LYSOZYME SEPARATION FROM TOBACCO EXTRACT BY AQUEOUS TWO-PHASE EXTRACTION

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(ABSTRACT)

Tobacco has long been considered as a host to produce large quantities of high-valued recombinant proteins. However, dealing with large quantities of biomass with a dilute concentration of product is a challenge for down-stream processing. Aqueous two-phase extraction (ATPE) has been used in purifying proteins from various sources. It is a protein-friendly process and can be scaled up easily. ATPE was studied for its applicability to recombinant protein purification from tobacco using egg white lysozyme as the model protein. Separate experiments with polyethyleneglycol(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 weight, PEG concentration, the concentration of phase forming salt, sodium chloride concentration, and pH on protein partitioning. The results showed that 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. The binodial curve and tie line corresponding to the optimal condition for lysozyme recovery for the PEG 3400/sodium sulfate system were developed. The selectivity at the optimal condition was experimentally determined to be 47 with a lysozyme yield of 79.6 % with a purification factor of 10 and a concentration factor of 20. From this study, ATPE was shown to be suitable for initial protein recovery and partial purification from transgenic tobacco.

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CHAPTER 1

INTRODUCTION

Production of recombinant protein for therapeutic use is gaining popularity in recent years. Sales of drugs of biological origin are projected to increase to approximately 40% of the pharmaceutical market in the year 2005 [1]. The production of therapeutic proteins has been made possible by new discoveries in biotechnology. Many researchers have successfully developed methods to express different types of proteins in various biological systems, such as microbial, plants, and animals. Introduction of this new technology necessitates innovation in process development for economic protein recovery and purification. There is the need to design new, as well as to improve existing processes associated with downstream processing for product isolation and purification.

1.1 Production of recombinant human proteins with tobacco

Tobacco is widely grown in the southeastern United States, but its production is declining due to the health issues associated with smoking and related federal regulations [2]. Using tobacco to produce therapeutic recombinant human proteins is a promising alternative. Genetic modification of tobacco is easy, and it is suitable for large-scale

production [3]. Each tobacco plant produces thousands of seeds which facilitates the propagation of the altered plant. Also, the leaf biomass production per acre is much higher than most other crops, which facilitates scale up. Many recombinant proteins such as lactoferin, cholera toxin B, and ricin B, have been expressed in tobacco [4, 5, 6]. However, the use of tobacco as host to produce therapeutic proteins on a large scale is dependent on the development of cost effective and efficient methods to handle huge amounts of biomass and to purify the expressed proteins. Since the overall cost of a protein production is mainly determined by the efficiency of the initial recovery and purification, extraction and initial purification procedures to obtain recombinant proteins from tobacco need to be developed.

1.2 Aqueous two-phase extraction

Aqueous two-phase extraction (ATPE) has been widely used for protein recovery and purification [7,8]. In ATPE, two immiscible phases are formed when polymers such as polyethylene glycol (PEG) are mixed with other polymers, such as dextran, or salts in particular concentrations. The equilibrium distribution (partitioning) of a protein in ATPE depends not only on its own surface properties such as charge and hydrophobicity but also on the physicochemical properties of the two phases [9], which can be manipulated by adjusting factors such as the polymer molecular weight 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 a single step as compared to the several steps involved in conventional down stream processing, including recovery, clarification, filtration, concentration, and initial 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 the use of ATPE to recover superoxide dimutase from bovine liver tissue have been demonstrated [10]. Among the 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 lower viscosity and lower cost [11, 12].

Production of mature transgenic tobacco plants that can be used to investigate down stream processing methods is very time consuming and difficult. Model experiments can be used instead to study the partitioning behavior of proteins in tobacco extract. In model experiments, a model protein with known properties is added to the native tobacco extract to simulate the protein being extracted from transgenic tobacco. 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 [7, 13]. The phase diagrams define the PEG and salt concentrations at which two-phase systems will be formed. They also aid 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 is utilized.

The selectivity is defined by

$$\alpha = K_P / K_C \tag{1.1}$$

where K_P = partition coefficient of the target protein, and

K_C = lumped partition coefficient of proteins other than the target protein.

Since the partition coefficients of proteins are the same on a tie line (See Sec.2.3.1), 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,

$$Phase \quad Ratio = \frac{Mass \quad of \quad Top \quad Phase}{Mass \quad of \quad Bottom \quad Phase} = \frac{DF}{ED} \tag{1.2}$$

where *DF* and *ED* are the tie line lengths illustrated in Fig. 1.1. The lower the phase ratio, the higher will be the purification factor (if the selectivity is greater than one). Increasing *ED* can decrease the phase ratio. It must be noted that 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.

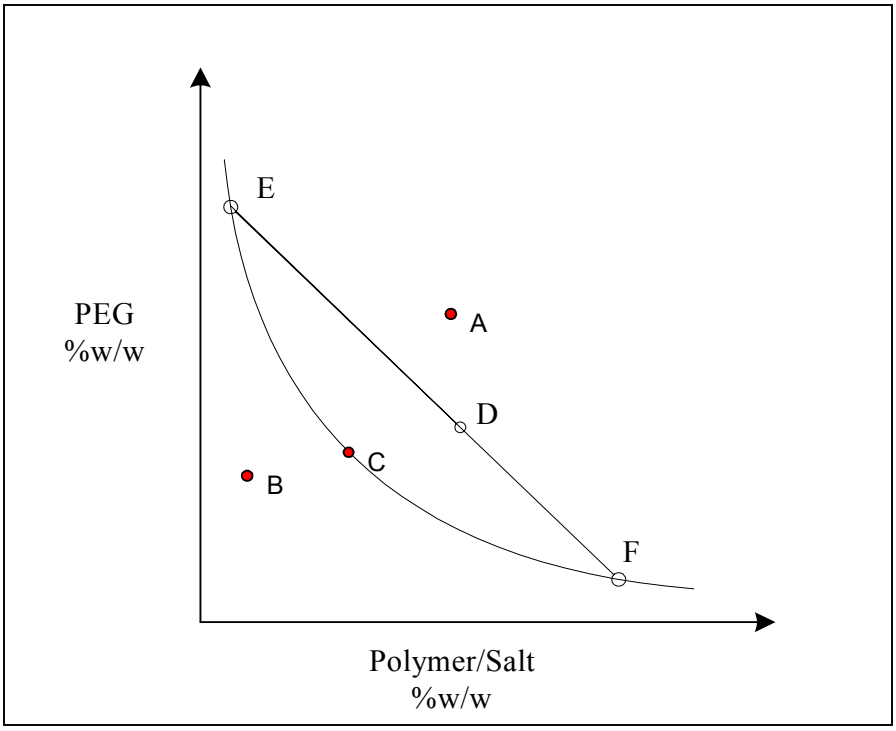


Fig. 1.1 Schematic phase diagram of ATPS.
A = two phases, *B* = one liquid phase, C = critical point, *EF*= tie line, *E* = composition of the top phase, F = compositions of the bottom phase, and D = total composition.

The scope of this study 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 designs are a convenient method to study the effects of a large number of factors and determine the significant effect the factors may have on the response of interest (protein partition coefficient in this case). The significant factors and their effects can be studied with fewer numbers of runs using fractional factorial runs. Statistical design of experimental procedures have been used to study protein separation by aqueous two-phase systems [14], and response surface designs have proven to be a very useful tool for optimization of bio-separation processes [14-16].

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 molecular weight of 14400. It was chosen as it is not natively produced in tobacco and can be quantitatively assayed easily and accurately in the presence of other proteins.

1.3 Objectives

1. Determine total soluble protein and effect of pH of the extraction buffer on total soluble protein extracted from leaves of flue-cured tobacco.

2. Study the partition behavior of native tobacco proteins in various two-phase systems.

3. Study the partition behavior of a model protein, lysozyme, in selected two-phase systems compared to native tobacco protein.

4. Select most suitable system and determine best conditions for recovery of model protein, lysozyme, spiked into tobacco extract.

5. Develop phase diagram and tie lines for the selected system, and use the data to determine the optimal recovery conditions for lysozyme from tobacco.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview

Protein recovery and purification are the greatest obstacles to overcome before any further development can be made to utilize tobacco for recombinant protein production [3]. The cost of recovery is a major factor influencing the recovery process to be used. Aqueous two-phase extraction (ATPE) has been widely used for protein recovery and purification. ATPE has been recognized as an economical and efficient downstream processing method and offers many advantages such as low process time, low energy consumption and a biocompatible environment [17]. This chapter deals with purification of protein expressed in tobacco, aqueous two-phase systems (ATPS), their properties and research conducted on the use of aqueous two-phase systems for the recovery of biomolecules such as proteins and enzymes.

2.2 Purification of recombinant proteins expressed in tobacco

Many recombinant proteins have been expressed in tobacco. A few examples are cationic peanut peroxidase [4], cholera toxin B subunit [5], insect chitinase [18], human lactoferrin [19], and lactate dehydrogenase [20].

Some researchers have tried to purify recombinant protein from tobacco leaves using chromatographic techniques. In the purification of cationic peanut peroxidase (CPRX) expressed in transgenic tobacco, histidine tags were added at its C-terminal to aid purification by single step metal affinity chromatography [18]. A similar technique was used to purify the enzyme lactate dehydrogenase [19]. In this study, metal affinity precipitation using chelating molecules like ethylene glycol-bis (β –aminoethyl ether) N, N, N', N'-tetra acetic acid (EGTA) charged with zinc or nickel ions was compared to affinity chromatography. This work also claims that affinity precipitation can be scaled up easily compared to chromatography, although the purification and yield may be slightly lower [19]. Both methods still involve adding genes to attach histidines to one end of the enzyme and use of expensive chemicals for purification, such as EGTA, Ni^{2+}-NTA resin, imidazole, and etc.

Cholera toxin B (CTB) expressed in tobacco was purified by a single step immunoaffinity chromatography using rabbit anti-CT IgG coupled with CNBrp

Sepharose 4B packed column [5]. Insect chitinase was purified by initial chromatography using anion exchange column followed by cation exchange chromatography for final purification [20]. However, purification methods based on chromatographic procedures for production of proteins on a large scale directly from crude extract are often less attractive due to high costs and column fouling [19].

2.3 Aqueous two-phase systems

The production of recombinant proteins and enzymes requires the development of an adequate separation technology at reasonable cost. Reducing the cost of protein purification has always been a challenge facing bioseparation scientists. For the past half century, researchers have been looking into liquid-liquid extraction systems for protein recovery and purification. Normally liquid-liquid extraction involves the use of organic solvents that are not suitable for protein recovery as proteins are either insoluble in organic solvents or denatured [21]. Liquid-liquid binary phases can also be formed using two polymers or polymer/salt solutions. Beijerinck first described this phenomenon in 1896 [7], when he observed the formation of two-phases after mixing agar with soluble starch or gelatin. These binary systems are called aqueous two-phase system and were first applied to recover biomolecules in 1956 by Albertsson [22]. Albertsson showed that microorganisms, cell walls, chloroplasts, chloroplast vesicles and other biological molecules partitioned selectively between the phases of an aqueous two-phase system

composed of either two polymers (PEG and Dextran) or a polymer and a salt (PEG and potassium phosphate). He further studied the effect of different polymers, their concentration and molecular weight, and developed phase diagrams for several systems. He developed procedures for the isolation of cell walls from *Chlorella* and *Aerobacter*. This work formed the basis for later applications to viruses, cells and cell organelles [22]. He also showed that the partition of proteins between the phases was reversible and that the partition coefficient could be adjusted by varying the salt composition of the two-phase system.

