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Tobacco protein separation by aqueous two-phase extraction

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Abstract

Tobacco has long been considered as a host to produce large quantity of high-valued recombinant proteins. However, dealing with large quantities of biomass is a challenge for downstream processing. Aqueous two-phase extraction (ATPE) has been widely used in purifying proteins from various sources. It is a protein-friendly process and can be scaled up easily. In this paper, ATPE was studied for its applicability to recombinant protein purification from tobacco with egg white lysozyme as the model protein. Separate experiments with poly(ethylene glycol) (PEG)–salt–tobacco extract and PEG–salt–lysozyme were carried out to determine the partition behavior of tobacco protein and lysozyme, respectively. Two-level fractional factorial designs were used to study the effects of factors such as, PEG molecular mass, PEG concentration, the concentration of phase forming salt, sodium chloride concentration and pH, on protein partitioning. The results showed that, among the studied systems, PEG–sodium sulfate system was most suitable for lysozyme purification. Detailed experiments were conducted by spiking lysozyme into the tobacco extract. The conditions with highest selectivity of lysozyme over native tobacco protein were determined using a response surface design. The purification factor was further improved by decreasing the phase ratio along the tie line corresponding to the phase compositions with the highest selectivity. Under selected conditions the lysozyme yield was predicted to be 87% with a purification factor of 4 and concentration factor of 14. From this study, ATPE was shown to be suitable for initial protein recovery and partial purification from transgenic tobacco.

Keywords: Aqueous two-phase systems; Extraction methods; Tobacco; Factorial analysis; Proteins; Lysozyme

1. Introduction

The expression of recombinant proteins such as lactoferin, cholera toxin B, and ricin in tobacco has been well documented [1–3]. Tobacco is abundantly grown in the southeastern USA, but its production is declining due to the health issues concerned with smoking and other federal regulations. Using tobacco to produce therapeutic recombinant human proteins is a viable alternative. Genetic modification of tobacco is easy and it is suitable for large-scale production [4]. Each tobacco plant produces thousands of seeds, and the biomass production per acre is much higher than most other crops, which facilitate scaled up production. However, commercialization of tobacco as a host for recombinant

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protein production is dependent on the development of cost effective and efficient methods to handle huge amounts of biomass. Since the overall cost of a protein's purification is mainly determined by the efficiency of the initial recovery and purification, extraction and initial purification procedures of recombinant proteins from tobacco need to be developed.

Aqueous two-phase extraction (ATPE) has been widely used for protein recovery and purification [5,6]. In ATPE, two immiscible phases are formed when polymers such as poly(ethylene glycol) (PEG) are mixed with dextran, other polymers, or salts in particular concentrations. The equilibrium distribution (partitioning) of a protein in ATPE not only depends on its own surface properties such as charge and hydrophobicity but also on the physicochemical properties of the two phases [7], which can be manipulated by adjusting factors such as the polymer molecular mass and concentration, type of phase forming salt, salt concentration, ionic strength, and pH.

ATPE has the potential to produce a concentrated and purified product in one step when compared to the number of steps involved in conventional down stream processing such as recovery, clarification, filtration, concentration, and purification. ATPE is a simple separation process, and it offers gentle non-toxic environments for biomolecules. It is cost effective and can be scaled up easily. Successful pilot scale studies of using ATPE to recover superoxide dimutase from bovine liver tissue have been demonstrated [8]. Moreover, among many two-phase systems studied, PEG–dextran–water and PEG–salt–water systems are most commonly used for protein separation. However PEG–salt–water two-phase systems have certain advantages over PEG–dextran–water systems such as low viscosity and lower cost [9,10].

Producing mature transgenic tobacco plants that can be used to investigate downstream processing methods is very time consuming and difficult. Spiking experiments can be used instead to study the partitioning behavior of proteins in tobacco extract. In spiking experiments, a model protein with known properties is added to the native tobacco extract to emulate the protein extract from transgenic systems. The partitioning characteristics of both tobacco protein and the model protein can be studied to provide insight into the use of ATPE.

Phase diagrams for a number of PEG–salt systems have been reported [11]. The phase diagrams are important in knowing the PEG and salt concentrations at which two-phase systems will be formed and in determining the optimum compositions of a system for the required level of purification. To select the optimal ATPE conditions for a protein's purification, the selectivity of a target protein over the contaminant proteins can be utilized, which, in parallel to protein separation in chromatography, is defined by:

$$\alpha = K_P / K_C \tag{1}$$

where K_P is the partition coefficient of the target protein and K_C is the lumped partition coefficient of proteins other than the target protein. Since the partition coefficients of proteins are the same on a tie line, the selectivity of a target protein is constant for all phase compositions on any particular tie line on the phase diagram. The importance of constant selectivity along a particular tie line is that the purification factor of the recovered protein (the mass ratio of the recombinant protein to the total protein in the top phase to that prior to separation) can be improved by varying the phase ratio:

$$\text{Phase ratio} = \frac{\text{Volume of top phase}}{\text{Volume of bottom phase}} = \frac{BC}{AB} \tag{2}$$

where BC and AB are the tie line lengths illustrated in Fig. 1. The lower the phase ratio, the higher will be the purification factor (if the selectivity is greater than one). The yield of the recombinant protein will decrease as the phase ratio decreases. Hence an optimum phase ratio chosen for the purification process would provide a balance between yield and purification factor.

