

A publication of
The Williamsburg BioProcessing Foundation

Spring 2006

BioProcessingTM JOURNAL

The Most Trusted Source of BioProcess TechnologyTM

Vol. 5 No. 1

www.bioprocessingjournal.com

Protein Recovery from Tobacco Extract by Non-Chromatographic Methods

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For more than a decade, transgenic plants have been investigated as alternatives to microbial, mammalian cell, and transgenic animal systems for recombinant protein production. The main advantages of using plants as “bioreactors” are that the cost of upstream production (*i.e.*, biomass creation) is low; plants do not carry viruses and other pathogens dangerous to humans such as human immunodeficiency virus (HIV), prions, hepatitis viruses and so on; and as eukaryotes, plants are capable of producing bioactive proteins. Numerous recombinant proteins have been expressed in various plant hosts, and some recombinant proteins are in various stages of clinical trials.^{1,2}

Many plant systems have been investigated for recombinant protein expression, and the pros and cons of each plant system have been well documented.³ However, it is increasingly obvious that tobacco will likely play an important role in “biopharming” (*i.e.*, recombinant protein production by transgenic plants). The favorable characteristics of tobacco include high biomass production, ease of genetic transformation, and because it is a non-food/non-feed crop, the risks for any potential food supply contamination is eliminated.

Despite its notorious reputation as a smoking material, tobacco is actually a good source for proteins. Its fraction I protein (identical to ribulose-1,5-diphosphate carboxylase-oxygenase),

which will not be extracted in typical aqueous extraction buffers, surprisingly, has nutritional values similar to those of egg or milk proteins, and its total protein content, albeit varying among different varieties, is comparable to that of barley, corn, wheat, and rice.^{3,4} The amount of protein extractable from tobacco by aqueous buffers is highly dependent upon the buffer pH.

As shown in Figure 1, protein extraction can vary more than 50% depending on the pH.⁵ The profile in Figure 1 shows that there are more acidic proteins than basic proteins in tobacco. Therefore, when considering tobacco for

recombinant protein production, a basic recombinant protein could be more favorable, from the purification perspective, since the purification burden is relatively low. However, purification of a target protein from tobacco extract is challenging, especially for large scale production because the recombinant protein expression level will likely be low, and the protein extract is a complex system containing components such as phenolics.

So far, most of the protein purification efforts from transgenic tobacco have been limited to bench scale operation. When cost is not a concern, all

