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The Art of Protein Purification

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1. Introduction

Describing, in words, the details of protein purification to a relative novice in the field is not unlike explaining on paper the steps required to turn a set of colored oils into a beautiful pastoral scene on sheet of stretched canvas. Playing the oboe in a sophisticated metropolitan orchestra or performing a solo aria in a Gilbert & Sullivan operetta are accepted artistic endeavors that command great mastery of technique. Each of these art forms requires years of experience and endless experimentation and refinement of technique. Protein purification is no different. It is an art form. Like all other art forms, perfecting the art of protein purification requires a long apprenticeship. But, like all other art forms, protein purification is aesthetically rewarding to the practitioner. Every day brings new challenges, new insights, new hurdles, and new successes. Art is a process, not a destination. Protein purification fits the same definition.

Perfecting the skills of protein purification can take many years of hands-on experience as well as periodic upgrading of those skills. Perhaps the most important part of protein purification is the set of pre-column steps that precede column chromatography. Pre-column steps are not covered as much in the protein purification literature as column chromatography, HPLC, and electrophoresis. So, I have chosen to focus much of my attention on the earlier stages of protein purification. More than column chromatography, pre-column steps are highly diverse and highly creative. Here the artistic aspects of protein purification are most apparent. But, still, there are basic guiding principles that can be communicated fairly effectively in written form. The purpose of this chapter is to outline many of these principles and techniques such that a relatively inexperienced biochemist can get started. Getting started is never easy. Inertia always seems to get in the way. When I think of the problem of overcoming inertia, I am reminded of the words of my first graduate school mentor. He chose to explain overcoming inertia with a metaphor based upon physical chemistry, "The function of education is to help others overcome their own energy barriers." In part, overcoming energy barriers is what I hope to accomplish in this chapter.

2. Protein purification in the analytical field

The words in my introductory paragraphs are more relevant to preparative techniques of protein purification than they are to analytical methods. Most of my research career has been focused upon preparative methods—the approach I liken to other art forms. Analytical

methods of protein purification are less likely to encompass the artistic range I ascribe to preparative methods.

The focus of analytical methods is usually to make a large number of precise measurements in a short period of time. One version of analytical methodology used extensively in the biopharmaceutical industry is called high throughput screening (HTS). Most commonly, HTS is used in drug screening. But HTS and other high throughput methods are applicable to analytical protein purification as well. But, as HTS is, by its very definition, a very rapid process, extensive protein purification is not possible by this method. Complex, multistep processes are almost always precluded. To meet time demands, just one simple and rapid purification step may be all that is permitted. Often this means that fast “sample cleanup” is the major goal of analytical processes. This “cleanup” may require nothing more than the removal of a particular interfering substance—an endogenous enzyme inhibitor, for example. External effector molecules may give falsely high assay values, or, more commonly, may inhibit enzyme activity, lowering an assay value, significantly. If, for example, one has a large number of relatively impure samples for which accurate values of the glucose oxidase activity is needed, it may be necessary to separate all other oxidoreductases from glucose oxidase. Alternatively, it may be sufficient to remove all endogenous sources of glucose. These types of separations are done routinely in clinical, medical, and pharmaceutical diagnostics laboratories. Sometimes, microliter samples are robotically introduced into small HPLC (high performance liquid chromatography) columns followed by on-line analysis of the protein of interest. On other occasions, machine-processed samples are introduced robotically into multi-well microtitre plates. Then, built-in robotic components introduce enzyme substrates and cofactors as the plates are stacked up by thousands to be measured after a precise incubation period.

In such analytical operations, the art is in the design of robust sample handling methods including electronic, mechanical, and robotic components. Optimization of protein separation may be an integral part of system design, but once the entire system is on-line, only routine validation tests along with periodic trouble-shooting of the overall system are required. Once the creative aspects of system design have been completed, everything now devolves into system maintenance.

3. Preparative protein purification

General Strategy

The greatest differences between analytical-scale and preparative-scale protein purification processes are that preparative methods (1) usually involve much larger volumes of starting material, (2) generally take much longer to carry out (days, weeks, or months), (3) usually require a variety of different purification methods or techniques (sometimes repeated), and (4) almost always have, as the primary goal, achieving very high purity (rather than high throughput). Sometimes, the amount of desired protein is so small, and the amount of macromolecular contaminant is so high, that one needs to employ nearly every “trick of the trade” to achieve high purity. Imagine wanting to isolate milligrams of a precious protein from thousands of liters of crude jellyfish extract. Our research group has done this for almost 3 decades (Roth, 1985, Johnson and Shimomura, 1972, Blinks, *et. al.*, 1976, and others). Sometimes, purifying a protein to homogeneity, from such large volumes of highly viscous starting material, may involve separating one milligram of the protein-of-interest (POI) from

100 mg of initial total protein. This is called a 100-fold purification. In other cases the required purification factor may be on the order of 1000-fold or 10,000-fold. My most difficult purification project was to isolate microgram amounts of green-fluorescent protein (GFP) from the homogenates of whole sea pens. In this instance, not only was the GFP present at about 1 part in 100,000 of total protein, but the proteoglycan-derived viscosity in the crude extract was so great that a magnetic stir bar failed to rotate (Ward and Cormier, 1978). So the issues facing a scientist working on a difficult protein purification project are many. Among these issues are those shown in Table 1.

1	Choosing or developing a sensitive, reproducible, and selective assay for the protein-of-interest (POI).
2	Establishing conditions under which the POI is stable and biologically active.
3	Finding conditions under which the POI can be stored safely between steps.
4	Choosing the best biological starting material (natural source or recombinant).
5	Developing or choosing appropriate methods for gross extraction.
6	Decreasing viscosity of crude extracts and removing particulates from those extracts.
7	Reducing volume.
8	Finding the substrate(s), inhibitors, activators, allosteric effectors, etc., if the protein-of-interest is an enzyme.

Table 1. Early steps in designing protein purification strategies

Some very useful information can be acquired, unambiguously, if a small sample of pure protein can be obtained. A former professor of mine said to our group of graduate students, "Don't waste clean thinking on a dirty enzyme." It is so easy to make major errors if you try to over-analyze a crude sample. Acquiring a pure sample of the protein-of-interest may be difficult (if the specific purification methods have not been optimized). But, obtaining a small amount of pure protein can be very useful for future optimization of purification. Table 2 lists a few of the characteristics of a pure POI that can be used to design a more effective purification strategy. Unless the protein-of-interest is pure, data on its characteristics can be very misleading (Karkhanis and Cormier, 1971).

a	Solubility in water, salt solutions, organic solvents, etc.
b	Presence of isoforms or isoenzymes.
c	Molecular weight.
d	Degree of oligomerization (monomer, dimer, tetramer, aggregation, etc)
e	Isoelectric point.
f	Partial amino acid sequence (needed if the mRNA is to be found).
g	Post translational alterations (phosphorylation, glycosylation, blocked N-terminus, etc).
h	Amino acid analysis.
i	Relative hydrophobicity (as determined by HIC trials or ammonium sulfate precipitation).
j	Antibodies to the protein-of-interest
k	Essential cofactors, prosthetic groups, stabilizing agents, etc.

Table 2. Physical and chemical properties of a pure sample that may be needed to effectively design a purification strategy.

Where to Begin

It is difficult to suggest a logical order of steps leading to a successful protein purification project. Proteins are very different from each other (and so are the mixtures of other components in which the protein-of-interest is found). So there is no common approach. Perhaps the best way to introduce protein purification is by example. I will do this by showing some of the intimate details of how one protein, *Aequorea victoria* GFP, has been purified in our academic lab at Rutgers University (Roth, 1985, Ward and Swiatek, 2009). In parallel, I will discuss the similarities and differences that accompany purification of another protein, soybean hull peroxidase. The latter has been purified in our Rutgers spin-off, start-up company, Brighter Ideas, Inc. (Holman, C., manuscript in preparation, Ward, 2012). I will not discuss, in detail, purification methods employed with recombinant proteins. These methods are much simpler and much more straight-forward (requiring considerably less “art” once the molecular biology has been completed).

The Assay

Before a protein purification process can begin, there must be a way to identify the protein-of-interest (POI). The means for identification is called an assay. For enzymes, the assay is usually a measure of enzyme activity. For proteins with distinctive chromophores, spectroscopic measurements of the chromophore help to distinguish the POI from other proteins. Sometimes all that one knows about the protein-of-interest is its molecular weight. In such cases the POI can be followed by SDS gel electrophoresis. Sometimes a protein is assayed by its immune response. Sometimes immune response is all that the scientist knows in the beginning. The protein, calmodulin, was discovered in brain tissue solely on the basis of its ability to bind radioactive calcium (Cheung, 1971). Binding calcium was all that was known about calmodulin in the earliest stages of its purification. But, the more one knows about alternate ways to detect the protein of interest, the easier the chore is likely to be.

GFP is not an enzyme, so there is no enzymatic assay. But, it has a spectroscopically measurable, covalently-bound chromophore (Fig. 1) that absorbs light maximally at 397 nm (Ward, 2005). GFP fluoresces brilliantly (emission peak at 509 nm) when excited in the UV. A hand-held, 365 nm, mercury vapor lamp (“black light”) becomes a convenient, portable detector. Molar extinction coefficient at 397 is 27,300, but that value varies 5-10% depending upon the degree of dimerization of the protein (Ward, 2005, Ward, *et. al.*, 1982). Fluorescence quantum yield is 80%. With all proteins, measurements by absorbance or fluorescence requires samples with VERY low turbidity (light scatter). Even partially clarified crude extracts have far too much scatter to measure any protein accurately by UV/Vis spectrophotometry (Fig. 2). Sometimes it takes a few purification steps before the level of GFP, for example, can be measured with any reliability.

Soybean peroxidase (SBP), like GFP, has a chromophore—a heme group that absorbs maximally at 403 nm. Absorbance at this wavelength can be used to quantitate the enzyme. But many other substances in crude soybean hull extracts absorb strongly at the same wavelength. So, the enzyme needs to be highly purified before this measurement is useful. Another assay is needed. Peroxidases, in general, bind to hydrogen peroxide, creating an active oxygen species that can then attack another molecule. In our case, the other molecule is ABTS (2,2'-azino-bis(3-ethylbenzthiazolene-6-sulfonic acid) available from the Sigma Chemical Co. ABTS, dissolved in a pH 5 buffer with added hydrogen peroxide, has only a

very slight visible absorbance. But in the presence of peroxidase, the active oxygen attacks the ABTS producing a teal colored solution. As with many other colorimetric assays, attention must be paid to the stability of the assay solution and the kinetics of the reaction.