The scope of this paper is to investigate the applicability of ATPE to purify proteins from transgenic tobacco using statistical design of experiments. Statistical design of experiments is a very widely used tool for process optimization and control. Factorial design is a convenient method to study the effects of a large number of factors and determine the significant effects the factors may have on the response of interest (protein partition coefficient in

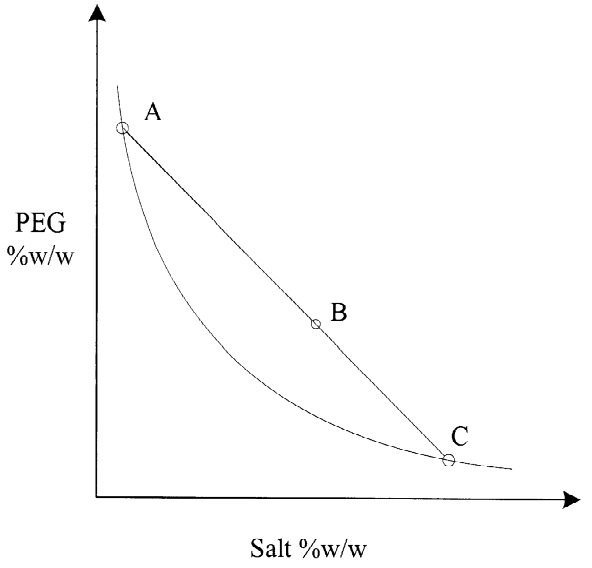

Fig. 1. Phase diagram schematic. *A* represents the compositions of the top phase, and *C* represents the compositions of the bottom phase, of a system with total compositions represented by *B*.

this case). The significant factors and their effects can be studied with fewer numbers of runs using fractional factorial runs. Statistical design of experiment procedures has been used to study protein separation by aqueous two-phase systems [12] and response surface designs have proven to be a very useful tool for optimization of bioseparation processes [12–14].

The partition behaviors of native tobacco proteins in different PEG–salt systems were investigated. Egg white lysozyme was used as a model protein to mimic the presence of a recombinant protein in tobacco. Lysozyme is a basic protein with an isoelectric point around 11 and a molecular mass of 14 400. It was chosen as it is not natively produced in tobacco and can be quantitatively assayed easily and accurately with the presence of other proteins. The partition characteristic of lysozyme in selected PEG–salt systems was studied, and the most suitable system for lysozyme purification from tobacco extract was determined. With the selected ATPE system, the system compositions at which the selectivity of lysozyme was the highest were determined using a response surface study. The theoretical yield and purification factors for various phase ratios with the same selectivity (phases on the same tie line) were calculated to determine an optimum system for lysozyme recovery.

2. Experimental

2.1. Materials

Flue-cured tobacco cultivar K 326 was grown at the Virginia Tech Southern Piedmont Agriculture Research and Extension Center, Blackstone, VA, USA during the summer of 2001. Seedlings were produced according to standard production practices [15]. Plots were fertilized with 282 kg N/ha of 6-12-18 analysis fertilizer prior to transplanting. Seedlings were transplanted at a rate of 118 311 plants/ha with a vegetable transplanter. Plants were harvested about 6 weeks after transplanting by cutting the stalk 10 cm above ground level. Plant tissue was stored on ice during transport to Blacksburg then stored at −80 °C.

PEG (molecular masses 3400 and 8000), potassium phosphate monobasic, potassium phosphate dibasic and ammonium sulfate were obtained from Sigma (St. Louis, MO, USA). Sodium phosphate monobasic, sodium phosphate dibasic, sodium chloride, citric acid, sodium citrate, Tris base, and sodium sulfate were obtained from Fischer Scientific (Pittsburgh, PA, USA). Bicinchoninic acid (BCA) assay reagents and bovine serum albumin (2 mg/ml) were obtained from Pierce Biotechnology (Rockford, IL, USA). Lysozyme from chicken egg white and *Micrococcus lysodeikticus* were obtained from Sigma. All statistical designs and analyses were assisted by MINITAB software (Version 13).

2.2. Methods

The unit operations involved in this study are illustrated in Fig. 2.