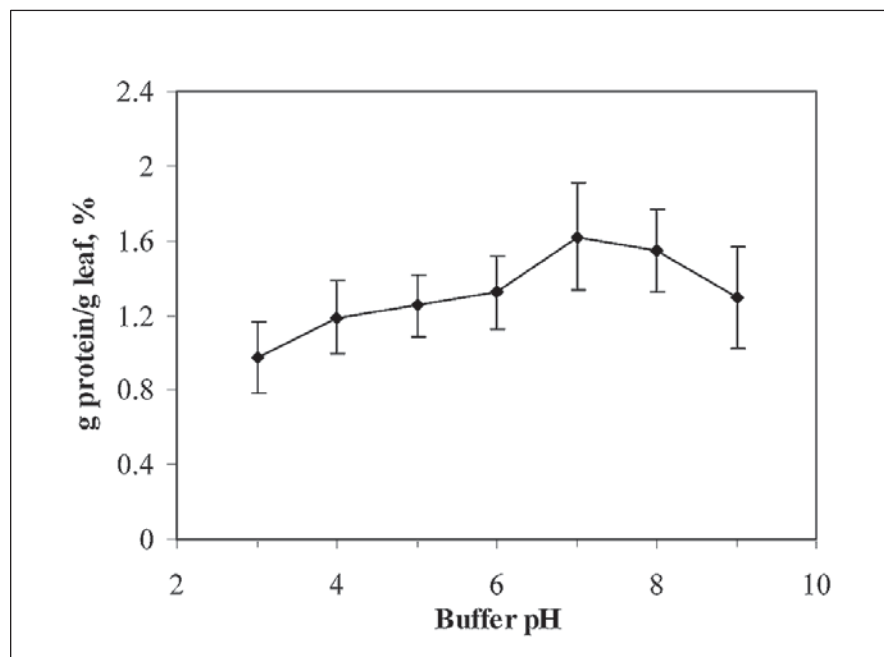


Figure 1. Percentage of g protein extracted per g leaf (flue-cured tobacco) versus buffer pH. The error bars represent the standard deviations. Buffer to biomass ratio = 10:1. All extraction buffers were 50 mM of corresponding salts, and the salts used were: pH 3-5, sodium citrate/citric acid; pH 6-8, sodium phosphates; pH 9, Tris base.

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processes have invariably used chromatographic methods for both recovery and purification. Expanded bed chromatography has been used to recover proteins from other plant sources, but direct application of this technology on leaf homogenate will be difficult, due to the asymmetrical nature of the particulates.^{6,7} Thus, developing non-chromatographic techniques, at least for protein recovery and the early stages of protein separation and concentration, would likely be the key for developing economical processes for protein purification from tobacco.

It is beyond the scope of this review to evaluate the economics of the processes anchored by non-chromatographic methods for protein recovery and initial purification. Rather, the focus is through several examples of model protein recovery from tobacco to demonstrate the utility of two widely used, inexpensive non-chromatographic methods: aqueous two-phase extraction (ATPE), and polyelectrolyte precipitation. The detailed experimental protocols are published in the references cited in each section.

Aqueous Two-Phase Extraction For the Recovery of Egg White-Type Lysozyme from Tobacco Extract⁵

ATPE, while not widely used in biopharmaceutical industrial settings currently, has the potential to lower the overall cost of a protein purification process. It can work with the presence of solid particulates and can be developed with relatively high selectivity because of the number of adjustable variables. The scale-up of this method is relatively straightforward and it is compatible with subsequent chromatographic techniques such as ion exchange chromatography, immobilized metal affinity chromatography, and size-exclusion chromatography.⁸⁻¹⁰ Furthermore, ATPE has been shown to stabilize protease-vulnerable proteins.¹¹

The partitioning behavior of tobacco-native protein was studied for three systems: PEG/potassium phosphate, PEG/sodium sulfate, and PEG/ammonium sulfate. The following variables were investigated in factorial design of

experiments: PEG molecular mass, PEG concentration, phase forming salt concentration, sodium chloride concentration (to adjust the ionic strength), and pH of the system. The partition coefficients of tobacco protein and the statistically important variables are shown in Table 1.⁵ Since a low partition coefficient of native tobacco protein will favor obtaining a high selectivity for recombinant proteins, PEG/potassium phosphate and PEG/sodium sulfate systems were chosen for further studies.

A parallel study with the pure model protein, egg white lysozyme, was also carried out. The results showed that the partition coefficient of lysozyme in PEG/potassium phosphate systems varied between 10 and 40 with no factors statistically significant, and 6 to 80 for PEG/sodium sulfate systems with sodium chloride and sodium sulfate concentrations being the important variables. The PEG/sodium sulfate system was thus selected for further studies because of the possibility of obtaining high lysozyme selectivity (lysozyme partition coefficient/tobacco protein partition coefficient). A response surface study was used to optimize the separation conditions (sodium chloride and sodium sulfate concentrations) to obtain the highest possible lysozyme

selectivity, and the results are shown in Figure 2.⁵ This figure indicates there are two possible approaches to improve lysozyme selectivity: 1) decreasing sodium sulfate concentration while increasing sodium chloride concentration, and 2) increasing sodium sulfate concentration but decreasing sodium chloride concentration. However, the adjustment of these two parameters is limited by: 1) the solubility of sodium sulfate in water being ~ 30% w/w, 2) the potential for lysozyme precipitation under high salt concentration (salting out), and 3) the requirement that the sodium sulfate concentration must be high enough for the overall phase compositions to fall into the two-phase region. Nevertheless, a selectivity of 57 was achievable, which was verified by two other independent experiments.

