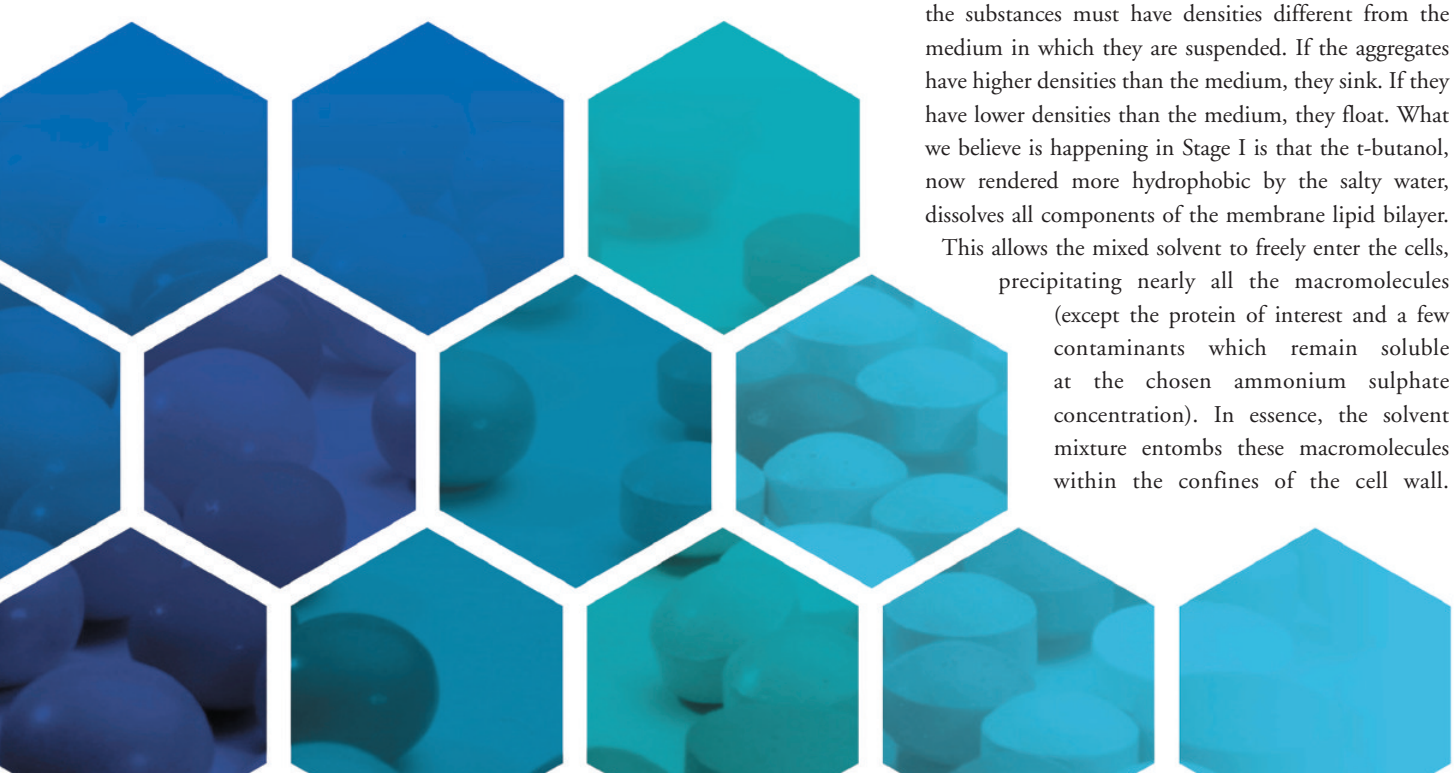
Going back to much earlier days, when we hand-collected and hand-dissected 100,000 Aequorea jellyfish or more per collecting season, it often took us six months to get to the same stage of purity that we can now achieve in just an hour or two with TPP.

UNRAVELLING THE MYSTERIES OF TPP

Ammonium sulphate is an anti-chaotropic salt that promotes protein stability by favouring hydrophobic interactions. But high concentrations of aqueous ammonium sulphate also favour hydrophobic interactions amongst otherwise water-miscible neutral solvents, such as alcohols. In the presence of high concentrations of ammonium sulphate, the effective dielectric constant of water greatly increases, such that alcohols – like t-butanol – act in a more lipophilic manner, associating with each other hydrophobically while being excluded from water.