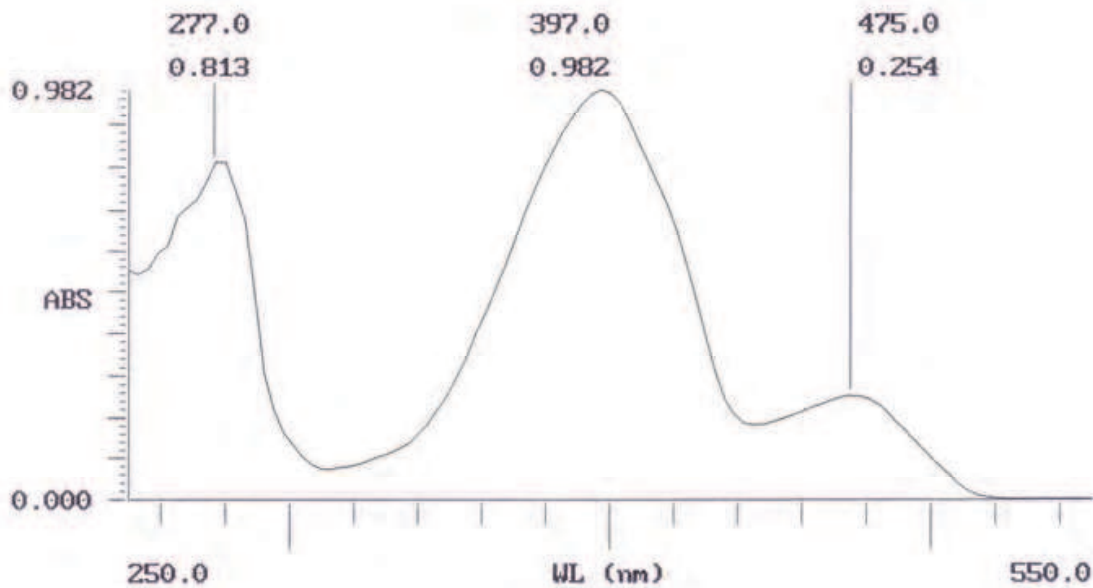


Fig. 1. Absorption spectrum of pure green fluorescent protein. Optical density vs. wavelength in nanometers.

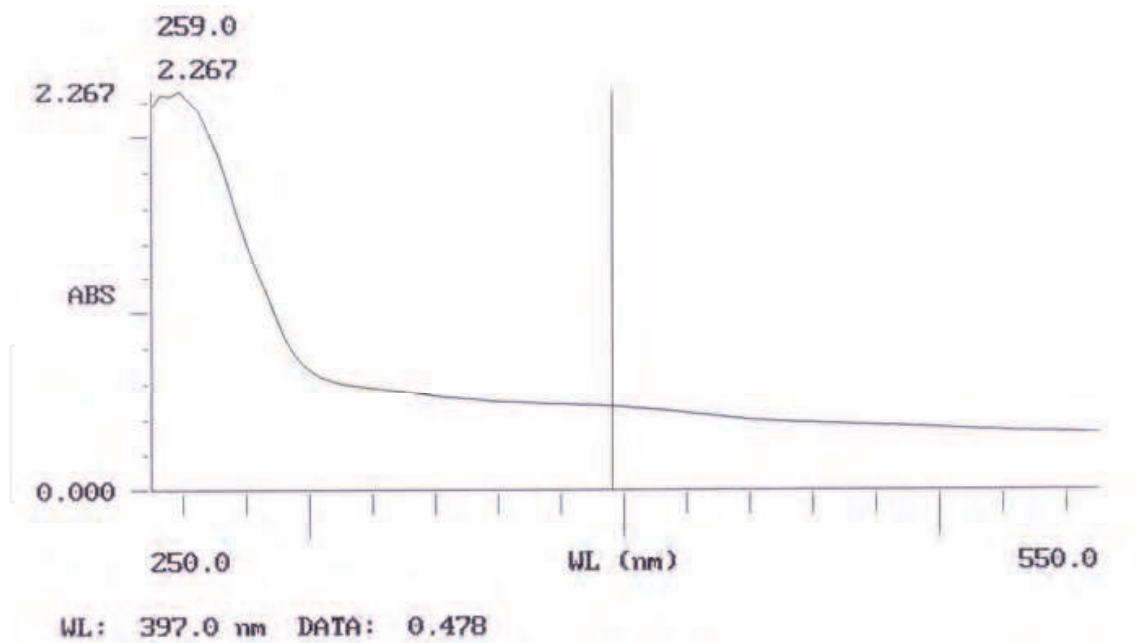


Fig. 2. Spectrum of diluted crude *E. coli* suspension. Apparent optical density (mostly scatter artifact) vs. wavelength in nanometers.

Stability

Probably the second most important characteristic for an effective protein purification scheme is the protein's stability, especially stability to heat and pH. But, just determining

conditions of high stability at the outset of purification is seldom sufficient. Some proteins are more stable in the crude form and others more stable when pure. So, at each step along the way, stability needs to be checked.

GFP and SBP are both thermally stable, up to 65 C for GFP (Bokman and Ward, 1981, Ward and Bokman, 1982) and nearly 90 C for SBP (Holman, C, manuscript in preparation). GFP is stable to proteases and aqueous alcohol solutions (Roth, 1985). The C-terminal 8 amino acid tail of native jellyfish GFP is protease labile, so we usually keep the crude extracts cold. We use sodium azide to inhibit microbial growth and phenylmethyl sulfonyl fluoride (PMSF) to inhibit the activity of serine proteases (Ward, 2005). Circular dichroism measurements confirm that the native secondary structure of GFP (predominantly beta pleated sheet and just a small amount of alpha helix) is directly proportional to the protein's fluorescence (Ward, *et. al.*, 1982). GFP retains its fluorescence and its secondary and tertiary structure at elevated pH (up to 12.2) but loses fluorescence at pH 12.3 (and simultaneously loses its CD signature) (Ward, 2005). Under acidic conditions (pH 6 and below) GFP fluorescence also fades as does the CD signal. Under the right conditions, GFP will recover most of its fluorescence after denaturation in acid, base, and guanidine hydrochloride (Bokman and Ward, 1981). The only known detergent to destroy GFP fluorescence, permanently, is sodium dodecyl sulfate (SDS).

Soybean peroxidase is stable over a wider range of pH and a wider range of temperature than GFP. But, its activity is inhibited by sodium azide and other agents that react with heme proteins. Instead of sodium azide, we use 10 % ethanol as a preservative for SBP. However, not all enzymes are stable in the presence of alcohol.

Storage Conditions

It is usually necessary, in multi-step purification protocols, to store the POI between steps. Generally, this is accomplished by freezing the protein solution. Freezing and cold storage work for both GFP and SBP, but not for all proteins. Some multisubunit proteins are cold labile. In such cases, the subunits are held together by hydrophobic interactions. Such hydrophobic bonding can be entropy driven, as structured water (surrounding the monomers in an ordered way) becomes released (and more disordered) when subunits bind to each other. The ΔS term in the equation: $\Delta G = \Delta H - T\Delta S$ increases with increasing temperature. GFP, SBP, and most monomeric proteins, are not cold sensitive. In addition, based upon its long-term retention of fluorescence, GFP appears to be stable for months at room temperature (Roth, 1985). But, isoelectric focusing of GFP may show extensive microheterogeneity after prolonged room temperature storage. The highly protease-sensitive eight amino acid C-terminal segment of native jellyfish GFP, (that extends from a protease-resistant beta barrel) is easily clipped by proteases – often in different places (Roth, 1985). When the recombinant protein is C-terminally tagged with hexa-histidine (for eventual immobilized metal affinity chromatography (IMAC), now both the naturally occurring octapeptide and the added hexapeptide are susceptible to cleavage at many sites by a variety of proteases.

Starting Material

In some cases, one has a choice of starting material. Luciferase, for example, can be isolated from a variety of fireflies and beetles. But, some firefly luciferases are very hard to purify while others are much easier. The sea pansy, *Renilla reniformis* (Wampler, *et. al.*, 1971,

Matthews, *et. al.*, 1977, Prendergast and Mann, 1978, Ward and Cormier, 1979) and the jellyfish, *Aequoria victoria*, (Morise, *et. al.*, 1974, Roth, 1985, Ward, 2005) were chosen as the starting materials for isolating and purifying GFP. In part, the selection of organisms was based upon their geographical locations, the availability of nearby laboratory facilities, and the means for collecting the animals. The shallow waters off the coast of Georgia proved to be a good location for collecting sea pansies and there was a local shrimper only too willing to do the collecting before the shrimp season began. The University of Georgia had a primitive laboratory on Sapelo Island, but early stage processing did not require sophisticated facilities. *Aequorea* jellyfish were abundant for decades at the University of Washington's Friday Harbor Labs (FHL) and the lab facilities were excellent. Excellent facilities were essential, as extensive floating docks were needed to provide close access to the water (so that the jellyfish could be scooped up with pool skimming nets). Processing involved holding the jellyfish (sometimes 10,000 per collection day) in large, circulating sea water aquaria. The FHL facilities include many circulating sea water aquaria, a walk-in coldroom, and a Sorvall centrifuge for further sample processing. The FHL staff was particularly supportive and encouraging.

While peroxidases can be isolated from many sources including horseradish, potatoes, sweet potatoes, and other plants, we chose soybean hulls as our starting material. The choice was based primarily upon easy access and low price. Perdue Farms processes huge quantities of soybeans for chicken feed. The hulls, a byproduct of their processing the more valuable soybean oil and soybean meal, are usually shipped to multi-grain bread manufacturers. To reduce storage and shipping volume, the hulls are crushed, on the Perdue site, into finer particles ranging down to the micrometer range. The bread producers apparently pay very little for an otherwise "throw-away" byproduct of the soybean. We, for example, ordered 2000 lbs of hulls, paying \$400 for hulls. The price included seven 55-gal barrels plus shipping. While access, ease of acquisition, and facilities were more than adequate for early, on-site processing of sea pansies, jellyfish, and soybean hulls, later laboratory processing was VERY demanding. This leads us into the next section, "Extraction".

Extraction

In the case of the sea pansy, extraction of GFP was accomplished by first anesthetizing the animals in a bath of the calcium-chelating agent EGTA plus magnesium sulfate. This was to preserve a luciferin binding protein, easily triggered to luminesce with calcium ions. Grinding the sea pansies with protein-saturating levels of ammonium sulfate came next, followed by acetone precipitation and rapid drying of the organic solvent. The powder that resulted, largely ammonium sulfate, was stored in chest freezers until processing time (Matthews, *et. al.*, Ward and Cormier, 1979).