One other advantage of utilizing ATPE for protein recovery is that once a set of optimized conditions is identified, a protein's recovery and purification can be further improved due to the fact that the selectivity of a protein will not change along a particular tie line in a phase diagram. The overall phase compositions and subsequently, the phase ratio, can be adjusted along the timeline, and the effect of this adjustability can be wonderful for bioprocess

Table 1: Factual studies of the partitioning of tobacco proteins in various two-phase systems: (+) denotes that the partition coefficient of tobacco protein increases with the increase of a factor and (-) denotes the partition coefficient decreases with the increase of a factor.

Two-phase system	Partition coefficient	Statistically significant factors
PEG/potassium phosphate	1 - 3.5	Potassium phosphate concentration (+), sodium chloride concentration (+), PEG molecular mass – pH interaction (-)
PEG/sodium sulfate	1 - 5	None
PEG/ammonium sulfate	3 - 5	Ammonium sulfate concentration (+), PEG molecular mass (-)

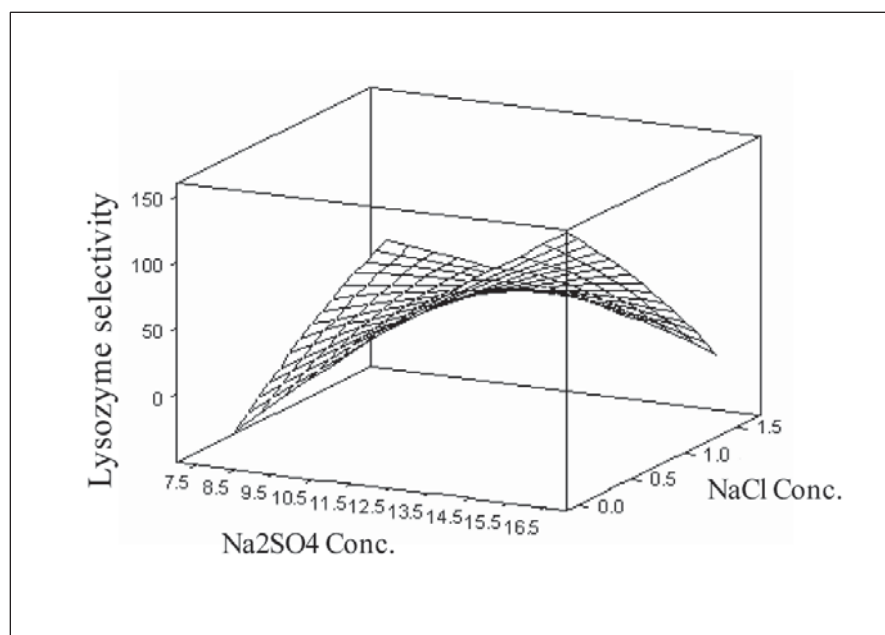


Figure 2. Response surface study for egg white lysozyme separation from tobacco extract. Lysozyme selectivity was used as the response estimate. Other conditions used in the study: PEG 3400 at 10% w/w and pH 7.

engineers, as demonstrated by the data in Table 2.

As shown in Table 2, the yield of lysozyme decreases with phase ratio because of the decreased top phase volume available, but that is more than compensated by much higher lysozyme purification and concentration factors. When the phase ratio is 1:15, 87% of lysozyme can still be recovered while more than 85% of tobacco protein is eliminated. When compared to those whose phase ratio is 1, the purification factor is improved 4 times and concentration factor, 7.⁵

This shows that ATPE, if developed properly, can be an effective first step for protein recovery and purification from tobacco extract. However, ATPE's many adjustable variables might, at the same time, prove disadvantageous. It can be quite time consuming to determine the optimal conditions for a protein's recovery from a particular expression system and unfortunately, it is often difficult to apply the results from system to system and from protein to protein. Nevertheless, the optimization effort may be well justified for large-scale manufacturing of a particular protein.