2.2.1. Tobacco extract preparation

The flue-cured tobacco leaves were thawed at room temperature washed with deionized water and dried using paper towels. The dried leaves were then cut using a Waring blender, and the required amount of blended leaves was weighed into a 50-ml conical tube. Buffers in the ratio of 10 ml for every gram of

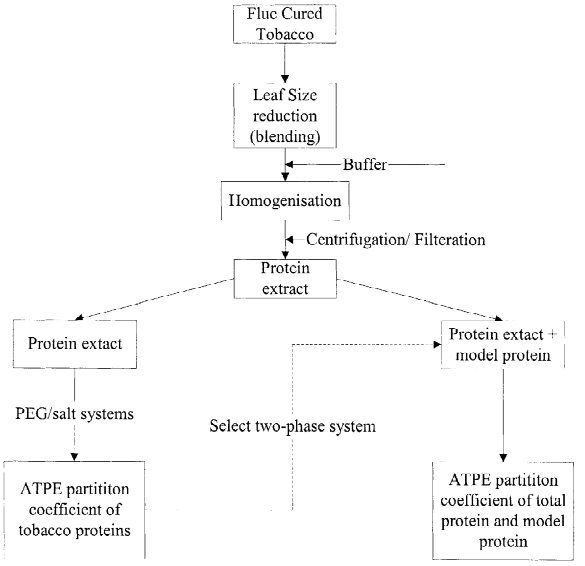

Fig. 2. Flow chart of unit operations involved in protein extraction from tobacco by aqueous two-phase extraction.

leaf were used to extract the protein from the leaf sample. The pH range considered was from 3 to 9. All extraction buffers were 50 m*M* of corresponding salts. Buffers of pH 3–5 were prepared using sodium citrate–citric acid. Buffers from pH 6 to 8 were prepared using sodium phosphate and pH 9 buffers was prepared using Tris base [16]. The blended leaves were then homogenized using a Power Gen 700 (Fischer Scientific). The homogenized extract was allowed to stand for 20 min at room temperature (20 °C) and then centrifuged at 4 °C, 12 857 *g* for 15 min. The extract was recovered by decanting the supernatant into a new tube. The volume of extract recovered was noted. The extract was then filtered using a syringe filter (45 μm) before further studies.

2.2.2. Bicinchoninic acid assay

Protein concentration was determined by the BCA assay [17]. Bovine serum albumin (BSA) was used as standard. A 50-μl volume of sample was mixed with 1 ml of the working reagent and incubated at room temperature for 2 h. The absorbance was measured at 562 nm.

2.2.3. ATPE

PEG–potassium phosphate, PEG–sodium sulfate, and PEG–ammonium sulfate systems were investigated to study the partitioning behavior of native tobacco proteins. Phase diagrams for these systems under certain phase conditions have been reported [11].

For PEG–potassium phosphate systems and PEG–ammonium sulfate systems, stock solutions of PEG 40 (w/w) were used. The potassium phosphate stock solutions at 40% (w/w) were prepared by dissolving calculated amounts of potassium phosphate (monobasic and dibasic) in deionized waster and titrated to the appropriate pH. The ammonium sulfate stock solutions at 40% (w/w) were prepared by dissolving the required amount of salt in 50 m*M* sodium phosphate buffers at the required pH. The stock solutions for the PEG–sodium sulfate system were PEG 50% (w/w) and sodium sulfate stock solutions 30% (w/w), which was made up using 50 m*M* sodium phosphate buffer at appropriate pH.

Systems of 5 g mass containing the required amounts of PEG, salt solution, sodium chloride and tobacco extract were prepared from appropriate stock solutions. The systems were thoroughly mixed first then centrifuged at 1157 *g* at room temperature for 10 min to expedite the phase separation. The centrifuged sample was allowed to stand for 30 min at room temperature. Then, the bottom phase was carefully pipetted out and weighed. The mass of the top phase was calculated by subtracting the bottom phase mass from the total (5 g). The density of each phase was estimated by measuring the mass of 100 μl of each phase in a preweighed microcentrifuge tube. The protein concentration in each phase was determined by BCA assay.

The factors affecting the partitioning of lysozyme were studied for the PEG–potassium phosphate and PEG–sodium sulfate systems. The same stock solutions were used for PEG and the salts. Two-phase systems (total mass 1 g) were prepared using required amounts of PEG, salt solution, sodium chloride, lysozyme (150 μl of 1 mg/ml) and deionized water. The top and bottom phases were separated and the amount of protein present in each phase was determined by BCA assay since lysozyme was the only protein present.

The experiments for response surface analysis were conducted only for the PEG–sodium sulfate system. A predetermined amount of lysozyme was

added into the tobacco extract, and the two-phase systems (total mass 5 g) were set up using PEG and salt stock solutions, sodium chloride and the tobacco extract with spiked lysozyme. The total protein content in each phase was determined using BCA assay. The amount of lysozyme in each phase was determined by lysozyme activity assay.