Both polymer/polymer and polymer/salt aqueous two-phase systems have advantages over conventional extraction using organic solvents. Aqueous two-phase systems provide an excellent environment for cells, cell organelles and biologically active proteins as they contain a large proportion of water in both phases. The interfacial tension for aqueous systems is low when compared to liquid-liquid extraction systems containing organic solvents, and that allows one phase to disperse into the other and thus create a high interfacial contact area for efficient mass transfer. The polymers are also known to have a stabilizing influence on the biological activity and structure of proteins and enzymes [7, 8]. The basis of separation is the selective distribution of substances between the two-phases. Small molecules are evenly distributed between the phases, but the partitioning of macromolecules is variable [7, 8]. A number of investigators have tried to study and explain the physical interactions influencing two-phase systems and model the

factors affecting protein partitioning [7, 13, 23]. However, the prediction of partition coefficients of proteins is still very difficult. The difficulties arise due to the complex and interrelated interactions between polymers, salts, buffers, water and protein present. Therefore, development of separation processes using aqueous two-phase systems relies upon extensive experimentation, which could be significantly simplified by using factorial experimental design methods [24].

Aqueous two-phase extraction involves two unit operations, equilibration and phase separation. Equilibration involves the mixing of the components that form the two-phase system and dispersion of the phases to obtain equilibrium between the two-phases. This process is generally very rapid. The phase separation under gravity is slower than in water-organic solvent systems and varies from a few minutes to a few hours. The main reason for this is the small difference in densities and viscosities between the two phases. However, the process can be enhanced by centrifugation at low speeds [8].

2.3.1 Phase Diagram

Each two-phase system has a unique phase diagram under a particular set of conditions such as pH and temperature. The phase diagram provides information about i) concentration of phase forming components required to form a two-phase, ii) the

concentration of phase components in the top and bottom phases, and iii) the ratio of phase volumes.

The diagram consists of the binodial curve and tie lines (Fig. 1.1). The binodial curve divides a region into component concentrations that will form two immiscible phases from those that will form one phase. The tie line connects two nodes on the binodial, which represent the final concentration of phase components in the top and bottom phases. Coordinates for all the potential systems that will have the same compositions for the top and bottom phases will lie on a tie line, but moving along the tie line coordinates specifies systems having the different total compositions and volume ratios. The point on the binodial at which the composition and volume of both phases are almost equal is called the critical point [25]. Phase diagram data is required for the design of ATPS extraction processes and development of models that predict partitioning of biomolecules [24].

Binodial curve

Developing the binodial curve is necessary for choosing system parameters for preliminary partition experiments. Phase diagrams for PEG/dextran and PEG/salt systems for various PEG molecular weights have already been published by [7, 13, 26]. One of

the following methods is generally used to develop the binodial curve for a polymer/polymer or polymer/salt systems.

1. Cloud point method [7]: A few grams of concentrated polymer solution are weighed into a test tube. A solution of known concentration of the second polymer or salt is added drop-by-drop to the test tube and mixed. The solution is clear at first, but after a certain amount of the second solution (polymer or salt) is added, one further drop makes the mixture turbid and the mixture separates into two phases. The mass of the mixture is noted and the composition of the two-phase system is determined. Adding a few drops of water makes the mixture clear again and the above procedure is repeated over a whole range of concentrations starting from both the solutions of the two-phase system.

2. Turbidometric titration method [25]: A series of systems of known total compositions and mass are prepared. These are then diluted with water or appropriate solvent, the mixture eventually becomes clear and a single phase is formed. The phase composition at the point of transition is calculated and plotted.

The above methods are relatively inaccurate when working with polydisperse polymers such as dextran/methylcellulose system [7] as they produce a gradual increase/decrease in turbidity rather than a sharp change.

Tie line

The tie line length has units of %w/w, same as the component concentrations. The length of the tie line is related to the mass of the phases by the equation

$$\frac{V_t \rho_t}{V_b \rho_b} = \frac{DF}{ED} \tag{2.1}$$

Where V and ρ are the volumes and densities of the top (t) and bottom (b) phases and DF and DE are the lengths of the tie line as shown in Fig. 1.1 [25]. Tie lines are commonly parallel, and hence the slope of the tie line can be used to construct other tie lines. Analysis of phase compositions, and the determination of amount of polymer and salt in each phase is carried out by using methods such as size exclusion chromatography, gravimetric analysis, refractive index measurement, optical rotation and conductivity measurements [7, 26].

2.3.2 Partition coefficient

The partition coefficient is defined as

$$K = \frac{c_t}{c_b} \tag{2.2}$$

where, c_t and c_b are the equilibrium concentration of the protein in the top and bottom phases. The partition coefficient of a protein is constant for all the systems on a particular

tie line provided there is no association or disassociation of oligomeric proteins in any of the phases. The partitioning is affected by a number of factors discussed below. Suitable conditions to obtain a particular partition coefficient have to be experimentally determined [21].

2.3.3 Factors that affect partitioning in ATPE

The properties of any two-phase system depend on the type and concentration of polymer and salt, the temperature and the presence of other salts and other low molecular weight substances such as detergents, sugars and pH.

1. Polymers

   Polyethylene glycol and dextran are the most commonly used polymers. Both polymers are non-toxic and have been thoroughly tested for food and pharmaceutical applications. This is of importance, as any substance to be used in recovery of therapeutic proteins needs to be tested and proved safe for use. A number of other polymers have been studied, and phase diagrams for dextran/polyethylene glycol, dextran/ficoll, polyethylene glycol/ficoll, polyethylene glycol/salt systems have been widely reported in literature [7, 26]. For industrial applications, highly purified dextran is expensive and the most suitable systems are the polyethylene glycol/ salt systems.

The molecular weight of the polymer used also influences partitioning of proteins. The greater the molecular weight, the lower the concentration required for phase separation [7]. As polymer concentration increases, differences in density, refractive index, and viscosity between the phases increase.

2. Time for phase separation

The time required for the two phases to separate varies from system to system. It depends on the density and viscosity of the phases and the time required for small droplets that are formed during mixing to coalesce into larger drops. The settling time is greatest near the critical point because density difference is small, and it is also great for a system far away from the critical point because polymer concentration and hence viscosity is high. The settling times are shortest at intermediate compositions [7]. The PEG/salt and PEG/dextran systems have the shortest settling times [7].

3. Effect of low-molecular weight substances

Systems containing nonionic polymers are hardly affected by the addition of substances such as 0.1-1M sugars or NaCl. The phase diagram and critical composition do not change much by the addition of such substances. Only high salt concentrations (greater than 1M) have any effect on the phase systems [7]. In PEG/salt systems, salting out effect is seen with increasing tie line length shifting

proteins from the salt phase to the PEG rich phase. If the solubility of the PEG rich phase is not high enough, protein precipitation will occur at the interface [21].

4. Temperature

It is important to maintain the temperature constant during any phase separation experiment as it affects the composition of the two phases and hence the protein partitioning. As temperature increases, the solubility of PEG in water decreases, and lower concentrations of PEG and salt can be used to obtain phase separation [27].

2.4 Product recovery in Biotechnology – Applications for ATPE

Biological molecules such as proteins are generally obtained in very dilute solutions; hence the first step in their recovery is usually concentration. An aqueous two-phase system can be used to concentrate samples by choosing the phase composition such that the desired substance is transferred into a phase with a smaller volume compared to the original solution. A single or multi-step procedure may be used depending on the partition characteristics of the desired and undesired substances [8].

The increase in applications of recombinant DNA technology for protein production has brought focus on downstream processing and product recovery. Traditionally, purification is done in steps involving clarification - using solid/liquid

separation techniques (centrifugation, filtration), followed by concentration. Product loss usually increases when the number of processing steps increases. ATPS circumvents many of the shortcomings of processes such as centrifugation and filtration that arise due to high viscosity and heterogeneous distribution of particle size. If optimized properly, ATPS provides integration of clarification, concentration and partial purification. This reduces the number of downstream processing steps and improves product yields and cost of product recovery.

The application of ATPS that has attracted the most interest is in recovery and isolation of proteins from crude feedstock. Pilot-scale studies to assess the feasibility of PEG/salt systems for extraction of enzymes (superoxide dimutase) from bovine liver tissue have been carried out. The enzyme was purified using 15% PEG/ 8% phosphate system and a 4-fold purification and recovery rate of 83% was obtained [10]. Though ATPS has a number of advantages, there is limited use due to poor understanding of the mechanisms involved in partitioning. The lack of knowledge has made the method wholly empirical.

The cost of phase forming substances and associated waste treatment are also to be considered. In the study to determine the partition of thaumatin in the presence of *Escherichia coli* contaminant proteins using the PEG/phosphate system, the enzyme was back extracted into a new phosphate phase from the polymer phase in a second step. This

allowed PEG to be recycled to the first extraction stage. Recycling will help in reducing the required chemical costs. Asenjo *et al.* [28] also showed that for phosphate systems an increase in NaCl concentration increased the partition coefficient of thaumatin from one to as high as 53.

ATPE has also been used to recover proteins from plant tissues. It has been used to purify peroxidase from the leaves of *Ipomoea palmetta* using a PEG/ammonium sulfate system, followed by gel filtration chromatography. Desired reduction in volume of the extract and selective partitioning was achieved by varying the phase composition and the NaCl concentration. Using a PEG/ammonium sulfate/NaCl (24/7.5/2 % w/v) system, the following performance was obtained, partition coefficient of 0.042, purification factor of 2.18, and volume reduction of 57.5% [17].

2.5 Summary

Many detailed studies have been conducted on the properties of aqueous two-phase systems. Researchers have tried to quantify the factors affecting partitioning and the mechanism of partitioning. However, it is very difficult to predict which factors affect the partitioning of proteins and enzymes, or to what extent they affect partitioning in two-phase systems. There is no work reported on using aqueous two-phase extraction to recover proteins from tobacco. This study concentrates on studying partitioning of native tobacco proteins and a model protein (lysozyme) in tobacco extract to determine the applicability of two-phase extraction for recombinant protein recovery from tobacco.

CHAPTER 3

EXPERIMENTAL

3.1. Materials

Flue-cured tobacco (*Nicotiana tabacum* L.) cultivar K 326 was grown at the Virginia Tech Southern Piedmont Agriculture Research and Extension Center, Blackstone, VA during the summer of 2001. Seedlings were produced according to standard production practices. Plots were fertilized with 282 kg N/ha of 6-12-18-analysis fertilizer prior to transplanting. Seedlings were transplanted at a rate of 118300 plants/ha with a vegetable transplanter [29]. Plants were harvested about six 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.

Polyethylene glycol (molecular weight 3400 and 8000), potassium phosphate monobasic, potassium phosphate dibasic and ammonium sulfate were obtained from Sigma (St. Louis, MO). Sodium phosphate monobasic, sodium phosphate dibasic, sodium chloride, citric acid, sodium citrate, Tris base, and sodium sulfate were obtained from Fischer Scientific (Pittsburgh, PA). Bicinchoninic acid (BCA) assay reagents and bovine

serum albumin (2mg/ml) were obtained from Pierce Biotechnology (Rockford, IL). Lysozyme from chicken egg white and *Micrococcus lysodeikticus* were obtained from Sigma (St. Louis, MO).

The fractional factorial experiments to study partition behavior of native tobacco protein and pure lysozyme, and response surface analysis to determine the conditions for maximum selectivity of the model protein, lysozyme, were assisted by the statistical software, MINITAB (version 13).

3.2 Methods

Fig 3.1 gives the flow diagram of the steps involved in study of recovery of a model protein from flue cured tobacco leaves.