Polyelectrolyte Precipitation

Polyelectrolyte precipitation is based on the charge-charge interaction between a protein and an oppositely charged polymer. The resulting charge-neutralized protein-polymer complex can readily flocculate and then precipitate out of a solution. The operation is straightforward, and if compared with other precipitation methods such as organic solvent or affinity precipitation, this method possesses the combined advantages of relatively high selectivity and low cost. More importantly, the bioactivity of a protein is typically maintained after product resuspension. The most commonly used poly-

anionic polyelectrolytes are polyacrylic acid (PAA), polyphosphate (GlassH), and carboxymethylcellulose (CMC) for precipitating positively charged proteins, and polyethyleneimine (PEI) for precipitating negatively charged proteins. All of these polymers are commercially available, however, this method must include a clarified solution if the product is to be recovered in the precipitate unless a "negative" precipitation is the designed unit operation (as explained later). Polyelectrolyte precipitation (for recovering proteins from transgenic tobacco extract) can work, but the researchers need to be prepared for unexpected challenges as shown below:

Polyelectrolyte Precipitation of a Basic Model Protein, Egg White Lysozyme, from Tobacco Extract¹²

PAA, GlassH, and CMC were used to precipitate lysozyme from tobacco extract. At pH 7, the model protein, egg white lysozyme (equivalent to 6% of the total extracted tobacco protein) was added ("spiked") into tobacco extract, but about 70% of the protein was lost due to instantaneous precipitation. The lost activity could not be recovered. Interestingly, the protein remaining in the solution did not respond to any of the added polyelectrolyte, although other reports have shown that PAA and GlassH can induce pure lysozyme precipitation.¹³ The failure of these experiments was largely due to the presence of phenolic compounds in tobacco extract which can modify lysozyme to shift from ~11 to ~6-7.¹⁴ On the other hand, tobacco protein was not precipitated by any of the polymers.

Table 2: Theoretical yield and purification factor of lysozyme at various phase ratios along the same timeline. Lysozyme selectivity used for the calculation is 57.

Phase Ratio	Lysozyme Yield	Tobacco Protein Yield	Purification Factor	Concentration Factor
1:1	0.99	0.77	1.16	1.98
1:15	0.87	0.14	3.99	14.01
1:30	0.77	0.07	5.44	24.16

This agrees with the results shown in Figure 1 in that most of the tobacco proteins are acidic in nature and they do not interact with polyanionic polyelectrolytes at neutral pH. However, lysozyme precipitation from tobacco extract was drastically improved when the experiments were carried out at pH 5. Not only did most of the spiked lysozyme (~75%) remain in the solution prior to the addition of the polyelectrolyte, but as shown in Figure 3, lysozyme could be effectively precipitated by PAA while less than 10% of tobacco protein was coprecipitated. An enrichment ratio of 8 was obtained. Since lysozyme is a basic protein, the change of the extraction buffer to a lower pH will not likely affect the effectiveness of the extraction. This improvement indicates that the amount of phenolic compounds extracted likely has been decreased and thus, if developed properly, basic recombinant proteins can be effectively recovered by polyelectrolyte precipitation from tobacco extract.

Polyelectrolyte Precipitation of an Acidic Model Protein, β -Glucuronidase (GUS), from Tobacco Extract¹⁵

PEI was used to precipitate GUS from tobacco extract. Precipitation studies with non-spiked tobacco extract and *E-coli*-derived GUS stock solution, respectively, indicated that the majority of tobacco proteins will interact with

PEI. GUS was shown to be preferentially precipitated because the percentage of GUS precipitated was higher than tobacco proteins at the same dosage of the polymer. However, the spiking experiments revealed a completely different story, as shown in Figure 4.