Depending upon the starting salt concentration in TPP, a portion of the water (10 to 30 per cent) migrates into the t-butanol in Stage I, increasing the concentration of salt in the aqueous phase. A subsequent addition of fresh t-butanol in Stage II further dehydrates the aqueous phase, increasing the salt concentration to the point that the protein of interest now comes out of solution. Protein has an intrinsic density of 1.30g/cc; nucleic acids and polysaccharides each have densities of 1.60g/cc; water has a density of 1.00g/cc; t-butanol a density of 0.76g/cc; and 4 M ammonium sulphate a density of about 1.25g/cc. Lipid, by definition, is less dense than water, and under the conditions of TPP, is soluble in the alcohol phase. So the question arises: 'Why, in Stage I of TPP, do the more dense proteins, nucleic acids and polysaccharides float upon an aqueous ammonium sulphate layer having a substantially lower density?' The only plausible explanation is that t-butanol, rendered more hydrophobic than usual by the presence of salt, binds to all these macromolecules, cooperating with salt to cause aggregation (precipitation) while lowering particle densities.

Two physical properties of substances dominate in moderate speed centrifugation (4,000rpm). The substances must be very large (that is, aggregates of proteins, aggregates of nucleic acids or whole cells) and the substances must have densities different from the medium in which they are suspended. If the aggregates have higher densities than the medium, they sink. If they have lower densities than the medium, they float. What we believe is happening in Stage I is that the t-butanol, now rendered more hydrophobic by the salty water, dissolves all components of the membrane lipid bilayer. This allows the mixed solvent to freely enter the cells, precipitating nearly all the macromolecules (except the protein of interest and a few contaminants which remain soluble at the chosen ammonium sulphate concentration). In essence, the solvent mixture entombs these macromolecules within the confines of the cell wall.



In very large scale operations, the TPP method can be applied in much the same manner, except that a large settling tank replaces centrifugation. Depending upon the nature of the starting material, a loose spongy mass will form in about 30 to 60 minutes at the solvent interface in Stage I

Trapping large molecular aggregates behind the cell wall makes clarification by moderate speed centrifugation quite easy.

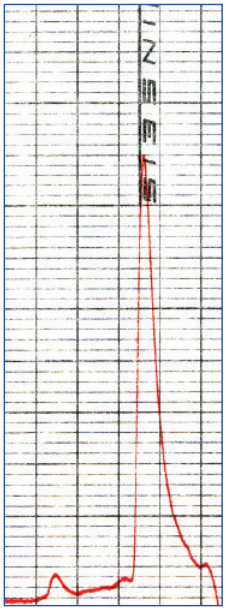
LARGE SCALE APPLICATIONS

In very large scale operations, the TPP method can be applied in much the same manner, except that a large settling tank replaces centrifugation. Depending upon the nature of the starting material, a loose spongy mass will form in about 30 to 60 minutes at the solvent interface in Stage I. An aspirator or siphon tube can then be used to collect the loosely formed disk so it can be clarified by centrifugation, separating any remaining aqueous liquid from the other unwanted phases. In Stage II, the protein of interest will precipitate in a similar way so that it too can be collected by an aspirator. To conserve salt and alcohol, pre-concentration of the crude extract by tangential flow ultrafiltration before the onset of TPP may be desirable.

TPP WITH OTHER PROTEINS

While GFP, expressed in *E coli*, has been used in this discussion as a model system to demonstrate the effectiveness of TPP, GFP is by no means the only protein amenable to three-phase partitioning. At least 25 other proteins, originating from a variety of biological materials, have been effectively purified by TPP (1). In our own lab, we have shown that a number of additional proteins make their way through all three stages of TPP with acceptable recoveries (see Figure 2). A few of the proteins we have successfully screened include: IgG, DsRed, Protein A, Protein G, ribonuclease, cytochrome c and five or six common proteases. Some of the haem-containing proteins (haemoglobin, myoglobin, horseradish peroxidase and catalase) do not fare as well, especially when taken as far as Stage III – they tend not to go back into solution.

Figure 5: HPLC size exclusion profile of TPP fraction following rapid Phenyl Sepharose FF chromatography



We expect TPP to achieve its greatest utility when many individual proteins and representatives of several individual classes of proteins have been carefully tested under a wide range of TPP conditions. Brighter Ideas, Inc, is currently mounting a large-scale screening project to demonstrate the universality of a protein mini-prep based upon TPP (3).

Dr Bill Ward is a Professor of Biochemistry at Rutgers University (New Brunswick, New Jersey) and a life-long expert on the chemistry of green-fluorescent protein (GFP). In 2003, he founded Brighter Ideas, Inc, a biotech company devoted to the biomedical and biopharmaceutical applications of GFP. Bill is a pioneer in the study of GFP, having begun his groundbreaking research in 1973. He has chaired three international symposia and amassed more than 100 publications on GFP, and is the first person to use GFP in college-level laboratory courses and hands-on corporate biotechnology training programmes and seminars. He is Founder of the Rutgers University Center for Research and Education in Bioluminescence and Biotechnology (CREBB) that has delivered two decades worth of hands-on short courses in biotechnology to over 2,000 individuals in the biopharmaceutical community.
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