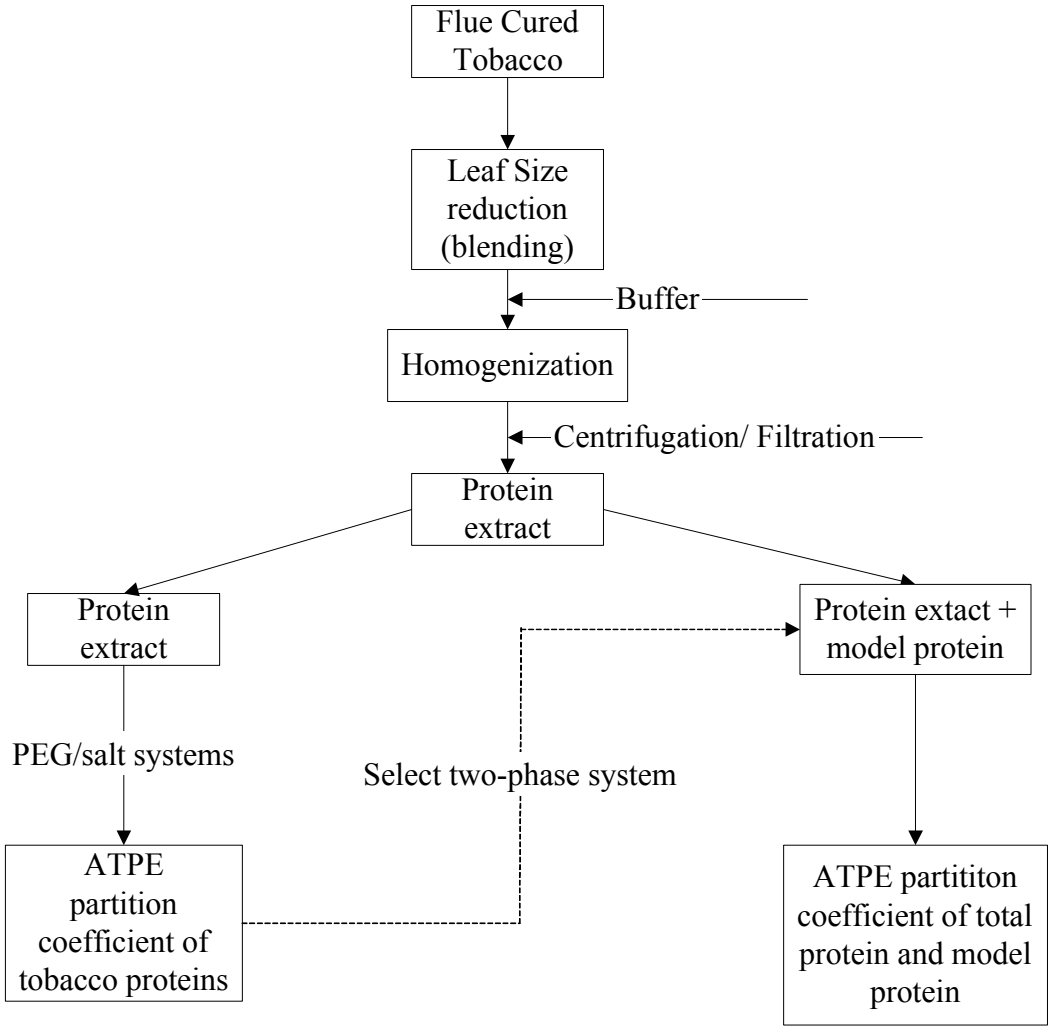


Fig. 3.1. Flow chart of operations involved in protein extraction from tobacco by aqueous two-phase extraction.

3.2.1. Tobacco extract preparation

The flue-cured tobacco leaves were thawed at room temperature, washed with de-ionized 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 leaf were used to extract the protein from the leaf sample. The pH range studied was 3 to 9. All extraction buffers were 50 mM of corresponding salts. Sodium citrate/citric acid was used to prepare pH 3 to pH 5 buffers. Buffers from pH 6 to 8 were prepared using sodium phosphate and pH 9 buffer was prepared using Tris base [30]. The blended leaves were then homogenized using a Power Gen 700 (Fischer Scientific). The homogenized extract was allowed to stand for 20 minutes at room temperature (20 °C) and then centrifuged at 4 °C, 12857 × g for 15 minutes. 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.

3.2.2. BCA assay

Protein concentration was determined by Bicinchoninic Acid (BCA) assay [31]. Bovine serum albumin (BSA) was used as standard. 50 μl 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 using a UV – 1601 spectrophotometer from Shimadzu (Kyoto, Japan)

3.2.3. 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, 18, 26]. Five gram systems were prepared using stock solutions of PEG dissolved in deionized water and the phase forming salt dissolved in buffer of appropriate pH. Tables 3.1 – 3.3 give the experimental conditions for each individual system studied. The systems were thoroughly mixed first, and then centrifuged at 1157 × g at room temperature for 10 minutes to expedite the phase separation. The centrifuged sample was allowed to stand for 30 minutes 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 pre-weighed micro-centrifuge tube. The protein concentration in each phase was determined by BCA assay.

Table 3.1 Experimental conditions for PEG/potassium phosphate/tobacco extract system.

PEG stock solution – 40 %w/w; potassium phosphate stock solution – 40 %w/w

Run Order	PEG MW	PEG conc. %w/w	Potassium phosphate conc. %w/w	NaCl conc. M	pH	PEG stock g	Potassium phosphate stock g	NaCl g	Tobacco extract g
1	3400	10	13	0.1	6	1.25	1.63	0.03	2.09
2	8000	10	13	0.1	8	1.25	1.63	0.03	2.09
3	3400	15	13	0.1	8	1.88	1.63	0.03	1.46
4	8000	15	13	0.1	6	1.88	1.63	0.03	1.46
5	3400	10	18	0.1	8	1.25	2.25	0.03	1.47
6	8000	10	18	0.1	6	1.25	2.25	0.03	1.47
7	3400	15	18	0.1	6	1.88	2.25	0.03	0.84
8	8000	15	18	0.1	8	1.88	2.25	0.03	0.84
9	3400	10	13	1.2	8	1.25	1.63	0.35	1.77
10	8000	10	13	1.2	6	1.25	1.63	0.35	1.77
11	3400	15	13	1.2	6	1.88	1.63	0.35	1.14
12	8000	15	13	1.2	8	1.88	1.63	0.35	1.14
13	3400	10	18	1.2	6	1.25	2.25	0.35	1.15
14	8000	10	18	1.2	8	1.25	2.25	0.35	1.15
15	3400	15	18	1.2	8	1.88	2.25	0.35	0.52
16	8000	15	18	1.2	6	1.88	2.25	0.35	0.52

Table 3.2 Experimental conditions for PEG/sodium sulfate/tobacco extract system.

PEG stock solution – 50 %w/w; sodium sulfate stock solution – 30 %w/w

Run Order	PEG MW	PEG conc. %w/w	Sodium sulfate conc. %w/w	NaCl conc. M	pH	PEG stock g	Sodium sulfate stock g	NaCl g
1	3400	10	13	0.1	8	1.0	2.17	0.03
2	8000	10	13	0.1	6	1.0	2.17	0.03
3	3400	15	13	0.1	6	1.5	2.17	0.03
4	8000	15	13	0.1	8	1.5	2.17	0.03
5	3400	10	18	0.1	6	1.0	3.00	0.03
6	8000	10	18	0.1	8	1.0	3.00	0.03
7	3400	15	18	0.1	8	1.5	3.00	0.03
8	8000	15	18	0.1	6	1.5	3.00	0.03
9	3400	10	13	1.2	6	1.0	2.17	0.35
10	8000	10	13	1.2	8	1.0	2.17	0.35
11	3400	15	13	1.2	8	1.5	2.17	0.35
12	8000	15	13	1.2	6	1.5	2.17	0.35
13	3400	10	18	1.2	8	1.0	3.00	0.35
14	8000	10	18	1.2	6	1.0	3.00	0.35
15	3400	15	18	1.2	6	1.5	3.00	0.35
16	8000	15	18	1.2	8	1.5	3.00	0.35

Table 3.3 Experimental conditions for PEG/ammonium sulfate/tobacco extract system.

PEG stock solution – 40 %w/w; ammonium sulfate stock solution – 40 %w/w

Run Order	PEG MW	PEG conc. %w/w	Ammonium sulfate conc. %w/w	NaCl conc. M	pH	PEG Stock G	Ammonium sulfate stock g	NaCl g
1	3400	10	13	1.2	6	1.25	1.63	0.35
2	8000	10	13	0.1	8	1.25	1.63	0.03
3	3400	15	13	0.1	8	1.88	1.63	0.03
4	8000	15	13	1.2	6	1.88	1.63	0.35
5	3400	10	18	1.2	8	1.25	2.25	0.35
6	8000	10	18	0.1	6	1.25	2.25	0.03
7	3400	15	18	0.1	6	1.88	2.25	0.03
8	8000	15	18	1.2	8	1.88	2.25	0.35

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 phase forming salts. Two-phase systems (total mass 1g) as shown in Table 3.4 and 3.5 were prepared using required amounts of PEG, phase forming salt solution, sodium chloride, lysozyme (30 µl of 1mg/ml) and de-ionized 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.

Table 3.4 Experimental conditions for PEG/potassium phosphate/lysozyme system.

PEG stock – 40 %w/w; potassium phosphate stock – 40 %w/w

Run Order	PEG MW	PEG conc. %w/w	Potassium phosphate conc. %w/w	NaCl conc. M	pH	PEG stock g	Potassium phosphate stock g	NaCl g	Lysozyme (1 mg/ml) µl	Water g
1	3400	10	13	1.2	6	0.25	0.33	0.070	30	0.35
2	8000	10	13	0.1	8	0.25	0.33	0.006	30	0.42
3	3400	15	13	0.1	8	0.38	0.33	0.006	30	0.29
4	8000	15	13	1.2	6	0.38	0.33	0.070	30	0.23
5	3400	10	18	1.2	8	0.25	0.45	0.070	30	0.23
6	8000	10	18	0.1	6	0.25	0.45	0.006	30	0.29
7	3400	15	18	0.1	6	0.38	0.45	0.006	30	0.17
8	8000	15	18	1.2	8	0.38	0.45	0.070	30	0.11

Table 3.5 Experimental conditions for PEG/sodium sulfate/lysozyme system.

PEG stock – 50 %w/w; sodium sulfate stock – 30 %w/w

Run Order	PEG MW	PEG conc. %w/w	Sodium sulfate conc. %w/w	NaCl conc. M	pH	PEG stock g	Sodium sulfate stock g	NaCl g	Lysozyme (1 mg/ml) µl	Water g
1	3400	10	13	1.2	6	0.2	0.43	0.070	30	0.30
2	8000	10	13	0.1	8	0.2	0.43	0.006	30	0.36
3	3400	15	13	0.1	8	0.3	0.43	0.006	30	0.26
4	8000	15	13	1.2	6	0.3	0.43	0.070	30	0.20
5	3400	10	18	1.2	8	0.2	0.60	0.070	30	0.13
6	8000	10	18	0.1	6	0.2	0.60	0.006	30	0.20
7	3400	15	18	0.1	6	0.3	0.60	0.006	30	0.09
8	8000	15	18	1.2	8	0.3	0.60	0.070	30	0.03

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) as shown in Table 3.6 were set up using PEG and sodium sulfate 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.

Table 3.6 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 weight: 3400
PEG Concentration: 10 %w/w
pH: 7

Run Order	Coded levels		Real values		PEG Stock g	Na_2SO_4 Stock g	NaCl g	Lysozyme (1 mg/ml) µl	Tobacco Extract g
	Na_2SO_4 Conc.	NaCl Conc.	Na_2SO_4 Conc. (%w/w)	NaCl Conc. (M)					
1	-1	-1	9	0.4	1.25	1.5	0.12	150	2.13
2	1	-1	15	0.4	1.25	2.5	0.12	150	1.13
3	-1	1	9	1.4	1.25	1.5	0.41	150	1.84
4	1	1	15	1.4	1.25	2.5	0.41	150	0.84
5	-1.414	0	7.8	0.9	1.25	1.3	0.26	150	2.19
6	1.414	0	16.2	0.9	1.25	2.7	0.26	150	0.79
7	0	-1.414	12	0.2	1.25	2.0	0.06	150	1.69
8	0	1.414	12	1.6	1.25	2.0	0.47	150	1.28
9	0	0	12	0.9	1.25	2.0	0.26	150	1.49
10	0	0	12	0.9	1.25	2.0	0.26	150	1.49
11	0	0	12	0.9	1.25	2.0	0.26	150	1.49
12	0	0	12	0.9	1.25	2.0	0.26	150	1.49
13	0	0	12	0.9	1.25	2.0	0.26	150	1.49

3.2.4. Lysozyme activity assay

Lysozyme activity present in each phase was measured by the clearing of a *Micrococcus lysodeiktikus* cell suspension [32]. *Micrococcus lysodeiktikus* cell suspension of concentration 0.5 mg/ml was prepared using potassium phosphate buffer at pH 6.2. 300 mM sodium chloride solution was prepared with de-ionized water. The rate of change of absorbance at 540 nm (using a UV- 1601 spectrophotometer, Shimadzu, Kyoto, Japan) for a mixture of 600 µl of 0.5 mg/ml *Micrococcus lysodeiktikus* cell

suspension, 200 µl of 300 mM sodium chloride and 400 µl protein sample was measured for every 10 s for one minute. 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 Abs_{540} change per minute (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.