Clearly, before a dosage of PEI equivalent to PEI:GUS = 30:1 (weight ratio between the added PEI and spiked GUS) tobacco protein was first precipitated by PEI. At a 15:1 PEI dosage, almost all GUS remained soluble while ~ 65% of tobacco protein was precipitated. Even at a 30:1 PEI dosage, more than 80% of GUS remained soluble, but only less than 20% of tobacco protein remained in the solution. GUS, obviously, is at a disadvantage in the competition for the available charged polymers during the precipitation. Not until most of the PEI-interacting tobacco proteins are precipitated, can GUS start to bind with PEI and in turn, form precipitate. After a 30:1 PEI dosage, tobacco protein solubility does not change significantly, and this shows that about 20% of the native tobacco protein is of basic nature (or weakly acidic).

In contrast to the lysozyme precipitation experiments, GUS precipitation is more challenging because of the coprecipitating acidic tobacco proteins. This, from the downstream processing point of view, shows again that tobacco likely will be a good expression system for basic recombinant pro-

teins. Nevertheless, Figure 4 presents a window of opportunity for recovering acidic proteins from tobacco extract by precipitation, and that is to add a small dosage of PEI to remove tobacco protein while the solubility of GUS is retained. This is the so-called “negative” precipitation which, for example, has been used to clear up nucleic acids and the other 90% of contaminating proteins during the first-step isolation of a basic protein, cysteine proteinase inhibitor stefin B, from *E-coli* lysate.¹⁶

This “negative” precipitation step enabled a single, subsequent chromatographic step to purify the protein to homogeneity.¹⁶ Also, as shown in Figure 4, an enrichment of 4.5 is achievable for “negative” precipitation of GUS, and this fact alone warrants the consideration of this technique during process development for acidic recombinant protein purification from tobacco. One possible advantage to using “negative” precipitation is that it is possible to work with unclarified leaf homogenate during precipitation, and the precipitate can be removed together with solid particulates during the clarification of the supernatant by centrifugation or filtration.

From the results presented in Figure 4, a two-step precipitation operation was developed in order to recover GUS in the precipitate. More than 60% of the tobacco protein was removed during the negative precipitation step with a 30:1 PEI dosage, and 90% of GUS was precipitated with 18% tobacco protein co-precipitated after the second precipitations step with a final overall 120:1 PEI to GUS ratio.

The enrichment ratio was slightly improved to 5, as compared with 4.5 by the single step “negative” precipitation mentioned above. However, the unexpected pitfall was that the resuspension of the precipitated GUS proved to be extremely challenging. A possible explanation is that GUS has been modified in the tobacco extract to some extent, which makes hydrophobic interaction the dominant force during protein-polymer complex flocculation. This further illustrates the importance of “negative” precipitation (pointed to previously), particularly for recovering

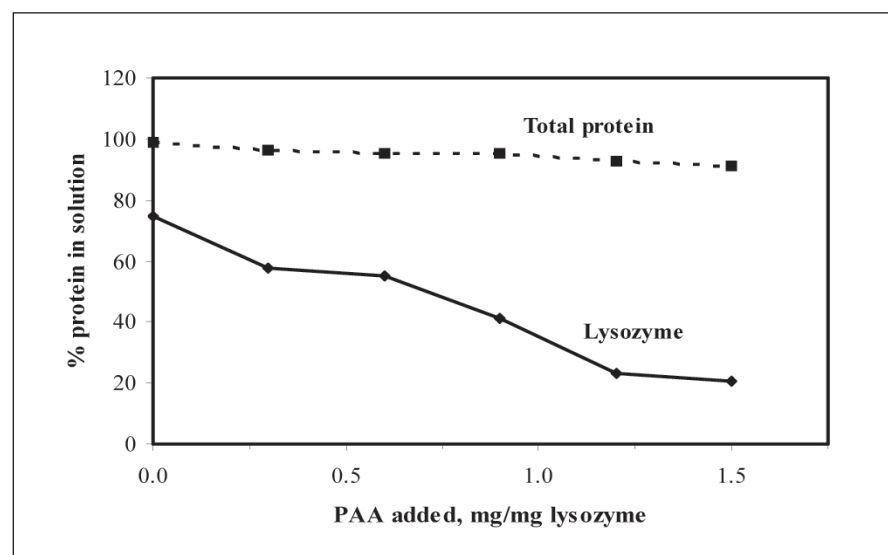


Figure 3. Precipitation of lysozyme by PAA at pH 5 with tobacco extract obtained at the same pH.