GFP isolation from the jellyfish was entirely different. A single jellyfish has a volume of about 35 ml. On days when we collected 10,000 animals, the volume we needed to process reached 350 liters. However, all of the luminescent tissue is found in a very narrow strip along the margin of the "bell" (Fig. 3). Special dissecting tables were constructed, allowing a small team of workers to dissect up to 10,000 animals in one collecting day. Dissection reduced the volume to about 15 liters. Next, the tissue was shaken vigorously, 500 ml at a time, in 3 liters of sea water (in a 4-liter flask). Seventy-five shakes released most of the photocytes into suspension. After crude filtration, the photocyte suspension was trapped in a large cake of celite (diatomaceous earth) held in a large Buchner funnel. After a wash with

75% saturated ammonium sulfate solution (containing EDTA to chelate calcium), the photocytes were lysed with dilute EDTA solution. A gentle vacuum applied to the suction flask released an amazingly bright stream of fluorescence that was captured in the 4-liter vacuum flask. The extract was precipitated with solid ammonium sulfate—the precipitated protein being trapped on a smaller cake of celite or collected by centrifugation. These procedures were developed by Dr. John Blinks (Blinks, *et. al.*, 1976).



Fig. 3. Underwater photograph of the jellyfish *Aequorea victoria*. Photograph is courtesy of R. Shimek of the University of Washington's Friday Harbor Laboratories.

Soybean peroxidase extraction just requires that the pulverized hulls be stirred in five volumes of distilled water for one hour.

Viscosity Reduction and Particle Removal

As one might imagine, extracts of whole coelenterates or coelenterate tissues (jellyfish or sea pansies) present a huge problem with viscosity. Aside from water, the animals are almost entirely composed of connective tissue and very high molecular weight proteoglycans. For 17 seasons, we solved the viscosity problem by passing crude extracts of jellyfish photocytes (and surrounding tissues) through an 8-liter gel filtration column of P-100 BioGel (our next step after ammonium sulfate precipitation). The void volume fraction (calibrated to have a molecular weight of 40 million Daltons or greater) contained most of the viscosity and none of the GFP. But, while this 3-day procedure worked quite well as a viscosity reduction method, each gel filtration run could handle, one at a time, only 5% of a season's collection. Larger amounts of extract invariably fouled the column. If one includes the frequent column washes, required to maintain reasonable flow, it takes 5-6 months to pass a season's worth of jellyfish extract through the column. It was not without trying many alternative methods that we settled on this highly unusual first chromatography step (Fig 4). Gel filtration is generally reserved as a late-stage polishing step. Much later in our work, we discovered that simple passage through a column of Celite easily solved the viscosity problem (W. Ward, unpublished). Diatomaceous earth is so inexpensive that the column contents could be

discarded after the desired protein easily passed through. The above example illustrates one of the great dilemmas in selecting steps for a protein purification protocol. When do you decide that you have spent enough time searching for a better way to do things? When do you give up trying to search for a better procedure by settling on a brute force method? The expression, “Are you going to fish or cut bait?” seems appropriate here.

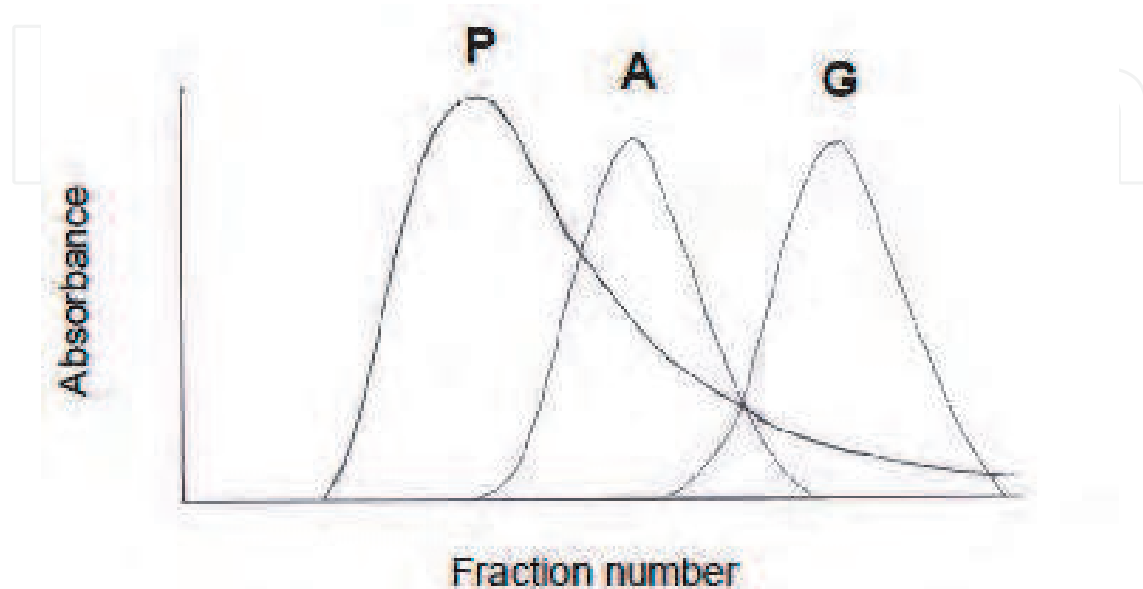


Fig. 4. P-100 Biogel profile of crude jellyfish extract. **P** marks the absorbance profile of total protein at 280nm. **A** marks the activity of Aequorin protein. **G** marks the GFP fluorescence.

Soybean peroxidase crude extracts are fairly low in viscosity, but the hull extracts present a very significant problem with particulates. The crude extracts include large particles (millimeter size) as well as tiny particles in the micrometer range—some as colloidal suspensions. Large fragments of hulls are easily filtered away with fine mesh nylon nets, but this leaves a very cloudy suspension of fine to very fine particles. Centrifugation has been ruled out because of the large volumes of extract produced and the high centrifugal forces needed to pellet the finest particles. Even continuous flow centrifugation trials have failed, repeatedly, because most of the particulates, including colloidal materials, have failed to sediment during the short interval of time it takes for liquid to traverse the centrifugation path. After trying everything we could imagine and after investing money in a variety of expensive filter devices (G. Swiatek and M. Browning, personal communication), we suspended this project for several years. Then we happened upon an ion exchange method normally applied to water purification. We found a company called ResinTech that provides, at very low cost, a high capacity polystyrene-based anion exchanger. The beads are large (1 mm) and dense, so, after stirring, they quickly settle to the bottom of a large container. Binding kinetics, however, are slow, because of the large size of the beads and relatively small pore size (access to the interior is slow and limited to proteins of MW 50 kdal or lower. So, notwithstanding the slow kinetics of binding and elution, these beads are useful for batch ion exchange applications—in our case, to trap the highly anionic soybean peroxidase (C. Holman, manuscript in progress, Ward, 2012). A provisional patent for our unique SBP purification method has been filed with Rutgers University. The fine particles of soybean hull extract (much too fine to settle on their own) are, however, too large to enter

the ResinTech pores. So the bound SBP can be separated from these fine particles. But, much to our surprise, we found that the fine particles, as soon as stirring ceases, immediately aggregate into a dense gelatinous mass that settles above the beads. By aspiration, this gelatinous mass is easily separated from the beads that now containing nearly all of the SBP.

Volume Reduction

In a typical academic or start-up corporate laboratory, the starting sample of crude protein might range in volume from a few milliliters to tens or hundreds of liters. In commercial operations, liquid volumes may reach thousands or hundreds of thousands of liters. Here, I focus on moderately large volumes that require much more effort than smaller volumes. The volume of starting sample dictates, in a sense, the methods that are appropriate for early stages of purification. Large aqueous volumes require an early stage trapping step—a step that eliminates large quantities of water while binding (or otherwise retaining) the protein-of-interest. The focus is not on separating a variety of macromolecules from each other. The focus is to reduce aqueous volume to a more reasonable level. Higher resolution methods can come later. Generic trapping can be accomplished by tangential flow ultrafiltration (Scopes, 1994), so long as the feed stock is not so viscous as to plug the membrane pores with large particles, colloidal materials, or slimy DNA or polysaccharides. Such membrane fouling will slow down (even halt, altogether) the trans-membrane penetration of water, salts, and small molecules.

Alternative methods include ion exchange or hydrophobic interaction. If ion exchange is chosen, the adsorbent should have relatively large particle size (several hundred micrometers to 1 millimeter in diameter). Large size ion exchange beads or fibers are preferable when trapping proteins from large volumes of dirty samples. It is advisable to save, for later, the higher resolution ion exchange materials, (such as positively charged DEAE Sepharose Fast Flow or negatively charged CM Sepharose Fast Flow—GE Healthcare). It is only after viscosity and the presence of particulates have been greatly reduced that high resolution ion exchangers can be expected to deliver superior flow with relatively little fouling. Crude starting materials are best processed in batch mode rather than by axial flow chromatography. Radial flow columns offer much greater surface area, but even these columns can clog if the feedstock has high viscosity (from DNA, polysaccharides, or lipid micelles). Turbid samples containing small particles or colloidal suspensions can be as troublesome as samples with high viscosity. Frequent stirring in batch mode overcomes this problem by allowing the POI to bind to the matrix, without the problems of column fouling. However, highly acidic DNA and sulfonated or carboxylated polysaccharides will also bind to anion exchange materials, such as DEAE. While batch adsorption to DEAE can work well, the viscosity problems may return if the POI and the highly acidic biopolymers come off the anion exchanger together. But, DNA and acidic polysaccharides generally bind to DEAE, or other anion exchangers, much more tightly than the POI. When this is the case, the desired protein will elute from the anion exchanger at much lower concentrations of aqueous salt solutions than the highly acidic biopolymers. DNA and anionic polysaccharides will remain bound to the anion exchange material, while the protein-of-interest elutes with greatly reduced viscosity.

Hydrophobic protein-binding materials, like Phenyl Sepharose (GE Healthcare), are excellent trapping agents for most proteins. This method is called hydrophobic interaction

(HIC). Just a few exposed hydrophobic amino acid R-groups are needed for binding to the phenyl group. The amino acids having R-groups that are strongly attracted to an HIC matrix include: phenylalanine, tyrosine, tryptophan, methionine, leucine, isoleucine, valine, proline, and lysine. It may be surprising that lysine is included as a very hydrophobic amino acid because lysine carries a positive charge at all pH values below 10. Hydrophobic interaction is not favored when charged residues are present. There is an exception when oppositely charged groups, within hydrophobic patches, are close enough to each other to bond electrostatically. Under these conditions, the electrostatic bond is exceedingly strong. Independent of electrostatic bonds, in which lysine could participate, the R-group of lysine is frequently exposed to the exterior (lysine has the greatest exposure of all amino acids, as its long string of methylene groups extends far into the aqueous medium). Hydrophobic interaction is not with the epsilon amine of lysine at the end of this string, but with the four methylene groups, themselves, to which the amine is attached. HIC and IEX media are available as very soft beads made of cross-linked dextran polymers or polyacrylamide, or they come in a more rigid form that is agarose-based. An agarose-based HIC medium, such as Phenyl Sepharose, is more pressure-tolerant and more robust than the older style, softer beads. Additionally, the agarose pores are larger, allowing very large proteins to enter the internal spaces. Despite the fact that some nucleic acids and some anionic polysaccharides could enter agarose beads, this does not happen with HIC media. In the case with ion exchange trapping chemistry, DNA and other acidic biopolymers may compete with, or displace, an anionic protein-of-interest. But, highly charged nucleic acids, as well as acidic and neutral polysaccharides, are not sufficiently hydrophobic to bind tightly to Phenyl Sepharose and related HIC materials. So, they easily separate from a protein-of-interest having a few exposed, hydrophobic amino acid side chains. On the downside, HIC, as a trapping step, can become very expensive if the volume of crude extract is large. HIC gels are expensive. There is an additional economic downside to HIC when large volumes must be processed. Highly purified ammonium sulfate is fairly expensive and the cost of disposal may be even higher. Many kilograms of ammonium sulfate may be required to trap proteins by HIC, especially if the protein of interest is fairly hydrophilic (highly water soluble). Proteins that are quite hydrophilic may require a very large amount ammonium sulfate to induce binding to the HIC resins.