3.2.5 Phase diagram of PEG 3400/sodium sulfate system

Binodial curve

The binodial was determined by the cloud point method [5]. About one gram of concentrated PEG 3400 (50 %w/w) solution was weighed into a polystyrene tube. The mass of PEG added was determined using an analytical balance. A solution of 30 %w/w of sodium sulfate solution made up in 50 mM, pH 7 potassium phosphate buffer was added drop-by-drop to the tube and vortexed. The point where the mixture became turbid or cloudy was noted and the amount of salt solution added was determined. 100 µl of deionized water was added and vortexed. The solution becomes clear on adding water and the mass of the mixture was noted. Sodium sulfate solution was added drop wise till mixture was turbid again and the above procedure was repeated over a whole range of concentrations starting with PEG and sodium sulfate solutions.

Tie Line

Phase composition was determined using the gravimetric method [26]. A five-gram system with phase compositions PEG 3400 10 % w/w and sodium sulfate solution 16.2 %w/w was prepared. The top and bottom phases were separated out carefully and weighed. About 100 mg of top phase and bottom phase was pipetted into separate pre-weighed glass vials. About 200 mg of deionized water was added and the total mass was noted. The samples were frozen on dry ice and freeze dried under vacuum for 24 h to remove all water. The mass of the samples was noted after freeze-drying. The samples were then placed in a furnace at 450 °C for 48 h. The PEG present was oxidized and volatilized while the salt remained as white ash. The tubes were weighed repeatedly until a constant value was reached. The phase composition and hence the tie line were determined.

Size exclusion chromatography was also used to determine the amount of PEG present in the top phase to construct the tie line. The mobile phase was 100 mM, pH 6.8 sodium phosphate buffer. Standards containing 1, 2, 5,7.5, 10 %w/w of PEG 3400 were used to obtain the calibration line using a size exclusion column (SEC S 2000, Phenomenex, Torrance, CA), and refractive index detector. The top phase samples were diluted five times and the sample was run through the column. The amount of PEG in the top phase was determined by comparison to the standard.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. 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 5-9, while it was higher than the pH of the buffer for the pH range 3-4 (Fig. 4.1). 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. 4.2. The total protein extracted varied from 1.0 to 1.6 %wt of the biomass (wet mass) at different pH. It increases with pH and maximizes at pH 7 and then decreases for pH > 7. Since the solubility of protein is minimum at the isoelectric point (pI) when it carries no net charge, it is less likely to be extracted when the buffer pH is at or near its pI. Fig. 4.2 thus implies that there are more acidic proteins (pI < 7) than basic

proteins (pI > 7) in tobacco. The tobacco leaves contained about 74.6 ± .04 % of moisture by weight.

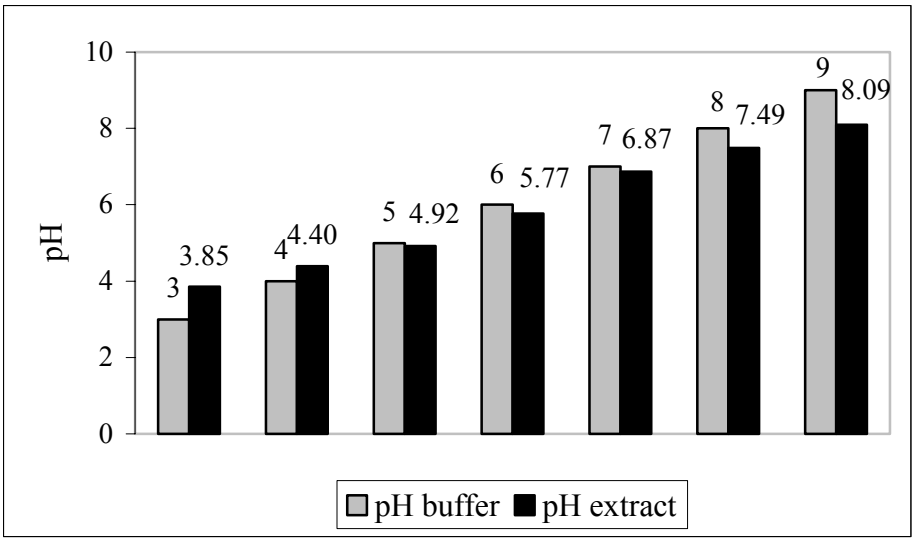


Fig. 4.1. Variation of the pH of tobacco extract compared to the pH of buffer.

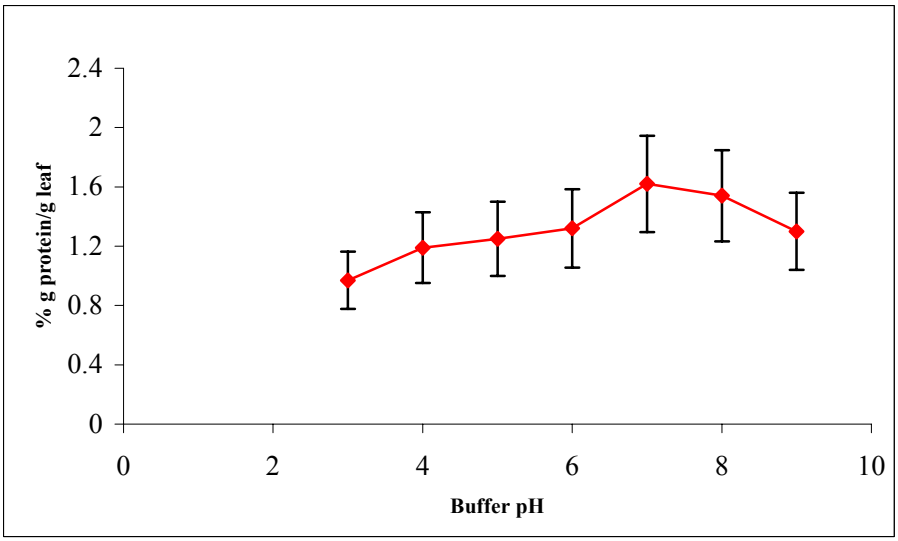


Fig. 4.2. Protein extraction from tobacco leaf.

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.

4.2. ATPE studies

4.2.1. 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 the following factors on the partitioning of tobacco protein: PEG molecular weight, PEG concentration, phase forming salt concentration, sodium chloride concentration, and pH of the system (Table 4.1). In fractional factorial experiments the effects due to some of the factors cannot be separated from the others. Such effects are said to be confounded with each other and form the alias set. The assumption in a fractional factorial experiment is that main effects and low-order interactions dominate most systems.

The level for the PEG molecular weight, 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 [21].

Table 4.1. Factors and levels for fractional factorial study of native tobacco partitioning.

Label	Factor	Low level	High level
A	PEG molecular weight	3400	8000
B	PEG concentration (% w/w)	10	15
C	Salt concentration (% w/w)	13	18
D	NaCl concentration (M)	0.1	1.2
E	PH of phase forming salt	6	8

All experiments were analyzed using MINITAB (Version 13) software for statistics with the protein partition coefficient as response. For PEG/potassium phosphate systems, the factors affecting the partition coefficient significantly were determined using the Pareto chart (Fig. 4.3a) and normal effects plot (Fig. 4.3b). 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 alpha (it is the value against 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 alpha 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.

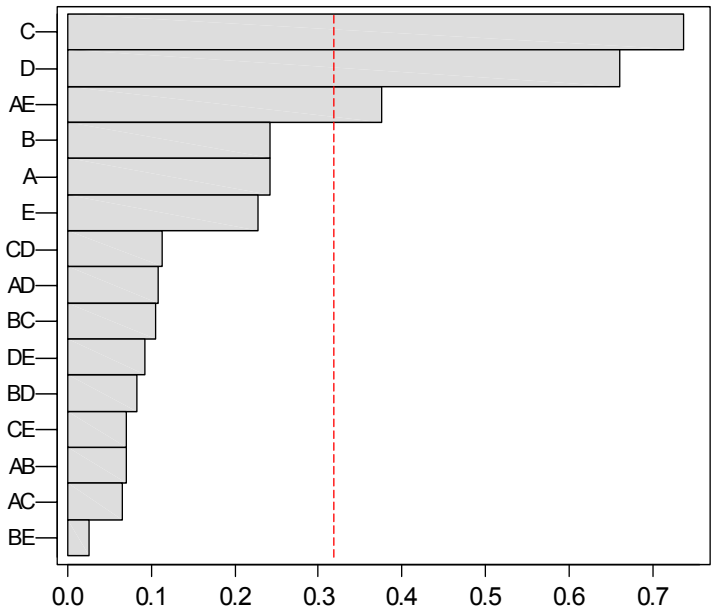


Fig. 4.3a. Pareto chart for PEG/potassium phosphate/tobacco system with alpha = 0.1.

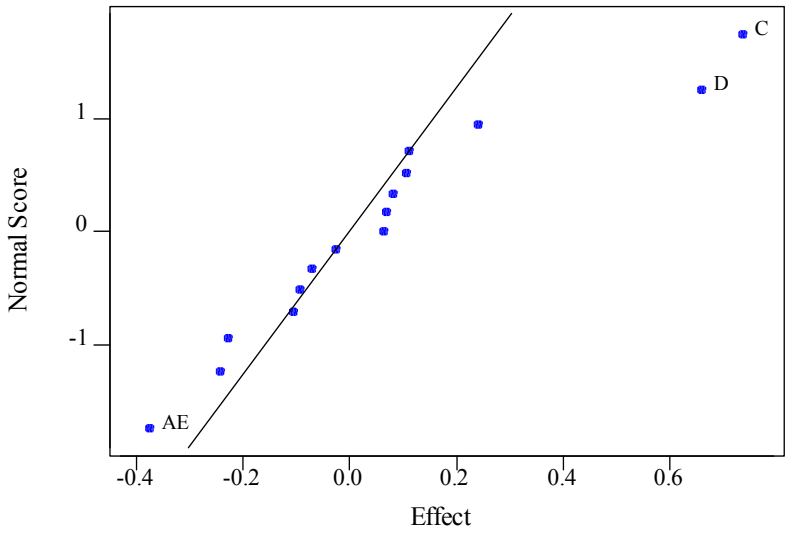


Fig. 4.3b. Normal effect plot for PEG/potassium phosphate system with alpha = 0.1.

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 4.2. The trend of each factor was determined from the main effect plots (See Appendices 1-3 for complete analysis of data.) For the PEG/potassium phosphate system, an increase in both the potassium phosphate concentration and sodium chloride concentration increases the partition coefficient, and for the PEG/ammonium sulfate system the partition coefficient increases with increase in salt concentration and decreases with increase in PEG molecular weight.

Table 4.2. 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 weight – pH interaction
PEG/sodium sulfate	1 - 5	None
PEG/ammonium sulfate	3 - 5	Ammonium sulfate concentration (+), PEG molecular weight (-).

As shown in Table 4.2, the minimum partition coefficient for tobacco protein was greater than 3 for the PEG/ammonium sulfate systems. Since a lower partition coefficient of native tobacco protein will favor obtaining a high selectivity for recombinant proteins, the PEG/ammonium sulfate system was not included in the subsequent studies. Moreover, the fact that no factor significantly affects the partitioning of tobacco protein in the 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.

4.2.2. 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 4.1. The partition coefficient of lysozyme in PEG/potassium phosphate systems varied between 10 and 40 with no factor statistically significant (See Appendix 4). For PEG/sodium sulfate systems, the most significant factor was sodium chloride concentration followed by sodium sulfate concentration (See Appendix 5). 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.