2.2.4. Lysozyme activity assay

Lysozyme activity present in each phase was measured by the clearing of a *Micrococcus lysodeiktikus* cell suspension [18]. *Micrococcus lysodeiktikus* cell suspension of concentration 0.5 mg/ml was prepared using potassium phosphate buffer at pH 6.2. A 300-m*M* sodium chloride solution was prepared with deionized water. The rate of change of absorbance at 540 nm for a mixture of 600 μl of 0.5 mg/ml *Micrococcus lysodeiktikus* cell suspension, 200 μl of 300 m*M* sodium chloride and 400 μl protein sample was measured for every 10 s for 1 min. The rate of change of absorbance was used to determine the activity. One unit of lysozyme activity is the amount of lysozyme that produces a 0.001 A_{540} change per min (units/ml). The specific activity of the enzyme (unit/mg) was calculated by dividing the activity of the pure enzyme by its concentration (using BCA assay). The concentration (mg/ml) of lysozyme in each sample was calculated by dividing the activity of the sample with the specific activity.

3. Results and discussion

3.1. Protein extraction from tobacco leaves

The amount of protein extracted was studied as a function of the pH of the extraction buffer. Interestingly, the pH of the extract varied from that of the buffer added. The pH of the extract was lower than that of the buffer for pH ranging from 5 to 9, while it was higher than the pH of the buffer for the pH range 3–4 (Fig. 3). There was a drop in the pH by almost one unit at pH 9 while at pH 5 the drop was very small (0.08 units). Similarly the increase in pH at pH 3 was about 0.85 units and at pH 4 was around 0.40 units. This suggests that the overall tobacco cellular environment is slightly acidic (pH~5).

The total protein extracted (g protein/g of leaf, %) as a function of the buffer pH is shown in Fig. 4; the total protein extracted varied from 1.0 to 1.6% (w/w) of the biomass at different pH values. It increases with pH and maximizes at pH 7 and then decreases when pH>7. Since a protein's solubility is at its minimum at its isoelectric point (p*I*) when it carries no net charge, it is less likely to be extracted when the buffer pH is at or near its p*I*. Fig. 4 thus implies that there are more acidic proteins (p*I*<7) than basic proteins (p*I*>7) in tobacco. When considering tobacco for recombinant protein production, a basic recombinant protein could be more favorable for purification since the purification burden (contaminants to be removed) is relatively low.

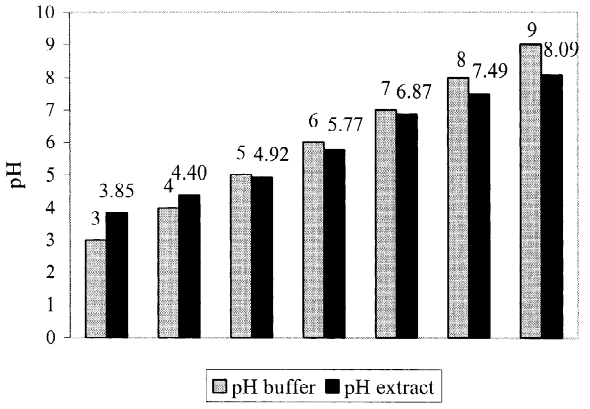

Fig. 3. Variation of the pH of extract compared to the pH of buffer. The gray bars represent the pH of the buffer, and the black bars represent the pH of the extract.

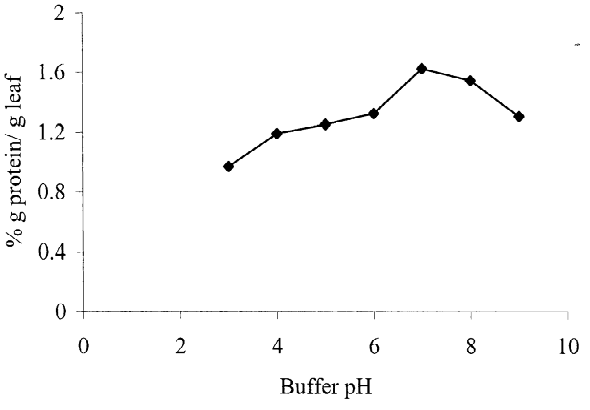

Fig. 4. Percentage of g protein extracted per g leaf vs. buffer pH. Each value is the average of 12 runs at each pH; extreme outliers were excluded from the data set.

3.2. ATPE studies

3.2.1. ATPE—tobacco extract

The partitioning behavior of native tobacco protein was studied for three systems, PEG–potassium phosphate, PEG–sodium sulfate and PEG–ammonium sulfate systems. Two-level fractional factorial experiments were conducted to study the effect of following factors on the partitioning of tobacco protein: PEG molecular mass, PEG concentration, phase forming salt concentration, sodium chloride concentration, and pH of the system (Table 1). The level for the PEG molecular mass, PEG concentration, and salt concentration were chosen based on the phase diagrams [10] for the three systems studied. Since protein extraction maximized at pH 7, the pH range chosen was close to pH 7. The phase diagrams for this pH range have been reported [10]. Sodium chloride was used to adjust the ionic strength of the system, which has been shown to have a significant effect on protein partitioning [19].