For a protein that is very stable at its isoelectric point (pI), isoelectric precipitation can provide an excellent, inexpensive trapping step (Scopes, 1994). Almost always this method requires a very low salt concentration, as electrostatically-driven protein-protein interaction is the mechanism that promotes precipitation. The flocculated protein may settle to the bottom of the container. If not, it may be pelleted in a centrifuge or collected by simple filtration on beds Celite. Resolubilization is accomplished by raising or lowering the pH or by adding salt. For proteins that remain soluble at their pI values, addition of a water-soluble organic solvent (generally a small aliphatic alcohol) may be used to promote isoelectric precipitation. Addition of a somewhat non-polar solvent lowers the dielectric constant of water, promoting charge-charge interactions among protein molecules. If this does not work, lowering the pH below the protein pI with simple addition of acetic acid, phosphoric acid, or HCl may cause precipitation. Occasionally, one finds that diatomaceous earth, alone, will bind certain proteins quite selectively. Because Celite is so inexpensive (available in 50 lb bags at pool supply stores), it makes sense to try Celite as a trapping agent.

With native *Aequorea* GFP, we never encountered a huge volume reduction problem because the dissection step and the trapping of whole photocytes on Celite greatly reduced the volume. But, soybean peroxidase is a different matter. See the section: “Viscosity Reduction and Particle Removal.” We found that one volume of soybean hull powder requires 5 volumes of water for efficient extraction. For 2000 lbs. of hulls, the amount of water required for extraction has been determined to be 16,000 liters (G. Swiatek, personal communication). Even if scaled down to 20 lbs. of hulls per batch, 160 liters of water would be required. Volume reduction is accomplished very effectively by trapping the SBP on ResinTech anion exchange beads. When we compared binding capacity of ResinTech beads with that of DEAE Sepharose Fast Flow, both exchangers bound the same amount of pure GFP (38 mg of protein per milliliter of swollen gel). Binding capacity of ResinTech beads with larger proteins, such as rabbit IgG, is considerably lower, as the ResinTech pores are much smaller than those of DEAE Sepharose.

Chromatographic Methods

On Table 1 and Table 2 are shown the categories of basic information generally needed to facilitate early stages of protein purification. The properties of a POI that should be known are listed here in no particular order of importance. In fact, almost never is the order of information discovery the same for any two proteins. In the course of developing a start-to-finish protocol for any given protein, unexpected information is uncovered along the way. Long after developing a working protocol, one may discover, for example, that the POI is glycosylated. Following this discovery, one might want to experiment with affinity chromatography using an immobilized lectin or may wish to try a boronate column that binds vicinal hydroxyl groups on sugar residues (Scopes, 1994). The message is that no purification protocol is ever final. There are always alternate ways that could improve or streamline an earlier protocol. This is one of many places that the artistry of protein purification comes into play.

Ion Exchange Chromatography (IEX)

Once viscosity has been largely eliminated and once the crude protein sample is particle free, it may be time to use ion exchange chromatography (IEX)—the most frequently employed chromatographic method for proteins. Early, small-scale testing with a relatively salt-free sample is advised. There are simple, syringe-operated ion exchange columns available from Pall Corporation or GE Healthcare—both anion exchange columns and cation exchange columns. These columns can be used to determine (within one-half of a pH unit) the isoelectric point of the protein. This is accomplished by equilibrating the two columns with low ionic strength buffers of varying pH values. The most common cation exchange functional group is carboxymethyl, abbreviated CM. CM is essentially immobilized acetic acid and, like acetic acid, CM takes on a negative charge at pH values of 4 and above. CM is designated a weak cation exchanger as it has little binding capacity below pH 4. Sulfonated or phosphorylated exchangers are called strong cation exchangers because they can be used at pH 2. For the POI to bind to CM, the protein must be positively charged (below its isoelectric point). CM is not satisfactory for GFP purification as GFP is unstable below its pI of 5.3. When GFP takes on a positive charge (below pH 5.3) the protein slowly denatures, losing its fluorescence. So, it is not possible to use CM with GFP in any slow process like column chromatography. But, if GFP exposure time is kept at a minimum, the pI of GFP can

be estimated by its binding to CM at pH's below 5.3. Diethylaminoethyl (DEAE) is the most commonly used anion exchanger. The DEAE functional group is a tertiary amine, protonated (and positively charged) at pH values below 10. DEAE is designated a weak anion exchanger as it cannot be used effectively above pH 10. But, a bead-bound quarternary amine extends the range of anion exchange to pH 12. So any medium designated Q (or QAE, for quarternary amino ethyl) is called a strong anion exchanger. All four of these types of these media (weak and strong cation exchangers and weak and strong anion exchangers) are available in small, syringe-operated columns. If one of these DEAE columns is equilibrated at a variety of pH values (10, 9, 8, 7, 6, 5, and 4), GFP will bind from pH 10 to pH 5.5, but not at pH 5, indicating that the pI of GFP is below 5.5.

Once the pI has been determined and the anion exchanger has been chosen, a preparative column can now be poured. Most ion exchangers can bind 30 to 50 mg of protein per 1 ml of swollen gel. One can estimate the total amount of protein in the sample by absorbance at 280 nm, ascribing one absorbance unit to one mg/ml of protein. But, high levels of DNA and moderate turbidity (Fig. 2) will artificially elevate this absorbance number (sometimes greatly). It is good practice to test, experimentally, the capacity of an ion exchange material in a small trial. Using 1 ml of swollen gel, add crude extract in successive 100 microliter volumes until the gel becomes saturated with protein. The saturation limit can be determined by taking POI activity measurements after each incremental addition of extract. For enzymatic measurement, remove just a few microliters of the supernatant after the gel settles (so the aqueous volume remains about the same). When the activity appears in the supernatant, you will have determined the saturation point in terms of mg of extract per ml of gel. Now fill a chromatography column with at least 5-times as much gel as your preliminary testing indicates you will need for total binding. Short, stout columns are usually better than long thin ones. Resolution comes not from column dimensions, but from the rate at which the eluting strength of the salt (usually sodium chloride) is raised in the elution phase. Take note of the fact that an ion exchanger is an excellent buffer, so pH equilibration of the gel requires many column volumes of dilute buffer solution. Alternatively, a very high concentration of buffer may be used to titrate the column, first. But, after titration, at least one column volume of the dilute (low ionic strength) buffer must be passed through the column. It is also necessary to use a buffering salt that has the same charge as the ion exchange gel. When using positively charged DEAE columns, positively charged Tris(hydroxymethyl aminomethane) buffer in the chloride form (generally abbreviated as Tris) is commonly used. For negatively charged CM, negatively charged sodium phosphate buffers are recommended. The protein of interest should be equilibrated in the same dilute buffer. For best resolution, a shallow, continuous gradient (50 column volumes or greater) from 0.0 M NaCl to 0.5 M NaCl is recommended. To achieve near base line resolution of 5 GFP isoforms (differing from each other by one or two amino acids), I have eluted a 100 ml DEAE column with 80 column volumes (8 liters) of sodium chloride solution from 0.05 to 0.25 M (Ward, 2009). In this case (and in all other cases) the salt solutions need to be prepared in the same buffer used to equilibrate the column.

Hydrophobic Interaction Chromatography (HIC)

HIC media are available in several strengths. The hydrophobic ligands are usually attached to the porous hydrophilic gels via a 3-carbon spacer based on epichlorohydrin

chemistry (Scopes, 1994). From strongest binding to weakest binding ligands, the order is Phenyl > Octyl > Butyl > Methyl. Strongly hydrophobic ligands are appropriate for weakly hydrophobic proteins and weakly hydrophobic ligands for strongly hydrophobic proteins. Early testing, calculation of gel volume, and choice of column dimensions are carried out in a similar fashion as the protocols used for ion exchange. Hydrophobic binding is favored by very high salt concentration (up to 3 molar ammonium sulfate, in some cases). Elution is accomplished by lowering the salt concentration in increments (step gradient) or by applying a continuous linear gradient of decreasing salt concentration. Be aware that gradients of ammonium sulfate produce gradients of refractive index, easily confused by a spectrophotometer as a higher UV absorbance value or a lower UV absorbance value. If precise 280 nm absorbance measurements are desired following gradient elution of proteins from an HIC column, it is necessary to have a continuously changing blank that closely matches the salt concentrations of the samples. An advantage to having HIC follow IEX is that one need not remove the NaCl in the fractions eluted from the IEX column. NaCl neither favors nor inhibits hydrophobic interaction nor does it interfere with spectroscopic measurements as much as ammonium sulfate. If the two steps are reversed, ammonium sulfate must be removed entirely before going on to IEX.

Affinity Chromatography

Some prefer to use affinity chromatography very early in a protein purification process – as a “one-step purification method” (Scopes, 1994). I use quotation marks because, despite frequent claims, affinity chromatography is seldom a one-step method. Often contaminants remain in affinity-purified proteins. Commonly, those contaminants are large protein aggregates that result from the almost inevitable leaching of “bound” ligand. That released ligand then forms a high molecular weight complex with the protein-of-interest. When we purify ‘anti-GFP’ antibodies on an immobilized GFP affinity column we almost always detect, by SEC-HPLC, a high molecular weight aggregate that is distinctly fluorescent, suggesting that an antigen(GFP)-antibody complex has formed. Because most affinity columns are quite expensive and could be plugged by crude starting samples, I prefer to use affinity chromatography late in a protocol. The principle is easy. Take for example, that a ligand, recognized by an enzyme, is covalently bound to the matrix (usually agarose). That ligand may be a pseudo-substrate, a cofactor, an inhibitor, or an antibody. Binding is easy, but elution may be difficult. It is preferable to use, as the eluting solvent, a solution containing a competing ligand (the pseudo-substrate, cofactor, inhibitor, or antibody). But, sometimes the competing ligand is very expensive, unavailable, or irreversibly bound to the enzyme. In such cases, other eluting solvents must be used. Dilute solutions of ethylene glycol in buffer are sometimes used. So are buffers of low pH, a variety of salts, metal chelators, etc. Many other forms of affinity chromatography exist. We purify anti-GFP antibodies on a column to which GFP is covalently immobilized. We normally elute with a concentrated pH 3.0 solution of sodium citrate. The pH 3 buffer temporarily denatures both the antibody and the GFP. Both column-bound GFP and the eluted antibody are rapidly renatured with a strong pH 8 buffer. Based upon analytical techniques (including size exclusion (SEC), HPLC, SDS gel electrophoresis, UV absorption spectroscopy and western blotting) purity of GFP-specific antibody can approach 99% (see Fig 5. a, b, c, d, and e).