The main effect plot for PEG/sodium sulfate systems are shown in Fig. 4.4. Each graph on Fig. 4.4 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 opposite effects on their partitioning. PEG molecular

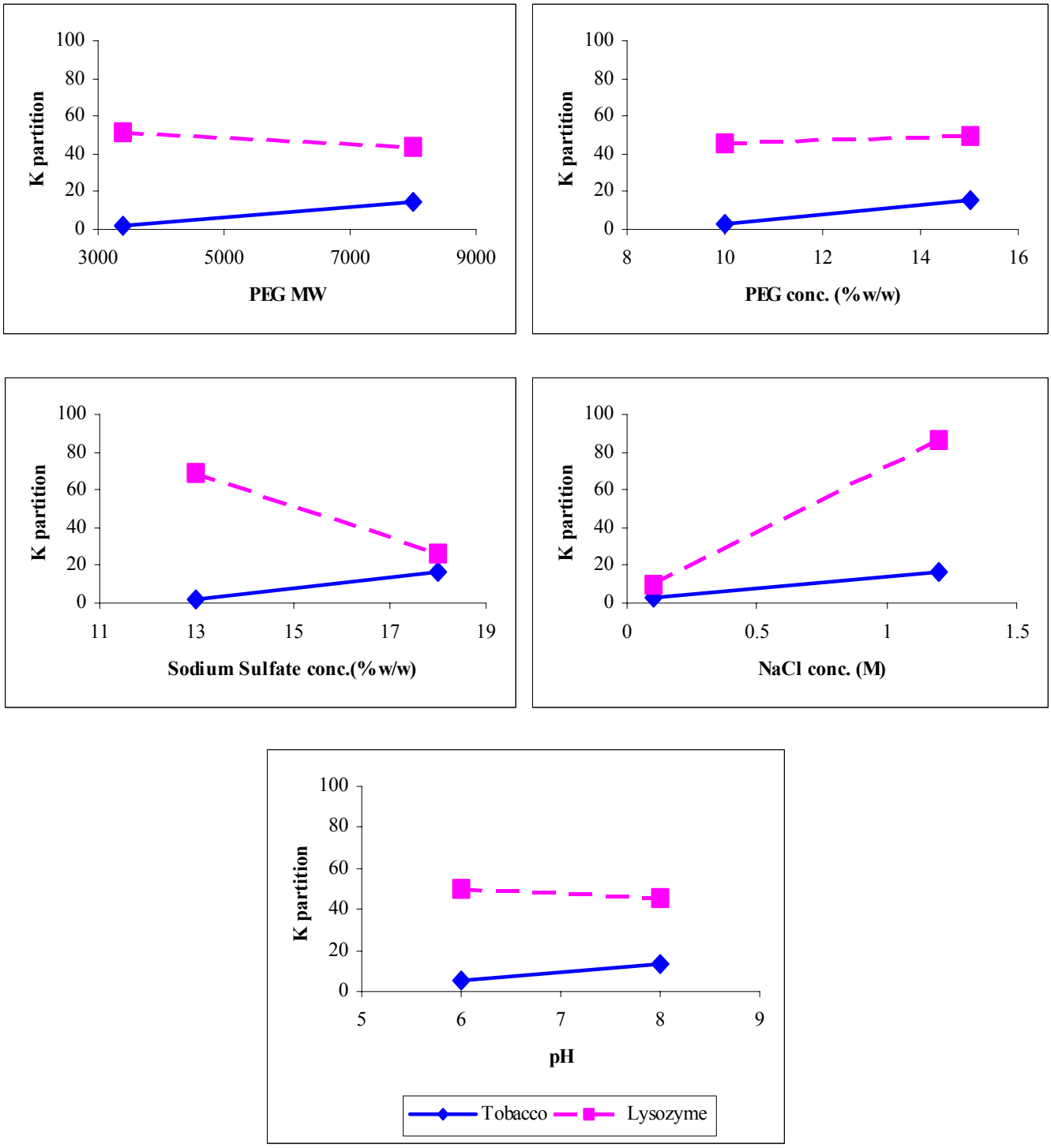


Fig. 4.4. Main effect plot for PEG/sodium sulfate system.

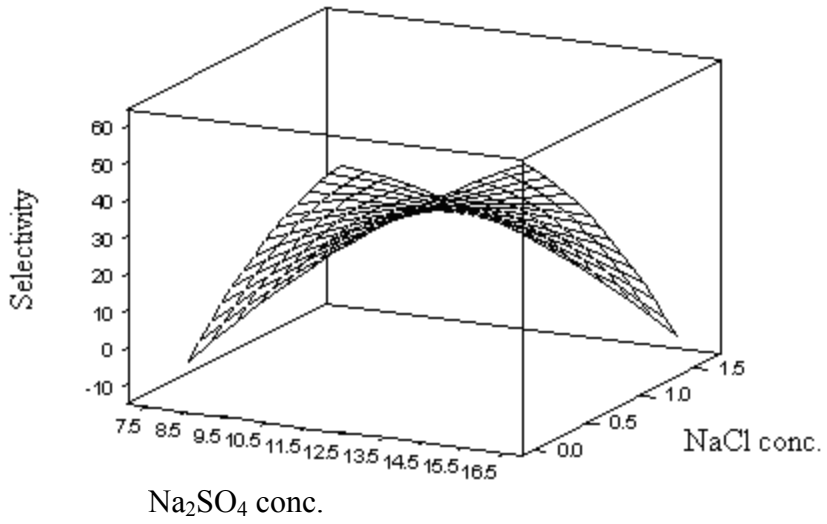
weight also has similar effect on partitioning of tobacco protein and lysozyme, to a different extent. PEG concentration and pH have opposite trends for both tobacco protein and lysozyme. Obviously, different factors have different effects on the partitioning of lysozyme and tobacco protein. These offer great opportunities to optimize the selectivity for lysozyme separation from tobacco extract.

4.2.3. 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 large number of 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 (Table 3.6). It can be seen from the main effect plots (Fig. 4.4) that lower PEG molecular weight and lower PEG concentrations were preferred for obtaining a lower partition coefficient for tobacco protein and higher partition coefficient for lysozyme. Hence, PEG molecular weight 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. Two level designs can be used to model only linear effects. Once near the optimal conditions, center points have to be added to the design to detect any curvature in the model.

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. 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 (See Appendix 6 for complete analysis.)

The partition coefficient for the total protein content was determined using the BCA assay and that for lysozyme was determined using lysozyme activity assay. It should be noted that 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 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 one minute of assaying time. The response surface design was analyzed using the selectivity as the desired response (Fig. 4.5). The conditions at which lysozyme selectivity over the native tobacco protein was highest (global solution) were determined using the response optimizer provided by MINITAB (Version 13) software.



Global Solution: Sodium sulfate conc. = 16.2 % w/w, NaCl conc. = 0.19 M, Selectivity = 57.7. (Other conditions used in the study: PEG 3400 at 10 %w/w and pH 7).

Fig. 4.5. Response surface design for egg white lysozyme spiked into tobacco extract.

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.4.5, lysozyme selectivity can be increased from two approaches: 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 the concentrations of sodium sulfate and sodium chloride are limited by the following factors: 1) the solubility of sodium sulfate in water is about 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, with average 47 ± 20.9. The large 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 retained in the bottom phase. Besides 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.

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 and protein concentrations obtained from the experiments with the conditions determined from the response surface study (Table 4.3). As shown in Table 4.3, the yield of lysozyme decreases with phase ratio, but that is more than compensated with much higher lysozyme purification and concentration factors. When 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 4.3. 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 of Lysozyme	Concentration Factor of Lysozyme
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

4.2.4 Phase diagram of PEG 3400/sodium sulfate system

The binodial curve for the PEG 3400/sodium sulfate system at room temperature and pH 7 was determined using the cloud point method as shown in Fig. 4.6 a. The curve was fit using piecewise regression models. The binodial curve is comparable to the PEG 3350/sodium sulfate phase diagram at 25° C developed by Kenneth Cole *et al.* [26].

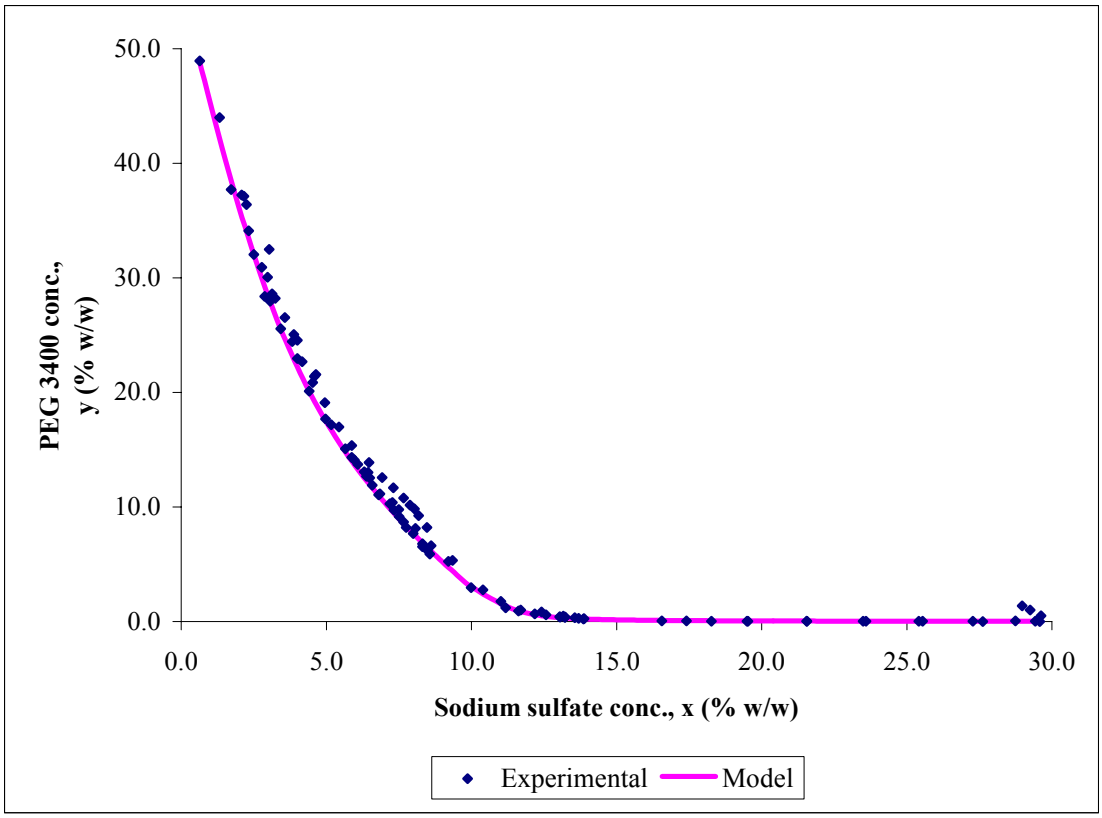


Fig 4.6 a. Binodial curve for PEG 3400/sodium sulfate system, at pH 7 and room temperature.
Equations for the fitted curve: ($x<9.99\%$) $y = -7.2009x + 0.3039 + (0.0632 - 0.7616x + 31.1623x^2)^{0.5}$; ($x>9.99\%$) $y = -0.93x + 0.1059 + (0.0115 - 0.1988x + 0.869x^2)^{0.5}$

An attempt was made to determine the tie line corresponding to 10% w/w PEG 3400 and 16.2 % w/w sodium sulfate using the gravimetric method and size exclusion chromatography. The top phase PEG concentration was determined to be 31.54% w/w using size exclusion chromatography and the tie line was developed by extrapolating the line to the bottom phase composition (Fig. 4.6 b – line TL1). The top phase PEG concentration is expected to be around 44 % PEG from the work by Kenneth Cole *et al.* [26]. The PEG used for these studies has an average molecular weight of 3400; this may

include some smaller or larger particles, leading to an error in the peak areas and calibration. There is also some error related to integration of peak area and determining base lines as peak tailing occurred. The tailing increased with increasing concentration of PEG. For the gravimetric method, we tried to determine the water content of the phases using freeze dryers and speed-vacs. Freeze dried samples had a top phase water content of 75 %w/w and bottom phase 69 %w/w (Fig.4.6b – line TL2). However, the top phase was expected to have around 55% water, and the bottom phase around 80% water [26]. A much higher percentage of water especially in the top phase was obtained in all the runs. The top phase samples contain mostly PEG, the increased weight loss while drying under vacuum could be due to PEG escaping along with the water vapor. To prevent loss of PEG or salt on removing all water from the samples, a speed-vac coupled with a centrifuge was used. Samples were pipetted into 1.5 ml micro centrifuge tubes and placed in a speed vac. The samples were centrifuged as they were dried. The results showed 56 %w/w water in the top phase and 79 %w/w water in the bottom phase. This was very close to the expected value, but complete recovery of these samples to determine the PEG concentration by oxidizing out all PEG present at 450° C was not feasible. Samples were transferred into a glass tube and the total percentage recovered was noted. The samples were placed in a furnace at 450° C for 72 h and the top phase and bottom phase PEG 3400 concentration was determined to be 42.5 % (w/w) and 0.0058 % (w/w). This tie line (Fig 4.5 b-TL3) was the closest fit that could be obtained.