All experiments were analyzed using MINITAB (version 13) software for statistics with the protein partition coefficient as response. As illustrated in Fig. 5 for PEG–potassium phosphate systems, the factors affecting the partition coefficient significantly were determined using the Pareto chart (Fig. 5a) and normal effects plot (Fig. 5b). The Pareto chart and the normal effects plot serve the same purpose. The Pareto chart is a bar chart with the effects plotted in decreasing order of magnitude. The line passing through the chart depends on the value of α (it is the value with which the P value of an effect is compared to determine whether an effect exists or not). Any factor with significance will extend beyond the line. The normal effect plot also detects significant factors based on the α values. The normality of the effects due to each factor is analyzed. Any factor that is significant does not conform to the normal plot and lies away from the normal line. Besides the main effects due to each factor, interactions among the factors can also affect protein partitioning. Among the interactions, two-factor interactions are most important. Higher order interactions are generally rare, and even when present they are usually insignificant. The factors of significance for each system considered and the range of the partition coefficients for these systems for native tobacco protein are shown in Table 2. The trend of each

Table 1
Factors and levels for fractional factorial study of native tobacco protein partitioning

Factor	Low level	High level
PEG molecular mass	3400	8000
PEG concentration (% w/w)	10	15
Salt concentration (% w/w)	13	18
NaCl concentration (M)	0.1	1.2
pH	6	8

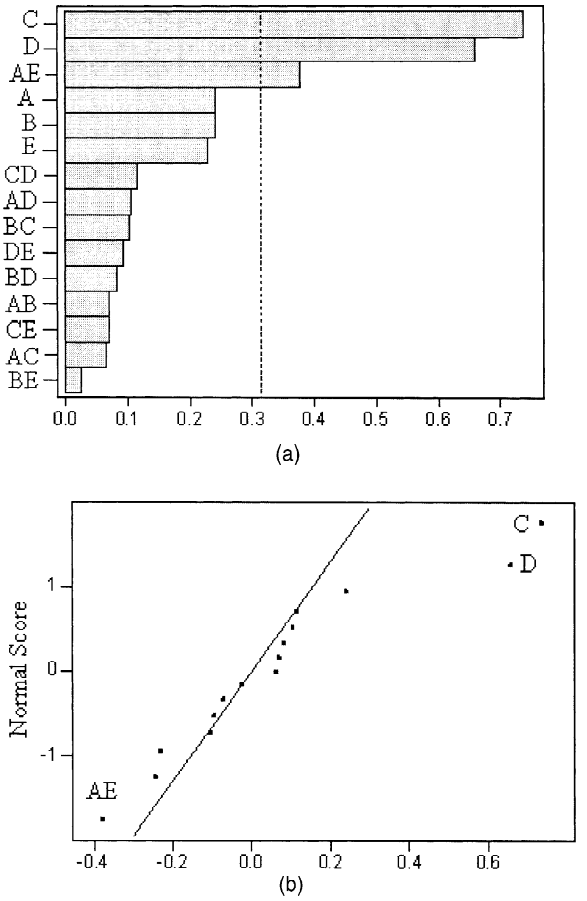

Fig. 5. Statistical analysis in determining the factors having significant effect on tobacco protein's partitioning. (a) Pareto chart for PEG–potassium phosphate system with $\alpha = 0.1$. A = PEG molecular mass, B = PEG concentration, C = salt concentration, D = NaCl concentration, E = pH. Two-letter symbols represent the interaction between two main factors. For example, AE is the interaction of PEG molecular mass and pH. (b) Normal effect plot for PEG–potassium phosphate system with $\alpha = 0.1$.

Table 2
Results of ATPE for tobacco extract: (+) 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 (−)

factor was determined from the main effect plots (data not shown). For the PEG–potassium phosphate system, increases in both the potassium phosphate and sodium chloride concentrations increases the partition coefficient, and for the PEG–ammonium sulfate system, the partition coefficient increases with an increase in salt concentration and decreases with an increase in PEG molecular mass.

As shown in Table 2, the minimum partition coefficient for tobacco protein was greater than 3 for PEG–ammonium sulfate systems. Since a lower partition coefficient of native tobacco protein will favor obtaining a high selectivity for recombinant proteins, PEG–ammonium sulfate system was not included in following studies. Moreover, the fact that no factor significantly affects the partitioning of tobacco protein in PEG–sodium sulfate system can be advantageous. If, for the same system, certain factors significantly influence the partitioning of the model protein, these factors can then be adjusted to increase the partition coefficient of the model protein while maintaining that of tobacco protein relatively constant. The selectivity could therefore be greatly enhanced.