However, if purity greater than 99% is desired, affinity chromatography requires a follow-up step. Most commonly we use preparative gel filtration to remove protein aggregates that may form when a small quantity of bound ligand leaches from the column.

For recombinant proteins, the favorite affinity column is an immobilized (chelated) metal ion column (abbreviated IMAC for immobilized metal ion affinity chromatography) (Scopes, 1994). In IMAC columns, nickel ions or cobalt ions are bound to the column in a chelation complex. The column-bound chelator is usually nitrilotriacetic acid. The metal ion, chelated to the IMAC column, can be co-chelated, non-specifically, by the R-groups of histidine, cysteine, and tryptophan. Binding may occur if one or more of these amino acids are exposed on the surface of the protein-of-interest (or any protein contaminant in the mixture). Almost universally, recombinant proteins that are subjected to generic affinity chromatography are processed by IMAC. But to achieve specificity (and tight binding), the recombinant proteins are genetically modified by the addition of a string of 6 histidine residues, sometimes on the C-terminus, sometimes on the N-terminus, and sometimes within exposed loop regions. The string of 6 histidines (the HIS-tag) is a strong co-chelator and the tag is sufficiently exposed that the His-tag almost always out-competes any naturally occurring co-chelators found in high abundance on the surface of a protein contaminant. The method is carried out at pH of 8 or higher and it must be performed in the absence of other metal chelators such as EDTA, citrate, oxalate, ammonium ion, etc. Concentrated solutions of imidazole are usually used for elution.

In my experience, all affinity chromatography columns, each time they are used, leach a bit of their covalently bound ligand, often as high molecular weight complexes with the POI. That ligand winds up in the fractions that have eluted from the column. So, in every case in which IMAC is used, it is wise to follow this step with a gel filtration run.

Gel Filtration Chromatography

Low pressure gel filtration is the easiest chromatographic method in principle, but it is the hardest method to administer properly. Because gel filtration seems so straight forward, liberties are sometimes taken in utilizing the method. For best results, attention to detail is essential. Gel filtration (or size exclusion as the method is called in HPLC) separates macromolecules by size. Size exclusion chromatography (SEC) is generally used as an analytical HPLC method while gel filtration is used primarily in preparative protein separations. Size exclusion HPLC utilizes small, rigid, uniform, spherical beads of 5 micrometer or 10 micrometer diameter. Small, porous, silica beads used in SEC provide higher resolution than low pressure gel filtration. But, the price per ml of HPLC column packing material is much higher than that of any soft gel used in low pressure applications. For further discussion of HPLC, refer to the HPLC section later in this chapter.

Low pressure gels are comprised of small (20 to 300 micrometers) porous beads which, unlike Fast Flow adsorption beads, have blind cul-de-sacs that provide differential flow paths through the column. The largest molecules are unable to enter any pores, so they must travel around the beads. This means that large molecules exit first while smaller molecules spend some time inside the beads, so they exit later. The volume in which the very large molecules exit (DNA, proteoglycans, ribosomes, lipid micelles, and protein complexes) is called the void volume. The void volume, usually 25% of the total column volume, is often

measured by the elution position of Blue Dextran (GE Healthcare), a covalently-dyed sugar polymer having a molecular weight of 2 million Daltons. So, if the column volume ($\pi r^2 h$) is 200 cubic centimeters (200 ml), the center of the Blue Dextran-calibrated void volume peak will appear close to the 50 ml mark. The next 25% of the column volume (the second 50 ml in this example) is the resolving zone, accessible to moderate size proteins. The final 50% of the column volume (100 ml) is the zone in which peptides, very small proteins, oligonucleotides, other small molecules and salt ions will elute. The total liquid volume in the column (salt volume) is accepted as being either the total volume of the column, as calculated from $\pi r^2 h$, or it is the volume measured by adding a measurable salt to the applied sample. The salt can be sodium chloride, detectable by conductivity, or sodium nitrite, detected by its fairly strong absorbance at 280 nm.

Gel filtration is, intrinsically, a low resolution separation method for proteins, yet it is frequently used in protein purification. Gel filtration is gentle to the sample and it is the best preparative method for fractionating native proteins by size and shape. Passage through the partially accessible pores in the beads will generate broad elution bands, each band lying within just 25 percent of the total column volume, thus the intrinsically low resolution of the method. Generally, the highest resolving columns, containing very small beads of soft gel materials, like Sephadex G-100 Superfine (GE Healthcare) or BioGel P-100 minus 400 mesh (BioRad Laboratories), operate under low gravitational force fields (50 cm pressure head, or smaller). Beads used for relatively large proteins must have low degrees of cross-linking, making the gels soft and highly compressible. For the most compressible beads (G-200 Sephadex, for example), pressure heads may need to be as small as 15 cm. In general, gel filtration columns are able to give baseline resolution for no more than 4 proteins, each differing in molecular weight by a factor of 2. So, under the best of conditions, a mixture of globular proteins of MW 200,000, 100,000, 50,000, and 25,000 Daltons can be baseline resolved.

Listed in Table 3 is a set of “best conditions,” – those that give maximum resolution by gel filtration.

1	Sample volume divided by column volume must be in the range of 1-2 %.
2	Sample must be applied very carefully to avoid channeling.
3	Beads must be very small (20-50 micron size range).
4	Flow rate must be very low (<2 ml/cm ² per hour), a rate which requires >47 hours for 121 cm X 1 cm columns.
5	The biological sample applied to the column must be low in viscosity.
6	At least 100 fractions should be collected, preferably in the protein resolving zone.
7	The pressure must be low, so as not to collapse the beads.

Table 3. Best conditions for maximum resolution in gel filtration

Physical Set-ups in Column Chromatography

Those new to column chromatography often ask, “What size column should I use and what are the most appropriate dimensions of length and width”? Clearly there is no one correct answer. But there are some appropriate generalities that can help with column selection in adsorption chromatography. Adsorption chromatography includes ion exchange,

hydrophobic interaction, affinity chromatography, and all other forms of chromatography in which the analyte binds to the stationary phase (all methods other than gel filtration and SEC). Protein resolution in adsorption chromatography depends upon the rate of change of the eluting solvent, not upon the length or width of the column. Better resolution results from gradual change in the strength of eluting solvent. The limit of "slow rate of change" is no change at all. In chromatography, we call "no change at all" isocratic elution. Isocratic elution, at the right solvent concentration, generates the highest possible resolution, but peak spreading will be greater in isocratic elution than in gradient elution. In general, the amount of adsorbent in a column should have the capacity to bind three-to-five-times the amount of protein being loaded. The length and width of the column are not critical. It is not unreasonable to use a short, stout column for adsorption chromatography – a column with length 2- to 3-times the column diameter, for example. Such columns allow very high flow rates, so a large volume of eluent can be used in a fairly short period of time. But, one should not greatly extend column width at the expense of length (eg. dimensions of a cake pan are problematic). A wide diameter column, where the eluent exits through a port at the center of the cylinder, provides early elution of protein that happen to migrate down the center of the column. Protein (of the same type) that migrates near the circumference of the column will exit significantly later. This differential elution (side vs center of the cylinder) produces smearing of a band that might otherwise be sharper (if the column had more "normal" dimensions). An exceedingly long and thin column is not desirable either. Flow rates will be very slow, especially if the gel is soft. If, in an attempt to speed up flow, pressure is increased, the gel may compress and flow will slow down. In extreme cases, flow may stop altogether. Even if the adsorbent is rigid and non-compressible (as in size exclusion HPLC) a column with a small cross-sectional area may over-pressure if particles collect on the surface. It is common to use long, narrow columns for gel filtration, but here as well, columns need not have such extreme dimensions. The problem of particles collecting on the surface of the column may still occur. But even if the sample is particle free, flow rate may be much slower than desired. A 50 cm column, with a diameter of 2.5 cm, can give excellent resolution in gel filtration as well as in adsorption chromatography. But, to achieve maximum resolution in gel filtration (in columns of such dimensions), the conditions listed in Table 3 must still be observed.

HPLC

The term HPLC stands for high performance liquid chromatography. Those with limited budgets prefer to substitute the word, "price," for "performance." Some use the word, "pressure." But, pressure is not what distinguishes HPLC from other forms of column chromatography. The fundamental difference between HPLC and columns containing relatively soft gels (Sephadex, BioGel, agarose, cellulose, etc.), is that the beads in HPLC columns are considerably smaller. HPLC beads are usually 5 micrometers in diameter. Columns with such small beads will not flow by gravitational pressure, nor will they flow with the pressure generated by a peristaltic pump. So, as a consequence of small beads, mechanical pumps capable of pressures as high as 7000 psi are needed. But, in practice, pressures greater than 2000 psi are seldom used. Even pressures of 2000 psi require very strong columns, usually of stainless steel. Tubing down-stream from the pump must also tolerate very high pressures. Very rigid gels are required or the beads will collapse under

the high pressures generated in HPLC. The most common of the rigid HPLC beads are made of porous silica.

More than 90% of HPLC columns in use are reverse phase columns (RPC). Reverse phase media is made of porous silica, but is functionally similar to low pressure hydrophobic interaction media made from soft gels. The greatest difference between RPC and HIC (other than tolerance for high pressure) is that reverse phase beads are much more hydrophobic than HIC beads. RPC beads have long aliphatic chains or aromatic groups bonded to the silicon dioxide media. The relative hydrophobicity of RPC columns is related to both carbon chain length and the carbon load. Carbon load (that can reach 20%) reflects the density of hydrocarbon substitution on the silica beads. The reported percentage is the ratio of the weight of bound hydrocarbon to the weight of silica. So, not only are the bonded phase hydrocarbons more hydrophobic than the ligands in HIC, but the density of hydrophobic ligands is also greater in RPC. The name "reverse phase" comes from the fact that the polarity of the mobile phase (the solvent) and that of the stationary phase (the beads) have been reversed. The original silica-based columns used unmodified silica that is polar and highly charged. So, this "normal phase" chromatographic method used a polar stationary phase and a non-polar mobile phase. RPC reverses the phases.