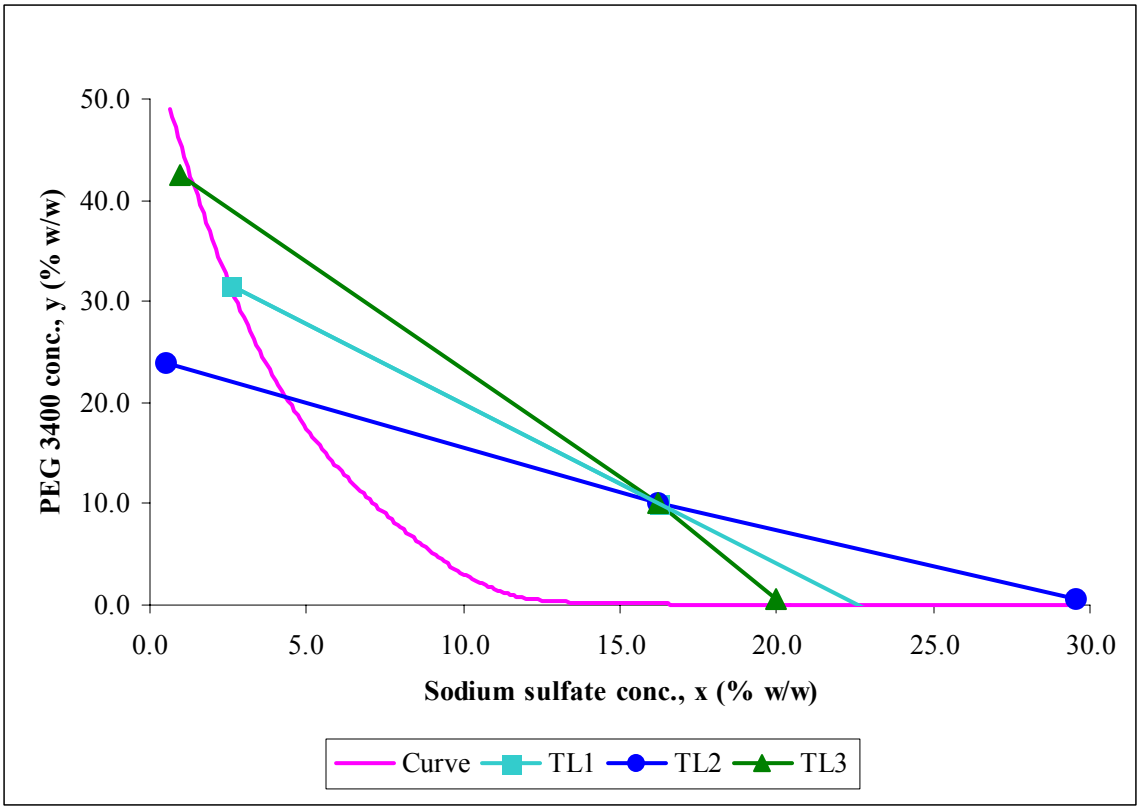


Fig 4.6 b. Phase diagram for PEG 3400/sodium sulfate system – with experimentally determined tie lines, at pH 7 and room temperature.
TL1 = tie line from size exclusion chromatography data, TL2 = tie line from gravimetric method using freeze dryer, TL3 = tie line from gravimetric method using speed-vac.

As accurate experimental determination of the tie line could not be achieved, the phase diagram reported by Kenneth Cole *et al.* [26] for the PEG 3350/sodium sulfate system was used to estimate tie line on the binodial curve developed. The tie lines Est. TL1 and Est. TL2 represented on Fig. 4.6 c. were determined. Using these tie lines as reference the tie line, Est. TL, corresponding to the optimal recovery conditions (10 % w/w PEG 3400/ 16.2 % w/w sodium sulfate) was estimated. The system corresponding to

the 1:15 phase ratio was determined to be around 2.8 %w/w PEG 3400 and 19.4 %w/w sodium sulfate.

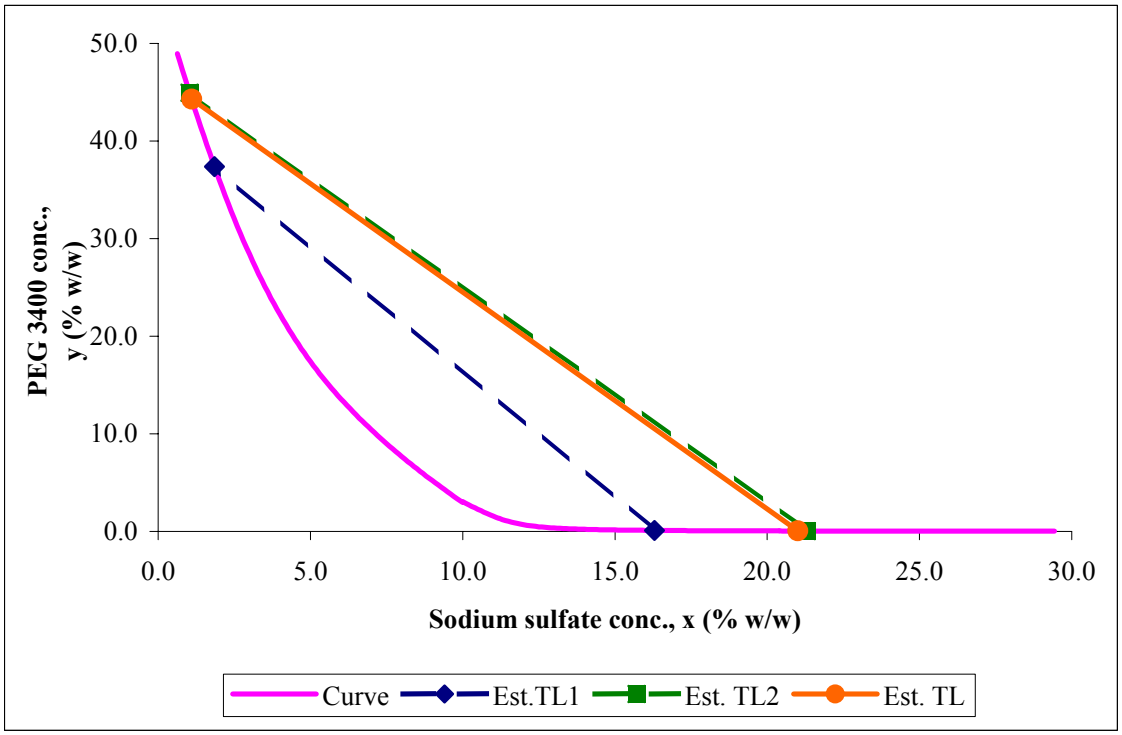


Fig. 4.6 c) Phase diagram for PEG 3400/sodium sulfate system at pH 7 and room temperature, with estimated tie lines.
Est. TL-1 and Est. TL2 = tie lines estimated using PEG 3350/sodium sulfate phase diagram and Est. TL = estimated tie line at maximum lysozyme selectivity condition.

4.2.5 Experimental verification at optimal recovery conditions

Three experiments were conducted at system conditions given in Table 4.4 to experimentally verify the theoretical predictions of 87% lysozyme yield and a purification factor 4 at a phase ratio of 1:15. The amount of tobacco extract and lysozyme added were maintained the same as for the experiments at 10 %w/w PEG 3400 and 16.2 % w/w sodium sulfate.

Table 4.4. System conditions for experimental verification of yield and purification factor at 1:15 phase ratio

PEG 3400	2.8 % w/w
Sodium sulfate	19.4 % w/w
NaCl	0.19 M
Tobacco Extract	1.23 g/5 g of system
Lysozyme	0.30 μg/ g of system
pH	7

The average selectivity from the experiments was 47.84 ± 12.6. The yield of lysozyme was 79.6 % and purification factor was 10.6. The selectivity was comparable to that obtained for the 10 %w/w PEG 3400 and 16.2 % sodium sulfate system. Yield was slightly lower than the predicted value of 87 % and the purification factor was higher by 6. The variability could be due to difference in the overall amount of tobacco protein used in the ATPE experiments. The protein concentration of tobacco extract does differ slightly from sample to sample, and thus the total protein in the system varies from sample to sample. The experimentally obtained data showed that it is possible to have

very high selectivity of lysozyme over tobacco protein and concentration at selected optimal conditions although lysozyme yield is slightly lesser than the predicted value.

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, statistical design of experiments can be used for system screening and process optimization to expedite the process development. It is our hope that the promises this study has shown for using ATPE in protein purification from tobacco will lead to more extensive application of this technique in protein recovery and purification from transgenic plants.

CHAPTER 5

CONCLUSIONS

1. The amount of soluble protein extracted from flue-cured tobacco leaves maximized at pH 7.

2. A PEG/sodium sulfate system proved to be the most suitable system for egg white lysozyme recovery among the three systems considered.

3. 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.

4. 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.

5. At a phase ratio of 1:15 (optimal condition), a yield of 87 % and purification factor of 4 with a concentration factor of 14 could be obtained for egg white lysozyme.

6. The phase diagram for the PEG 3400/sodium sulfate system at room temperature and pH 7 was developed and the PEG and sodium sulfate concentrations corresponding to 1:15 phase ratio were determined.

7. Experiments at the optimal condition for lysozyme recovery from tobacco extract produced a yield of 79% with a purification factor of 10 and concentration factor of 20.

8. It is possible that the optimum ATPE conditions for lysozyme purification from tobacco extract might be further improved using PEG with different molecular weight or other PEG/salt systems.

9. 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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APPENDIX 1

PEG/Potassium Phosphate/Tobacco extract system

This system was used to study the partition behavior of native tobacco proteins. The concentration of protein in the top and bottom phases was determined using the BCA assay and the partition coefficient calculated was used as response to analyze the fractional factorial experiment. When using fractional factorial experiments to study the effect of various factors on a response of interest, in our case the partition coefficient, the effects due to some of the factors cannot be separated from the others. Such effects are said to be confounded with each other and form the alias set. The assumption in a fractional factorial experiment is that main effects and low-order interactions dominate most systems.

Table A1.1. Factors and levels for fractional factorial study of native tobacco partitioning.

Label	Factor	Low level	High level
A	PEG molecular weight	3400	8000
B	PEG concentration (% w/w)	10	15
C	Salt concentration (% w/w)	13	18
D	NaCl concentration (M)	0.1	1.2
E	pH	6	8

Factorial Design

Factors: 5 Base Design: 5,16 Resolution: V

Runs: 16 Replicate: 1 Fraction: ½

Blocks: none Center points: 0

Table A1.2. Experimental conditions and observed Partition Coefficient.