3.2.2. ATPE—lysozyme

Egg white lysozyme was used as a model protein to mimic the presence of a recombinant protein in transgenic tobacco. Before conducting spiking experiments, the partitioning behavior of pure lysozyme was first determined in PEG–potassium phosphate and PEG–sodium sulfate systems. Two-level fractional factorial experiments were run using the same factors and levels as listed in Table 1. The partition coefficient of lysozyme in PEG–potassium phosphate systems varied between 10 and 40 with no factor statistically significant. For PEG–sodium sulfate systems, the most significant factor was sodium chloride concentration followed by sodium sulfate concentration. The partition coefficient of lysozyme ranged from 6 to 80. As the PEG–sodium sulfate system had a higher partition coefficient for egg white lysozyme under certain conditions, it was selected for further optimization studies using response surface design.

Fig. 6 gives the main effect plot for PEG–sodium sulfate systems. Each graph on Fig. 6 gives the trend of the partition coefficients for both tobacco protein and lysozyme for all five factors between the two levels considered. Examining the details of the main effect plots reveals that sodium chloride affects the partitioning of both tobacco protein and lysozyme in the same way to different extent, but on the other hand, sodium sulfate concentration has the opposite effect on their partitioning. PEG molecular mass also has a similar effect on partitioning of tobacco protein and lysozyme, to different extents. PEG concentration and pH show opposite trends for both tobacco protein and lysozyme. Obviously, different factors have different effects on the partitioning of lysozyme and tobacco protein. These offer great opportunities to optimize the selectivity for lysozyme separation from tobacco extract.

3.2.3. ATPE—spiking experiments

Lysozyme was spiked into tobacco extract to examine if adjusting the factors in PEG–sodium sulfate systems can enhance its purification. Performing a response surface study with five factors would be a very tedious task involving a huge number of

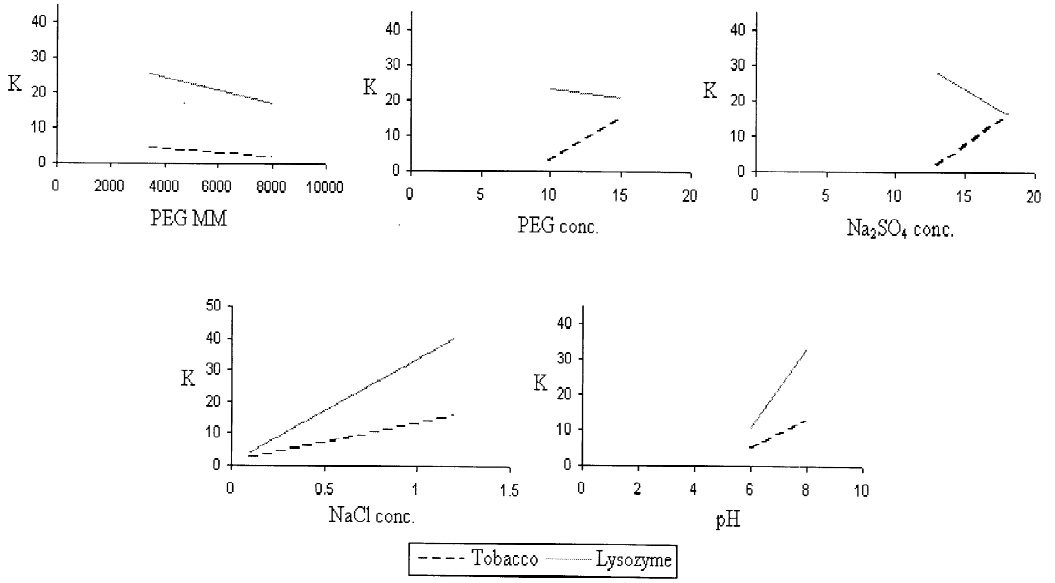

Fig. 6. Main effect plots for the PEG–sodium sulfate system. All plots have the partition coefficient (K) plotted against the levels of each factor i.e. PEG molecular mass (PEG MM) in g/mol, PEG and Na_2SO_4 concentrations in % (w/w), and NaCl concentration in molarity (M) and pH.

experiments. Since the significant factors affecting lysozyme partitioning in PEG–sodium sulfate system were sodium chloride and sodium sulfate concentrations, a response surface study was thus carried out only for these two factors while the other three factors were held constant. It can be seen from the main effect plots (Fig. 6) that lower PEG molecular mass and lower PEG concentrations were preferred for obtaining lower partition coefficient for tobacco protein and higher partition coefficient for lysozyme. Hence a PEG molecular mass was chosen as 3400 and the PEG concentration was maintained at 10% (w/w). The pH of the system was selected at the median, 7. Thirteen experiments were planned for response surface study of the two factors at two levels. The thirteen experiments correspond to four cube points, four axial points and five center points. Table 3 gives the list of conditions at which the response surface experiments were conducted. Sodium sulfate concentration levels were chosen based on the phase diagrams and the sodium chloride levels were altered to see if a higher concentration would increase the selectivity.