Typically, samples are introduced into an HPLC column through an injection valve that maintains atmospheric pressure on the outside and high pressure down-stream. So, a standard syringe can be used to load a sample while solvent continues flowing at very high pressure. RPC is more appropriate for small polar molecules (amino acids, peptides, oligonucleotides and polar lipids) than for native proteins. Most proteins bind too strongly and may bind irreversibly or become denatured.

Batch Methods for Protein Purification

Occasionally one finds a batch method that works as well in purifying a particular protein as a variety of chromatographic methods. Batch methods are particularly useful in early stages when a sample is highly viscous or full of fine colloidal material. Such batch methods include ammonium sulfate precipitation, precipitation from other salt solutions, from aqueous solutions at low pH, or from organic solvents (usually acetone, ethanol, ethylene glycol, or polyethylene glycol). In some cases, recrystallization from salt solutions is possible. Even if crystals do not form, differential precipitation can be an effective purification method. A particularly effective batch method is isoelectric precipitation in which the pH of a dilute aqueous buffer is adjusted to the isoelectric point (pI) of the POI. The protein-of-interest, or contaminants in the POI mixture, can be adsorbed to Celite, alumina gels, calcium phosphate gels (hydroxyapatite), and other media. If antibodies are available, the protein of interest can be selectively bound to those antibodies. If aggregates form upon such treatments, the aggregate can be collected by centrifugation and then dissociated into free antibody and free POI by a variety of methods including application of low pH buffers.

A-Free IgG

Recently, I have been exploring, with repeated rounds of ammonium sulfate precipitation, the purification of rabbit-derived antibodies, goat anti-rabbit IgG, and chicken IgY. Because

this process does not utilize Protein-A, I call the method “A-Free.” For rabbit-derived antibodies, the “A-free IgG” procedure works at least as well as chromatography on columns of Protein-A. Goat-derived antibodies, that are not as amenable to purification on Protein-A columns, and chicken-derived IgY, that cannot be purified on Protein-A at all, respond equally well to the “A-Free” method. Although very commonly used in purifying therapeutic monoclonal antibodies, Protein-A is quite expensive. Despite its being covalently bound to the affinity column matrix, Protein-A is able to leach from the column matrix during the elution phase. Traces of Protein-A in therapeutic monoclonals could present a health hazard, as Protein-A may bind to other essential antibodies in the patient. We have not found formal regulations limiting the use of Protein-A in purifying therapeutic monoclonals, but manufacturers might prefer a safer, more cost-effective method. We have a satisfactory replacement for Protein-A in the method we call “A-Free IgG.” This method has been submitted, through Rutgers University, as a provisional patent application.

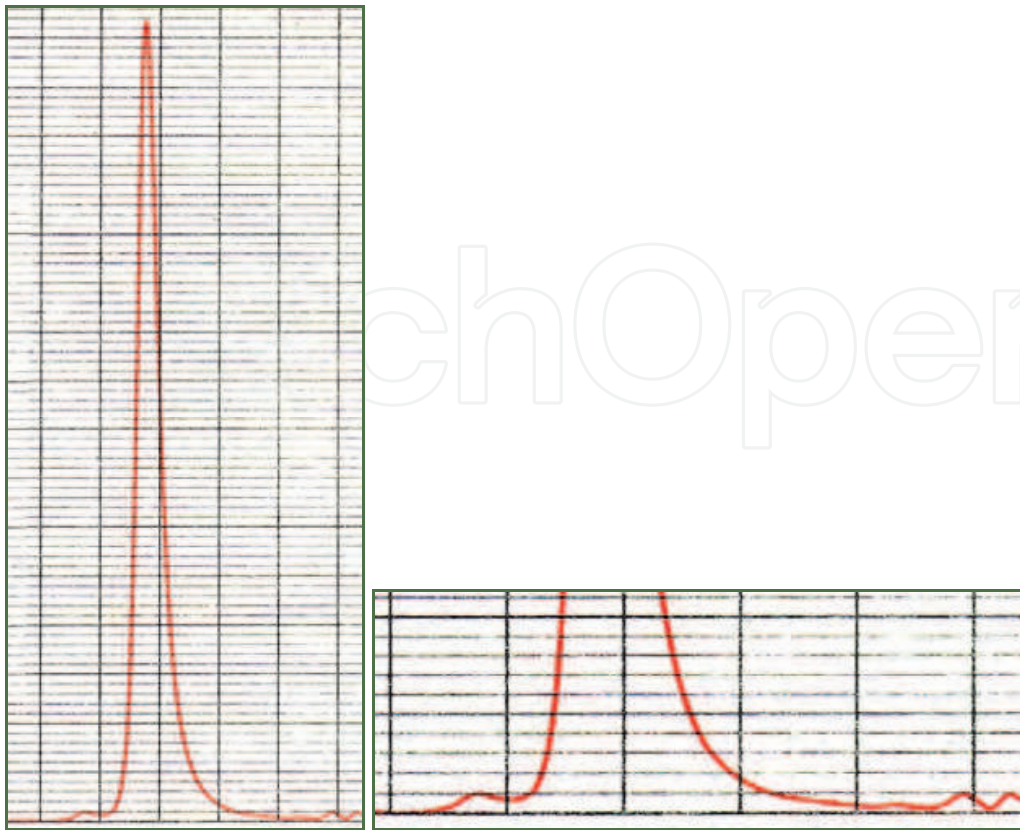
As often occurs in experimental science, the “A-Free IgG” method of antibody purification arose from an accident. I am primarily a bench scientist. But, with all the other things I must do, I get too little bench time to satisfy my urges to discover and create. As a consequence, when I have a bit of research time, I tend to rush through projects, sometime binging well into the night. Often this means cutting corners to save time. Such was the case with developing the “A-Free IgG” method – an accident created by my hasty experimentation.

In the course of purifying IgG from rabbit serum by a traditional single round of ammonium sulfate fractionation, I made a mistake that was picked up by size exclusion HPLC. The SEC profile showed more contaminants than I had seen previously. So, to remove those additional contaminants, I repeated the entire process. To my surprise, the second round of ammonium sulfate precipitation produced a cleaner IgG sample than I had previously seen with just one round of precipitation. But the redissolved pellet was still slightly pink (not all the transferrin was removed), and the HPLC profile still showed a tiny shoulder of albumin. So, I did the same precipitation process a third time. This time, the HPLC profile showed 99% pure IgG – virtually no high molecular weight contaminant and no indication of any albumin (Fig. 5 a, b, c, and d). The SDS gel profile showed strongly stained heavy chain and more weakly stained light chain (normal for IgG) and a very weakly staining contaminant or two (Fig. 5 e). These side-by-side experiments show that the “A-Free IgG” method actually out-performs Protein-A affinity chromatography. The time involvement is similar and the price is much lower. On occasion we perform a 4th ammonium sulfate precipitation, obtaining a sample marginally cleaner than that resulting from three rounds of precipitation.

The method works equally well with goat anti-rabbit IgG, an antibody less amenable to Protein-A purification. We have a large supply of chicken egg yolk containing anti-GFP antibodies (IgY) for which Protein-A is totally ineffective. The A-Free method is suitable with IgY so long as the large amount of lipid has been removed by a freeze-thaw method.

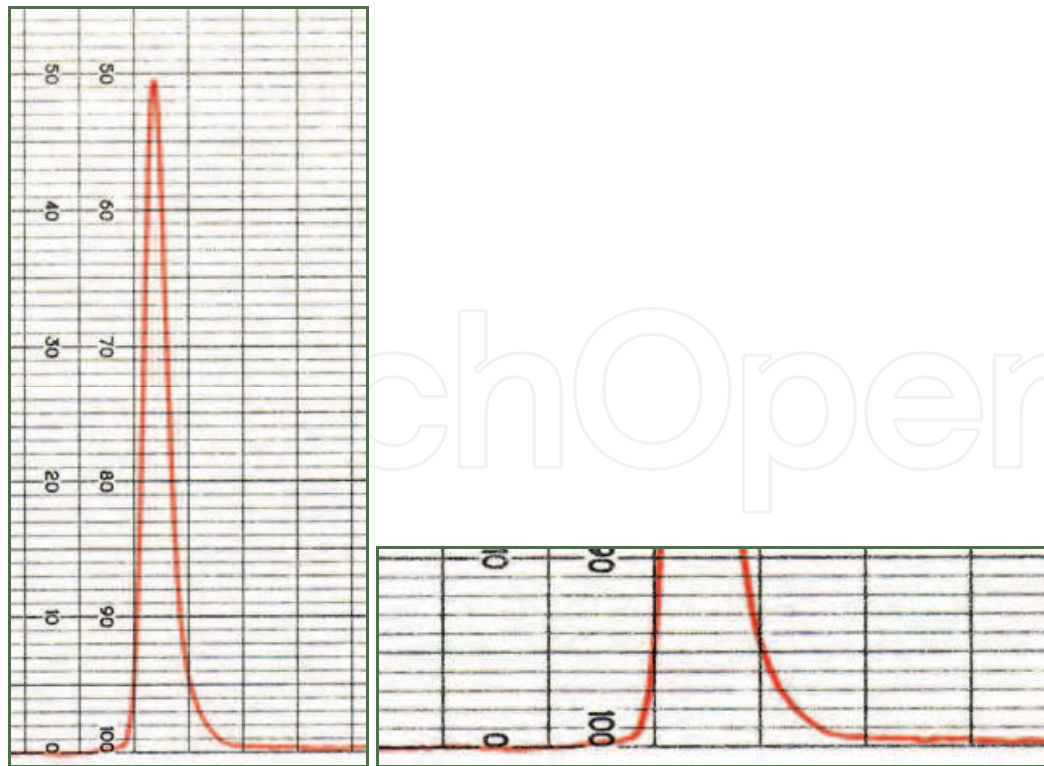
Three-Phase Partitioning (TPP)

The most exciting method we have used for protein purification is three-phase partitioning (TPP). TPP was developed in the 1990's (Dennison and Louvrien, 1997) and rediscovered in



a)