Run Order	PEG MW	PEG conc. %w/w	Potassium phosphate conc. %w/w	NaCl conc. M	pH	K partition
1	3400	10	13	0.1	6	1.33
2	8000	10	13	0.1	8	1.29
3	3400	15	13	0.1	8	1.35
4	8000	15	13	0.1	6	1.58
5	3400	10	18	0.1	8	2.26
6	8000	10	18	0.1	6	2.57
7	3400	15	18	0.1	6	1.59
8	8000	15	18	0.1	8	1.63
9	3400	10	13	1.2	8	1.93
10	8000	10	13	1.2	6	2.37
11	3400	15	13	1.2	6	1.68
12	8000	15	13	1.2	8	1.76
13	3400	10	18	1.2	6	2.74
14	8000	10	18	1.2	8	2.72
15	3400	15	18	1.2	8	2.39
16	8000	15	18	1.2	6	3.29

Design generators = E=ABCD

Alias Structure

I +ABCDE
A+BCDE
B+ACDE
C+ABDE
D+ABCE
E+ABCD
AB+CDE
AC+BDE
AD+BCE
AE+BCD
BC+ADE
BD+ACE
BE+ACD
CD+ABE
CE+ABD
DE+ABC

Table A1.3 Estimated effects and coefficients for K (coded units).

Term	Effect	Coefficient
Constant		2.03
A	0.2425	0.12
B	-0.2425	-0.12
C	0.7375	0.37
D	0.6600	0.33
E	-0.2275	-0.11
A*B	0.0700	0.04
A*C	0.0650	0.03
A*D	0.1075	0.05
A*E	0.3750	-0.19
B*C	-0.1050	-0.05
B*D	0.0825	0.04
B*E	-0.0250	-0.01
C*D	0.1125	0.06
C*E	-0.0700	-0.04
D*E	-0.0925	-0.05

Table A1.4. Analysis of variance for K (coded units).

Source	DF	Seq. SS	Adj. SS	Adj. MS	F	P
Main Effects	5	4.5955	4.5955	0.91910	*	*
2-Way Interactions	10	0.8235	0.8235	0.08235	*	*
Residual Error	0	0.0000	0.0000	0.00000		
Total	15	5.4190				

where,

DF - degrees of freedom, Seq.SS – Sequence sum of squares, Adj. SS – Adjusted sum of squares, Adj. MS – adjusted mean squares, No estimate of F and P-value as the number of replicates is one and there is no estimate of error for the analysis.

Table A1.5. Estimated Coefficients for K using data in uncoded units.

Term	Coefficient
Constant	-0.73
A	6.23 E-4
B	-0.12
C	0.53
D	0.52
E	0.35
A*B	1.50 E-5
A*C	1.40 E-5
A*D	2.30 E-5
A*E	-8.15 E-05
B*C	-0.05
B*D	0.04
B*E	-0.01
C*D	0.06
C*E	-0.04
D*E	-0.05

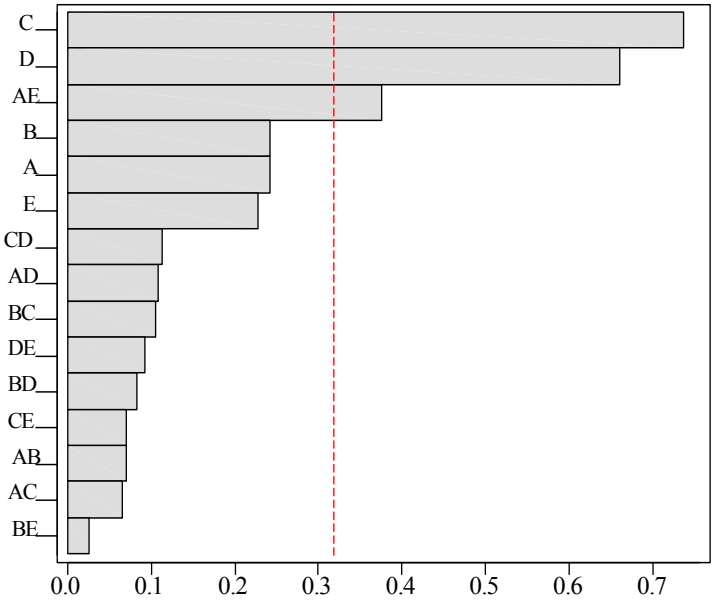

Fig A1.1 Pareto chart of the effects (response = K partition; alpha = 0.10)

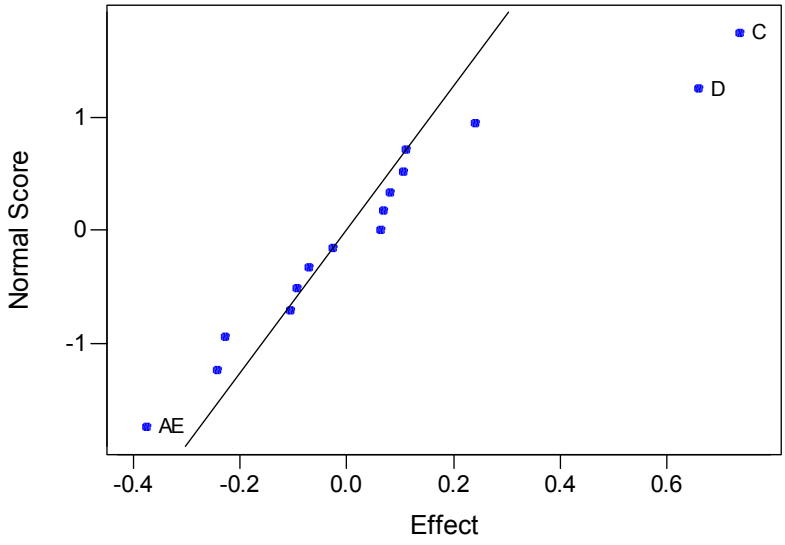

Fig A1.2 Normal probability plot of the effects (response = K partition; alpha = 0.10)

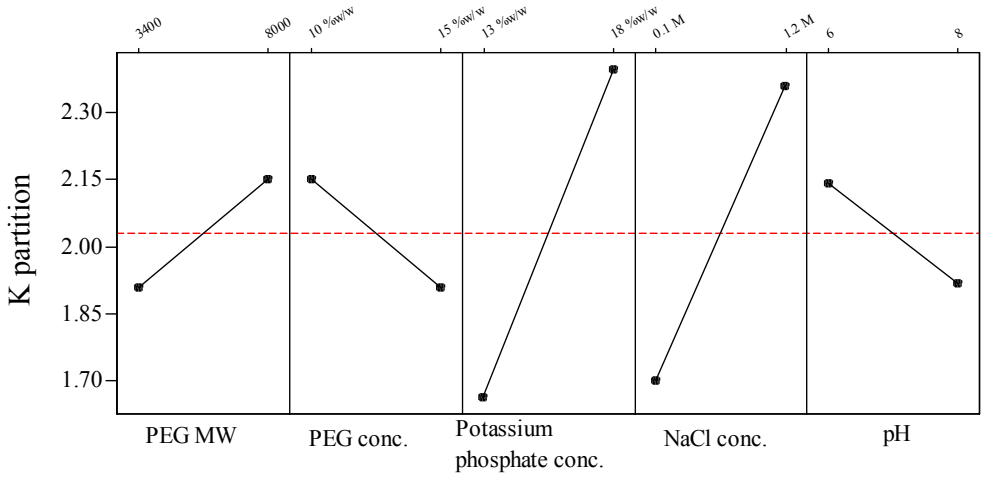

Fig A1.3 Main effects plot for K partition

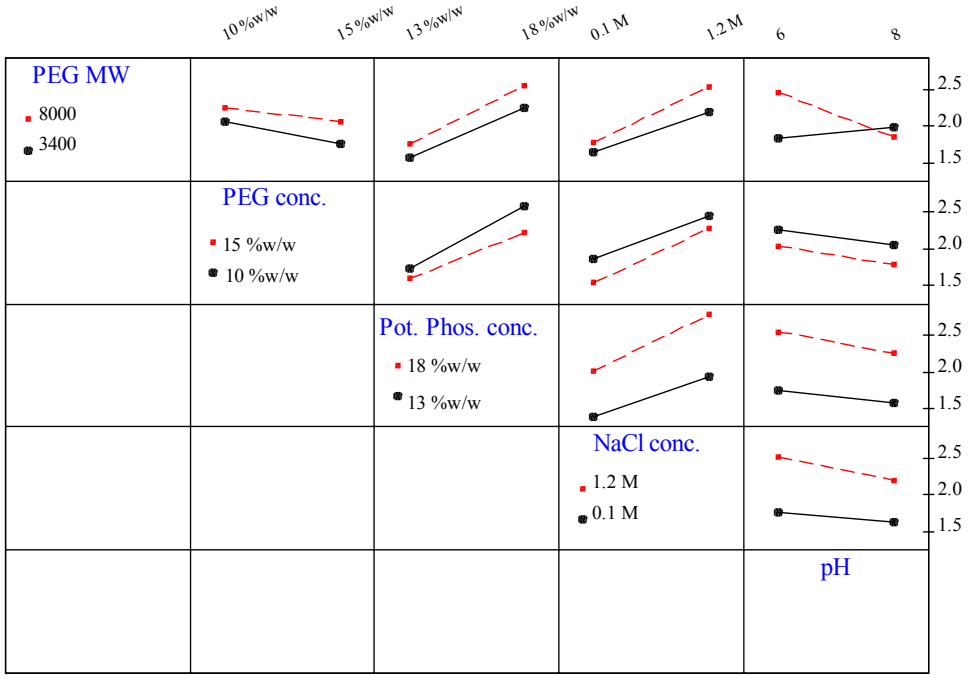

Fig A1.4 Interaction plot for K partition

Significant Factors

C – potassium phosphate concentration: partition coefficient increases as potassium phosphate concentration increases.

D – sodium chloride concentration: partition coefficient increases as sodium chloride concentration increases.

APPENDIX 2

PEG/Sodium sulfate/Tobacco extract system

This was the second system used to study the partition behavior of native tobacco proteins. The concentration of protein in the top and bottom phases was determined using the BCA assay and the partition coefficient calculated was used as response to analyze the fractional factorial experiment.

Table A2.1. Factors and levels for fractional factorial study of native tobacco partitioning.

Label	Factor	Low level	High level
A	PEG molecular weight	3400	8000
B	PEG concentration (% w/w)	10	15
C	Salt concentration (% w/w)	13	18
D	NaCl concentration (M)	0.1	1.2
E	pH	6	8

Factorial Design

Factors: 5 Base Design: 5,16 Resolution: V

Runs: 16 Replicate: 1 Fraction: ½

Blocks: none Center points: 0

Table A2.2. Experimental conditions and observed Partition Coefficient.

Run Order	PEG MW	PEG conc. %w/w	Sodium sulfate conc. %w/w	NaCl conc. M	pH	K partition
1	3400	10	13	0.1	8	1.91
2	8000	10	13	0.1	6	2.49
3	3400	15	13	0.1	6	1.02
4	8000	15	13	0.1	8	2.00
5	3400	10	18	0.1	6	3.25
6	8000	10	18	0.1	8	4.22
7	3400	15	18	0.1	8	2.00
8	8000	15	18	0.1	6	3.95
9	3400	10	13	1.2	6	2.73
10	8000	10	13	1.2	8	2.29
11	3400	15	13	1.2	8	1.16
12	8000	15	13	1.2	6	2.98
13	3400	10	18	1.2	8	4.24
14	8000	10	18	1.2	6	5.39
15	3400	15	18	1.2	6	20.29
16	8000	15	18	1.2	8	89.70

Design generators = E=ABCD

Alias Structure

I +ABCDE
A+BCDE
B+ACDE
C+ABDE
D+ABCE
E+ABCD
AB+CDE
AC+BDE
AD+BCE
AE+BCD
BC+ADE
BD+ACE
BE+ACD
CD+ABE
CE+ABD
DE+ABC

Table A2.3.Estimated effects and coefficients for K (coded units).

Term	Effect	Coefficient
Constant		9.35
A	9.552	4.78
B	12.072	6.03
C	14.558	7.28
D	13.492	6.75
E	8.177	4.09
A*B	8.988	4.49
A*C	8.817	4.41
A*D	8.433	4.22
A*E	12.673	6.34
B*C	12.638	6.32
B*D	12.798	6.40
B*E	8.478	4.24
C*D	13.058	6.53
C*E	8.643	4.32
D*E	8.323	4.16

Table A2.4. Analysis of variance for K (coded units).