The partition coefficient for the total protein content was determined using the BCA assay and that for lysozyme was determined using lysozyme activity assay. The concentration of lysozyme in the bottom phase was very small. The absorbance values for the bottom phase did not change over the time for the activity assay. In order to obtain meaningful partition coefficients, the lysozyme activity was assumed to be equal to the minimum activity, which corresponds to 0.001 of absorbance change at 540 nm over 1 min of assaying time. The response surface design was analyzed using the selectivity as the desired response (Fig. 7). The conditions at which lysozyme selectivity over the native tobacco protein was highest were determined using the response optimizer provided by MINITAB (version 13) software.

The global solution of the response surface study predicted a selectivity value of 57 when sodium sulfate concentration was maintained at 16.2% (w/w) and sodium chloride concentration at 0.19 M with PEG 3400 at 10% (w/w) at pH 7. Nevertheless, as shown in Fig. 7, lysozyme selectivity can be increased by two approaches: (1) decreasing sodium sulfate concentration while increasing sodium chloride concentration, and (2) increasing sodium sulfate concentration but decreasing sodium chloride con-

Table 3
Central composite design for response surface study of the effect of sodium sulfate concentration and sodium chloride concentration on the selectivity of lysozyme; PEG molecular mass: 3400; PEG concentration: 10% (w/w) pH 7

Run order	Coded levels		Real values	
	Na_2SO_4 conc. (%, w/w)	NaCl conc. (*M*)	Na_2SO_4 conc. (%, w/w)	NaCl conc. (*M*)
1	−1	−1	9	0.4
2	1	−1	15	0.4
3	−1	1	9	1.4
4	1	1	15	1.4
5	−1.414	0	7.8	0.9
6	1.414	0	16.2	0.9
7	0	−1.414	12	0.2
8	0	1.414	12	1.6
9	0	0	12	0.9
10	0	0	12	0.9
11	0	0	12	0.9
12	0	0	12	0.9
13	0	0	12	0.9

centration. However, the adjustment of the concentrations of sodium sulfate and sodium chloride are limited by the following factors: (1) the solubility of sodium sulfate in water is around 30% (w/w), (2) high concentration of either salts might cause protein precipitation, (3) sodium sulfate concentration has to be high enough to produce two phases.

Two experiments at the globally selected conditions were carried out and produced selectivity values of 62.1 and 32.5, which averaged at 47. The huge variation in the selectivity values probably comes from the inaccuracy of lysozyme activity assay for the bottom phase. Since almost all lysozyme is partitioned into the top phase, the activity assay is not sensitive enough to accurately determine the amount of lysozyme stayed in the bottom phase. Besides the lysozyme activity assay, another factor that could contribute to the difference between the theoretically predicted and the experimental selectivity values could be the amount of extract added to the experimental systems. The tobacco extract could affect the formation of the two-phase systems and the compositions of each phase, and consequently the partition coefficients of lysozyme and tobacco protein. This effect is being studied in our research laboratory.

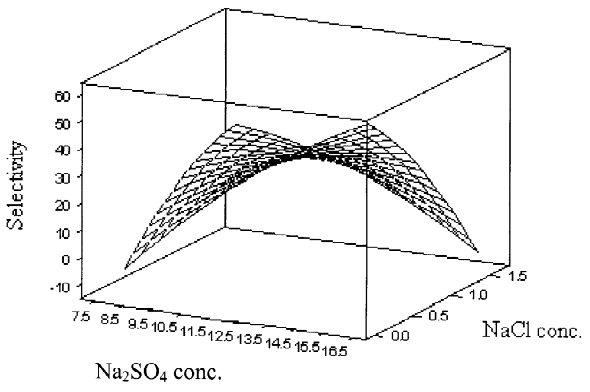

Fig. 7. Response surface design for egg white lysozyme spiked into tobacco extract. Lysozyme selectivity was used as the response estimate. Other conditions used in the study: PEG 3400 at 10% (w/w) and pH 7.

Since the selectivity of lysozyme would not change along the tie line, the yield and purification factor were calculated for various phase ratios based on the selectivity obtained from the experiments with the conditions determined from the response surface study (Table 4). As shown in Table 4, the yield of lysozyme decreases with phase ratio, but that is more than compensated for with 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. The purification factor and concentration factor are improved four and seven times, respectively, compared to when the phase ratio is 1.