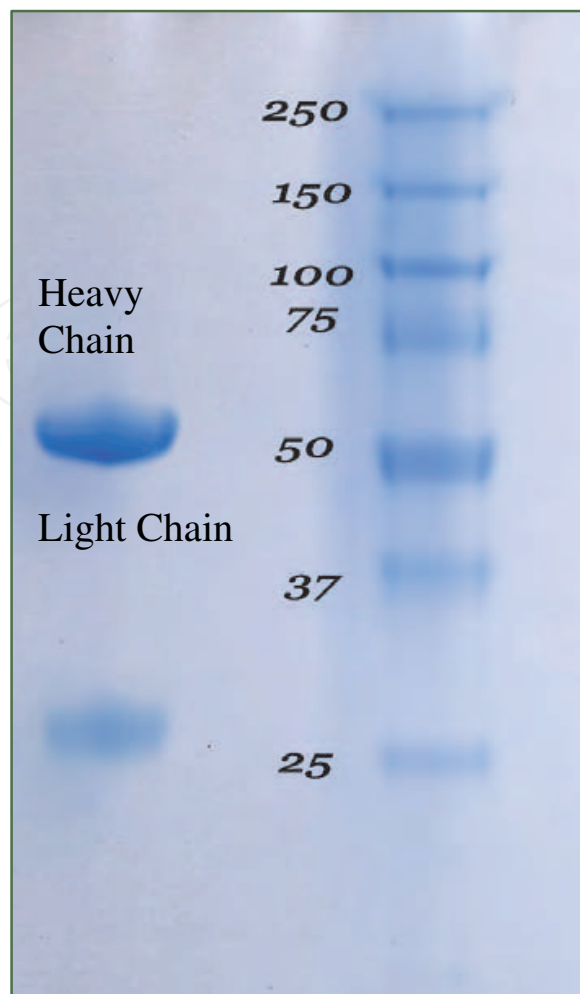
b)



c)

d)

Fig. 5. Continued



e)

Fig. 5. **(a)** HPLC profile of rabbit IgG purified by standard Protein-A chromatography. **(b)** Expanded view of **Fig. 5 (a)** showing small contaminants. **(c)** HPLC profile of rabbit IgG purified by Brighter Ideas Inc's new proprietary method, "A-Free IgG". **(d)** Expanded view of **Fig. 5 (c)** showing no visible contaminants. **(e)** SDS- PAGE gel showing heavy chain and light chain bands of rabbit IgG prepared by "A-Free IgG". Right hand lane shows molecular weight markers in kDa.

our Rutgers University lab in 1998. We happened upon this method by accident in 1998 – not by reading the paper, but by experiencing the method ourselves. The process is so elegant that we can purify recombinant or native jellyfish GFP to 80% purity in less than half a day. In the early years of our research, we purified GFP from jellyfish extracts by traditional methods, spending 6 months to reach 80% purity. TPP provides about a 3000-fold savings in time and significant savings in equipment use and materials expenses. What is the magic?

Our adaptation of the TPP method for purifying recombinant GFP begins with whole, unlysed *E. coli* cells transformed with the gene for GFP. Three-phase partitioning works very well with GFP-containing cell extracts, but it works even better if the process begins with unlysed cells. Entire companies are built around releasing recombinant proteins from whole

E. coli cells (Glens Mills, for example). Huge French presses, sonication baths, day-long, repeated freeze-thaw cycles, treatment with lysozyme, or use of a bead mill are some of the standard methods for rupturing *E. coli* cells (Scopes, 1994). Fig. 6 shows an SDS gel electrophoretic profile for a sample prepared by TPP as compared to identical samples extracted by three other standard methods. TPP accomplishes the release of recombinant proteins in seconds, using the simplest of standard equipment. Described below are three stages in the process:

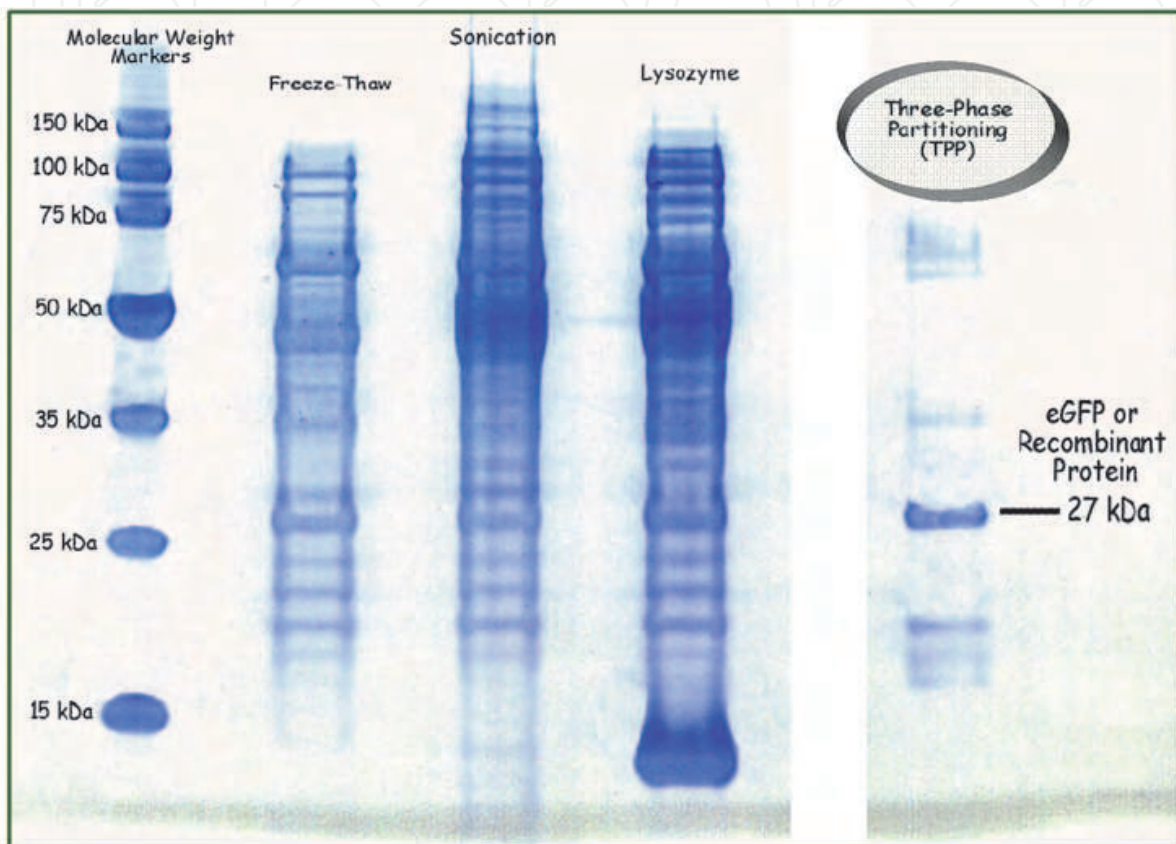


Fig. 6. SDS- PAGE gel showing the released *E.coli* proteins resulting from routine lysis methods compared with the non-lysis Three-Phase Partitioning method as applied to the same amount of starting material.

Stage I. To release GFP and to perform the first stage of TPP purification, we treat whole *E. coli* cells with 1.6 M ammonium sulfate with shaking. Then we add one volume of tertiary butanol. If we do this in a 50 ml Falcon tube, we pour a suspension of the cells (in 25 ml of 1.6 M ammonium sulfate, pH 8.0) into the tube and then we add 25 ml of t-butanol. After about 1 minute of vigorous shaking, the Falcon tube is centrifuged in a moderate speed (3000 rpm) table-top centrifuge for 15 minutes at room temperature. Although t-butanol is completely miscible with water, it is quite insoluble in aqueous solutions having high concentrations of salt, especially ammonium sulfate solutions. Three phases separate during centrifugation (Fig. 7) (or, for large scale operations, by settling in a tank by gravity alone). The upper phase contains t-butanol which expands to 30 ml, having taken up 5 ml of water. Release of 5 ml of water from the lower aqueous phase raises the ammonium sulfate concentration from 1.6 M to 2.0 M.

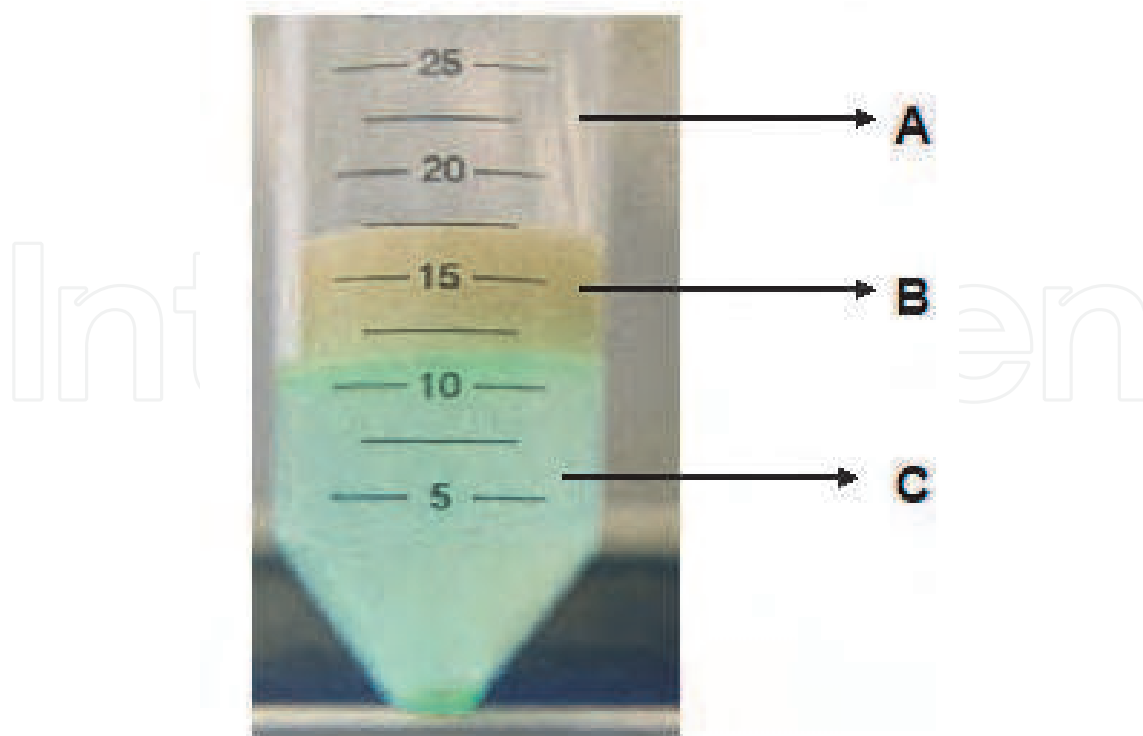


Fig. 7. Tube showing Stage- I of Three-Phase Partitioning. Three phases are formed after centrifugation. Layer A contains t- butanol, B is a thick “pancake” layer of precipitated material and C is aqueous ammonium sulfate solution containing GFP.