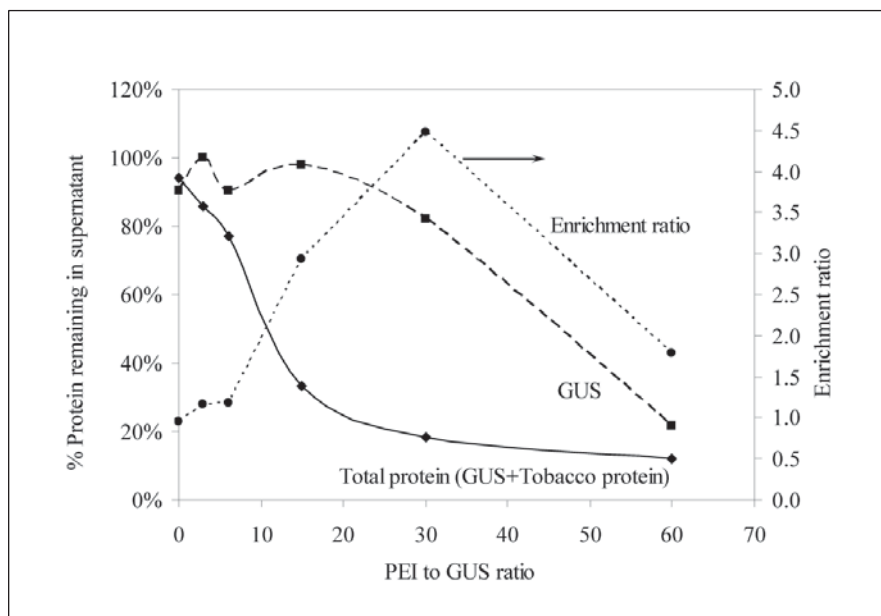


Figure 4. Precipitation of spiked GUS from tobacco extract by PEI-750k. Total protein was determined by protein assay, and GUS was determined by activity assay of the supernatant. The dotted line indicates the enrichment ratio at different dosage of PEI. At and before PEI: GUS = 30:1 (weight ratio), most of GUS remained soluble and the enrichment ratio was calculated for soluble GUS. When PEI:GUS = 60:1, the enrichment ratio was calculated for GUS recovered in the precipitate.

proteins from plant materials containing significant amounts of phenolics.

In addition to the advantages mentioned earlier, “negative” precipitation minimizes the risk of losing protein activity from protein resuspension. Therefore, even though a protein will not be concentrated by “negative” precipitation, maintaining its solubility while removing a majority of the impurity proteins should still provide enough incentives for bioprocess engineers to consider its inclusion in process development.

Conclusion

Recombinant protein production from transgenic plants will be a technology to be reckoned with. However, the low upstream production cost could be offset by the high cost incurred during downstream processing. Developing nonchromatographic methods for recovering and partially purifying the recombinant protein may help to reduce the overall purification cost. Aqueous two-phase extraction and polyelectrolyte precipitation are two promising tech-

niques. It was shown that ATPE can be effective in recovering egg white lysozyme from tobacco extract with a high enrichment ratio. Anionic polyelectrolyte can also be effective in recovering lysozyme.

Sometimes a simple adjustment of the operation pH might aid in recovery and activity but the recovery of acidic protein (such as GUS) by precipitation is more challenging and might require a different strategy. Instead of collecting the protein in the precipitate to risk the loss of the protein activity, “negative” precipitation should be considered. Both ATPE and “negative” precipitation can be employed with an unclarified leaf homogenate, and this gives them the added advantage for eliminating unit operations such as centrifugation or filtration to further improve the overall economics of a protein purification process.

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