Table 4
Theoretical yield and purification factor of lysozyme at various phase ratios along the same tie line

Phase ratio	Lysozyme yield	Tobacco protein yield	Purification factor	Concentration factor
1:1	0.99	0.77	1.16	1.98
1:5	0.95	0.34	2.23	5.72
1:10	0.91	0.20	3.24	10.05
1:15	0.87	0.14	3.99	14.01
1:20	0.84	0.11	4.58	17.66
1:30	0.77	0.07	5.44	24.16

The above work has demonstrated that ATPE is a very useful tool in lysozyme recovery and purification from tobacco extract. Given the bountiful choice of phase forming salts and the fact that various factors can be adjusted to influence protein partitioning, ATPE is shown to be a versatile and promising technique for protein recovery and purification from transgenic plants. Even though the development of an ATPE process is protein- and expression-system dependent due to the lack of predictability of protein partitioning, a statistical design of the experiments can be used for system screening and process optimization to expedite the process development. It is our hope that the promise this study has shown for using ATPE in protein purification from tobacco will lead to more extensive applications of this technique in protein recovery and purification from transgenic plants.

4. Conclusions

The amount of soluble protein extracted from flue-cured tobacco leaves maximized at pH 7. A PEG–sodium sulfate system proved to be the most suitable system for egg white lysozyme recovery among the three systems considered. The study using the model system showed that it is possible to have high selectivity of lysozyme over the native tobacco protein for this system. The response surface study predicted that selectivity values as high as 57 could be obtained when the sodium sulfate concentration was maintained at 16.2% (w/w) and sodium chloride concentration at 0.19 *M* with PEG 3400 at 10% (w/w) at pH 7. Experiments at these conditions resulted in selectivity values that averaged around 47, and that was used to calculate the theoretical purification factor and yield under various phase ratios. At a phase ratio of 1:15, a yield of 87% and a purification factor of 4 with a concentration factor of 14 for egg white lysozyme could be obtained. It is possible that the optimum ATPE conditions for lysozyme purification from tobacco extract might be further improved using PEG with different molecular masses or other PEG–salt systems. Nevertheless, this study has clearly shown that ATPE can be successfully used for recombinant protein recovery and initial purification from transgenic tobacco.

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References

[1] V. Salmon, D. Legrand, M. Slomianny, I.E. Yazidi, G. Spik, V. Gruber, P. Bournat, B. Olagnier, D. Mison, M. Theisen, B. Merot, Prot. Expr. Purif. 13 (1998) 127.
[2] X.G. Wang, G.H. Zhang, C.X. Liu, Y.H. Zhang, C.Z. Xiao, R.X. Fang, Biotechnol. Bioeng. 72 (4) (2001) 490.
[3] P.C. Sehnke, L. Pedross, A.L. Paul, A.E. Frankel, R.J. Ferl, J. Biol. Chem. 269 (1994) 22473.
[4] C.L. Cramer, D.L. Weissenborn, K.K. Oishi, E.A. Grabau, S. Bennett, E. Ponce, G.A. Grabowski, D.N. Radin, Ann. NY Acad. Sci. 792 (1996) 62.
[5] P.A. Albertsson, Partition of Cell Particles and Macromolecules, 3rd ed, Wiley, New York, 1986.
[6] R.H. Kaul (Ed.), Aqueous Two-phase Systems, Methods and Protocols, Humana Press, Clifton, NJ, 2000.
[7] D.E. Brooks, K.A. Sharp, D. Fisher, in: H. Walter, D. Brooks, D. Fisher (Eds.), Partitioning in Aqueous Two-Phase Systems, Academic, New York, 1985, p. 12.
[8] M.J. Boland, P.G.M. Hesselink, N. Papamicheal, H. Hustedt, J. Biotechnol. 19 (1991) 19.

[9] A. Salabat, Fluid Phase Equil. 187–188 (2001) 489.
[10] A.D. Diamond, J.T. Hsu, Adv. Biochem. Eng./Biotechnol. 47 (1992) 89.
[11] B.Y. Zaslavsky, Aqueous Two-Phase Partitioning, Marcel Dekker, New York, 1995.
[12] C. Li, J. Bai, W. Li, Z. Cai, F. Ouyang, Biotechnol. Prog. 17 (2) (2001) 366.
[13] Y. Shi, P.J. Weimer, Appl. Environ. Microbiol. 58 (1992) 2583.
[14] S. Oh, S. Rheem, J. Sim, S. Kim, Y. Baek, Appl. Environ. Microbiol. 61 (1995) 3809.
[15] T.D. Reed, J.L. Jones, C.S. Johnson, P.J. Semtner, C.A. Wilkinson, Virginia Cooperative Ext. Publ. 436-048, (2001).
[16] V.S. Stoll, J.S. Blanchard, Methods Enzymol. 182 (1990) 31.
[17] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Anal. Biochem. 150 (1985) 76.
[18] R.M. Parry Jr., R.C. Chandan, K.M. Shahani, Proc. Soc. Exp. Biol. Med. 119 (1965) 384.
[19] J.A. Asenjo, R.E. Turner, S.L. Mistry, A. Kaul, J. Chromatogr. A 668 (1994) 129.