Meanwhile, membrane phospholipids, triacylglycerols, pigments, dyes, cholesterol and other steroids, fats, oils, and miscellaneous lipids become dissolved in the upper layer. Exposure of a complex macromolecular mixture to both t-butanol and the now higher salt concentration causes massive precipitation. The precipitate settles below the organic layer as a thick “pancake” of congealed protein, nucleic acids, cell walls, and other unwanted materials (Fig. 8). While the t-butanol, under the influence of high salt, has dissolved the cell membrane, it has not affected the cell wall. Normally, with membrane dissolved, nearly every macromolecule in the cell can escape to the outside through the cell wall. But, “stressed” by the high concentration of ammonium sulfate, t-butanol binds to anything that is even slightly hydrophobic, causing massive precipitation of most of the proteins, and virtually all chromosomal DNA. These aggregates are too large to exit through the cell wall pores, so they remain entombed inside the cell, behind the cell wall barrier. The binding of t-butanol to these macromolecules (whether they have remained within the cell or escaped to the outside) lowers the density of the precipitated macromolecules to such a point that the still intact cells, with their entombed macromolecules, easily float above the ammonium sulfate solution below. The whole cell mass forms a thick rubbery mat that floats above the salt solution (Fig. 8). As centrifugation simply speeds up the formation of three layers, the process can be scaled up to almost any volume by simple gravitational settling in a large tank. There is no limit to the scale-up potential. After loss of 20 % of the water to the overlying alcohol layer, GFP is still soluble in the aqueous ammonium sulfate (now at a concentration of 2.0 M. Because GFP remains soluble, it escapes easily through the pores in the cell wall and enters the aqueous layer.



Fig. 8. Recovered “plugs” of precipitated material from Stage I of Three-Phase Partitioning.

Stage II. The alcohol layer is removed by aspiration and the floating disk of precipitated cells and macromolecules is also removed (almost as easily as flipping a pancake with a spatula). To the 20 ml of aqueous solution remaining in the tube, we add 30 ml of fresh t-butanol, (with vigorous shaking once again). Fresh t-butanol causes further dehydration of the lower liquid phase, creating a saltier aqueous solution. The saltier solution now favors precipitation of the GFP, already coated with many molecules of t-butanol. So, like the aggregated molecules in stage one, the now precipitated GFP (with its bound cage of t-butanol) moves into the organic-aqueous interface as a fine disk, compressed by centrifugal force between the alcohol layer and the aqueous ammonium sulfate layer. Both liquid layers are then carefully removed.

Stage III. The GFP disk, remaining after the liquid phases have been removed, is taken up in a series of very small volumes of 25% saturated ammonium sulfate which, when added to the very salty GFP disk, raises the ammonium sulfate concentration to 1.6 M. One at a time, these suspensions of GFP are serially transferred into one or more microfuge tubes. Serial transfer allows virtually 100% of the GFP to be transferred in a minimum volume. Volume is kept at a minimum because GFP is incredibly soluble, even in 1.6 M ammonium sulfate. When GFP has just barely gone into solution, the tube(s) is spun in a microcentrifuge. Those remaining contaminants, having lower solubility than GFP, now collect as a pellet at the bottom of the tube(s). There is usually a tiny floating disk of contaminant and a small volume of overlying alcohol. In a sense, Stage I of TPP has been repeated in Stage III. The final GFP product of TPP is pipetted from the microfuge(s) as a bright, crystal-clear green liquid. On average, the GFP has been purified from its original milieu by a factor of 100-fold and concentrated by a factor of 50. The amazing effectiveness of TPP is also shown in the before and after absorption spectra seen in Fig. 9.

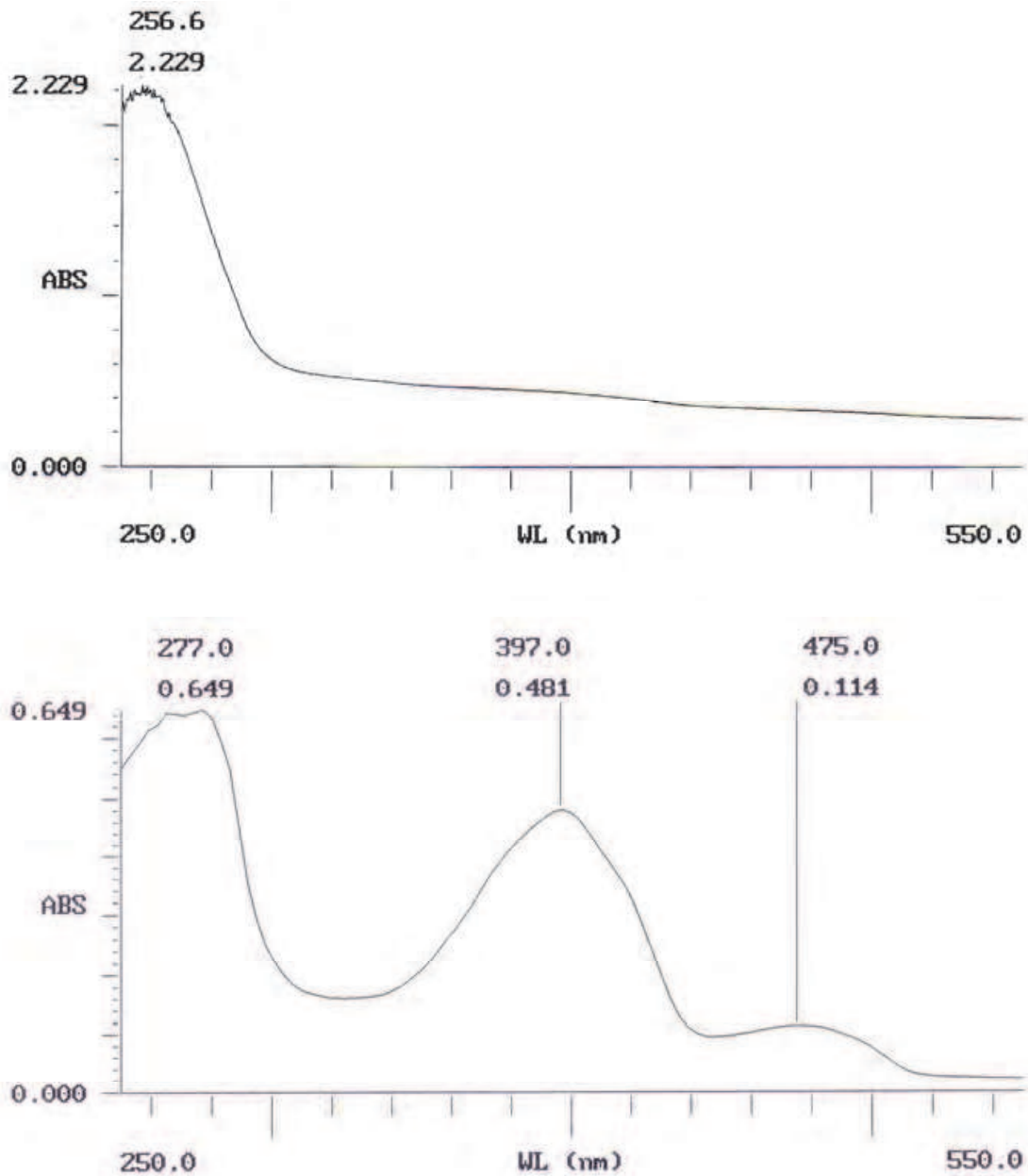


Fig. 9. Spectrum of GFP-containing crude cell extract (a) before and (b) after Three-Phase Partitioning, now 60% pure. Final purity has reached 60%.

A Rutgers University patent was issued in 2008 for a protein mini-prep kit (based upon our work with TPP). The patent calls for a mixture of two organic solvents (a mixture of t-butanol and isopropanol). Included in the patent description is the use of a microbiological dye, previously added to a very concentrated ammonium sulfate stock solution. The purpose of the dye is to facilitate detection of the solvent interface, as all of the dye leaves the aqueous layer and travels into the alcohol layer. We have explored sixteen water-soluble microbiological dyes, each of which partitions effectively into the organic phase. The boundary between the colored organic layer and the colorless aqueous layer provides a visible means for separation of the two layers. Visualization of this boundary is especially useful when tiny quantities of protein are being prepared. Surprisingly, TPP works almost equally well when small

concentrations of protein are processed. Such very dilute protein solutions of protein are almost never amenable to ordinary ammonium sulfate precipitation. What's more, TPP works, not only on crude extracts and whole *E. coli* cells, but it works well as a polishing step. Even 90% pure protein can be taken to near homogeneity by a second round of TPP.

Other proteins may be purified by TPP, but often the initial ammonium sulfate concentration must be adjusted on a protein-by-protein basis so as to maximize both recovery and purity of the protein-of-interest. The salt concentration over which TPP is effective ranges from about 0.6 M to 2.6 M ammonium sulfate. Below 0.6 M, the two solvents are miscible. Above 2.6 M, the salt begins to precipitate.

Criteria for Protein Purity

Demonstrating purity of a given protein is not an easy task. But, without achieving protein homogeneity, serious errors and experimental artifacts may arise. Even a 1% contaminant may contribute to erroneous observations. A minor contaminant (protein or otherwise) could significantly raise or lower an enzyme's apparent activity level. If an impure protein-of-interest is used to generate antibodies, a very immunogenic contaminant could induce more antibody than the POI. Some biochemists and some journals will accept, as the sole criterion of purity, a photograph or a densitometry trace of a Coomassie-stained SDS polyacrylamide gel that shows one stained band. But, I know of a case in which a "single band" on an SDS gel, accepted by a prominent journal as proof of purity, turned out to be a 97% contaminant of the protein-of-interest. The actual POI represented only 1% of the total "pure protein" (Karkanis and Cormier, 1971). Errors of this magnitude can be avoided by using a variety of different criteria for evaluating protein purity.

1	Constant specific activity across a broad portion of the peak in the final preparative chromatography column.
2	Single, symmetric band by size exclusion HPLC.
3	Single band, in the correct MW region, on an SDS gel (or, for hetero-oligomers, the appropriate number of bands in the correct positions).
4	Unambiguous, single amino acid detected in N-terminal amino acid analysis. Inability to detect an N-terminal amino acid may also be taken as evidence of purity – not a very strong criterion as many other proteins have blocked N-terminal amino acids.
5	Single band on a native polyacrylamide gradient gel (or appropriate number of bands of correct MW for heterodimers, heterotetramers, etc).
6	Single, sharp band by isoelectric focusing in an acrylamide gel or in a capillary isoelectric focusing system (or the appropriate number of bands for hetero-oligomeric proteins).
7	Unambiguous N-terminal peptide sequence by Edman degradation.
8	Single band by Western blot, if antibodies are available.
9	Single MW form by Maldi TOF (matrix assisted laser desorption time-of-flight mass spectrometry).

Table 4. Criteria of purity

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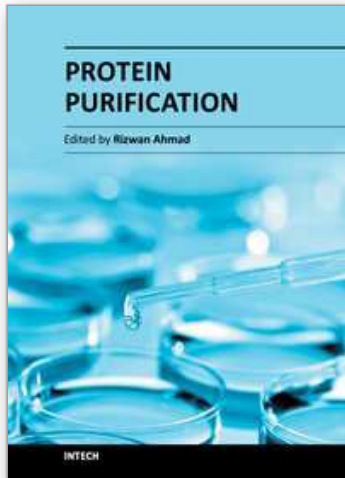
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