Source	DF	Seq. SS	Adj. SS	Adj. MS	F	P
Main Effects	5	2791	2791	558.3	*	*
2-Way Interactions	10	4400	4400	440.0	*	*
Residual Error	0	0.0000	0.0000	0.00000		
Total	15	7191				

Table A2.5. Estimated Coefficients for K using data in uncoded units.

Term	Coefficient
Constant	78.81
A	-0.02
B	-34.77
C	-33.90
D	-32.83
E	-11.61
A*B	2.00 E-3
A*C	1.90-3
A*D	1.80 E-3
A*E	2.80 E-3
B*C	6.32
B*D	6.40
B*E	4.24
C*D	6.53
C*E	4.32
D*E	4.16

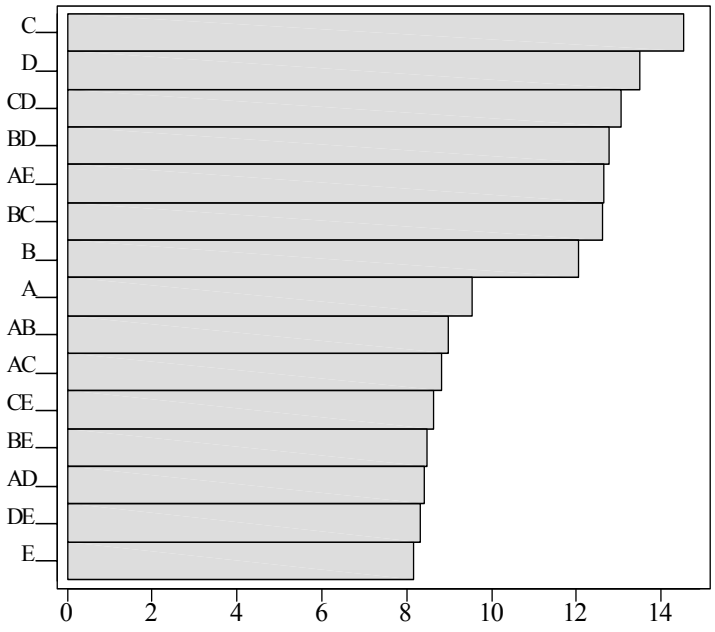

Fig A2.1. Pareto chart of the effects (response = K partition; alpha = 0.10).

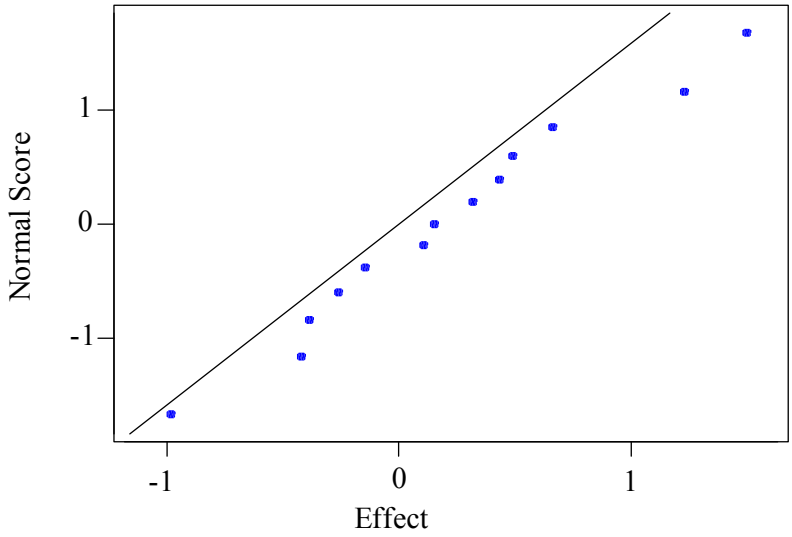

Fig A2.2 Normal probability plot of the effects (response = K partition; alpha = 0.10).

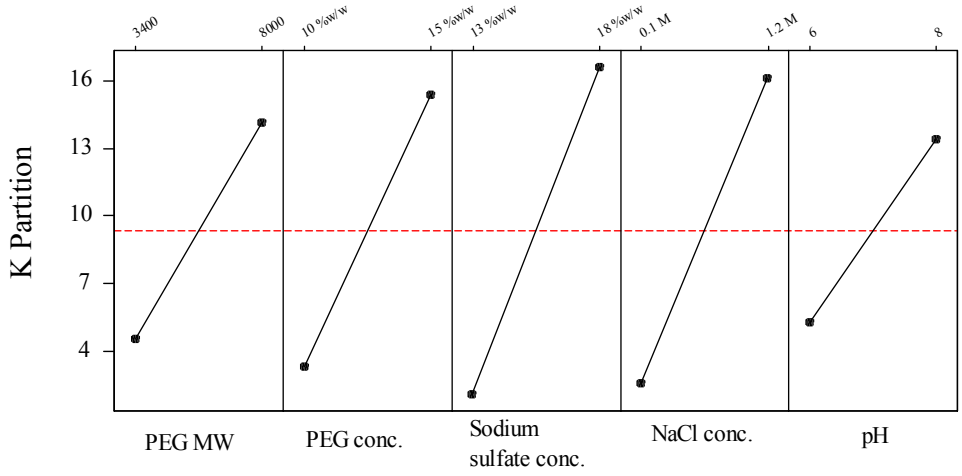

Fig A2.3 Main effects plot for K partition

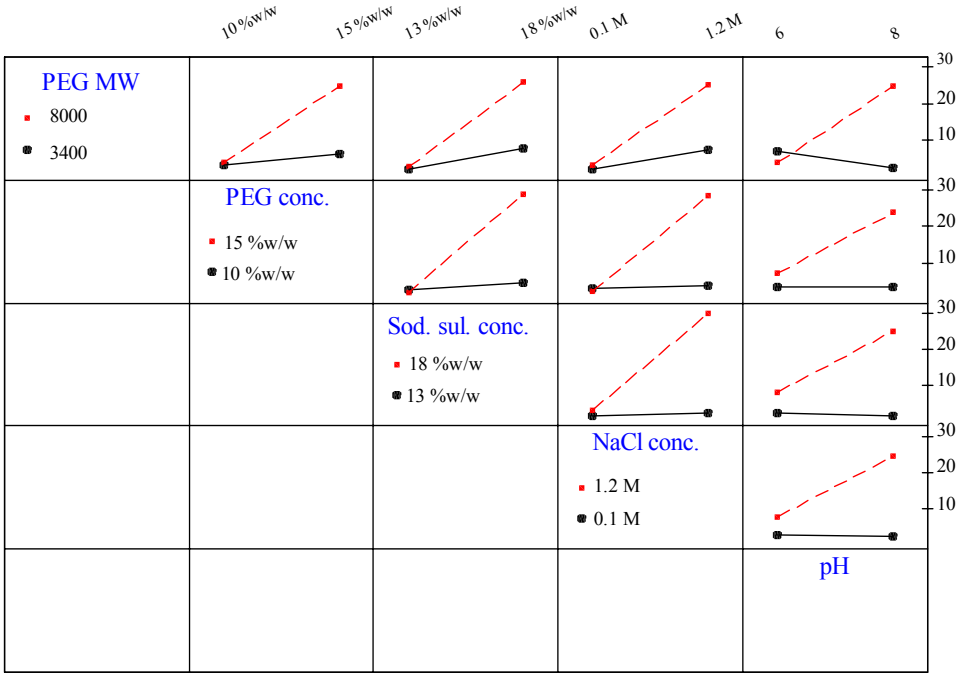

Fig A2.4. Interaction plot for K partition.

Significant Factors

No factor statistically significant. Partition coefficient seems to increases with increase in every factor.

APPENDIX 3

PEG/Ammonium sulfate/Tobacco extract system

This was the third system used to study the partition behavior of native tobacco proteins. The concentration of protein in the top and bottom phases was determined using the BCA assay and the partition coefficient calculated was used as response to analyze the fractional factorial experiment. This was a ¼ fractional factorial experiment and only 8 experiments out of the 32 were studied. Some of the second order interactions are confounded with main effects in this design.

Table A3.1. Factors and levels for fractional factorial study of native tobacco partitioning.

Label	Factor	Low level	High level
A	PEG molecular weight	3400	8000
B	PEG concentration (% w/w)	10	15
C	Salt concentration (% w/w)	13	18
D	NaCl concentration (M)	0.1	1.2
E	PH	6	8

Factorial Design

Factors: 5 Base Design: 5,8 Resolution: III

Runs: 8 Replicate: 1 Fraction: ¼

Blocks: none Center points: 0

Table A3.2. Experimental conditions and observed Partition Coefficient.

Run Order	PEG MW	PEG conc. %w/w	Ammonium sulfate conc. %w/w	NaCl conc. M	pH	K partition
1	3400	10	13	1.2	8	3.54
2	8000	10	13	0.1	6	2.84
3	3400	15	13	0.1	8	3.41
4	8000	15	13	1.2	6	2.50
5	3400	10	18	1.2	6	4.89
6	8000	10	18	0.1	8	4.47
7	3400	15	18	0.1	6	4.28
8	8000	15	18	1.2	8	3.45

Design Generators: D = AB E = AC

Alias Structure

I + ABD + ACE + BCDE

A + BD + CE + ABCDE
B + AD + CDE + ABCE
C + AE + BDE + ABCD
D + AB + BCE + ACDE
E + AC + BCD + ABDE
BC + DE + ABE + ACD
BE + CD + ABC + ADE

Table A3.3. Estimated effects and coefficients for K (coded units).

Term	Effect	Coefficient
Constant		3.67
A	-0.7150	-0.38
B	-0.5250	-0.26
C	1.2000	0.60
D	-0.1550	-0.08
E	0.0900	0.05
B*C	-0.2900	-0.15
B*E	-0.0500	-0.03

Table A3.4. Analysis of variance for K (coded units).

Source	DF	Seq. SS	Adj. SS	Adj. MS	F	P
Main Effects	5	4.5180	4.5180	0.90359	*	*
2-Way Interactions	2	0.1732	0.1732	0.08660	*	*
Residual Error	0	0.0000	0.0000	0.00000		
Total	7	4.6912				

Table A3.5. Estimated Coefficients for K using data in uncoded units.

Term	Coefficient
Constant	4.24
A	-1.65 E-4
B	-0.09
C	0.60
D	-0.08
E	0.05
B*C	-0.15
B*E	-0.03

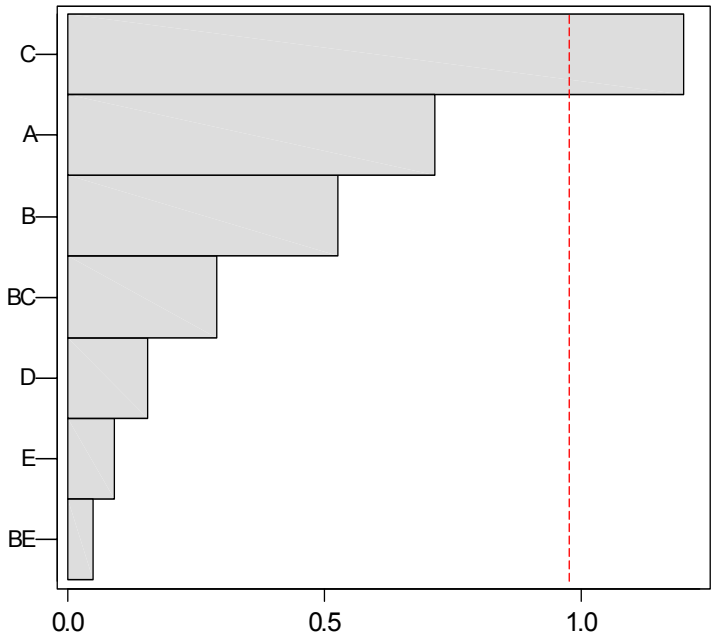

Fig A3.1 Pareto chart of the effects (response = K partition, alpha = 0.10)

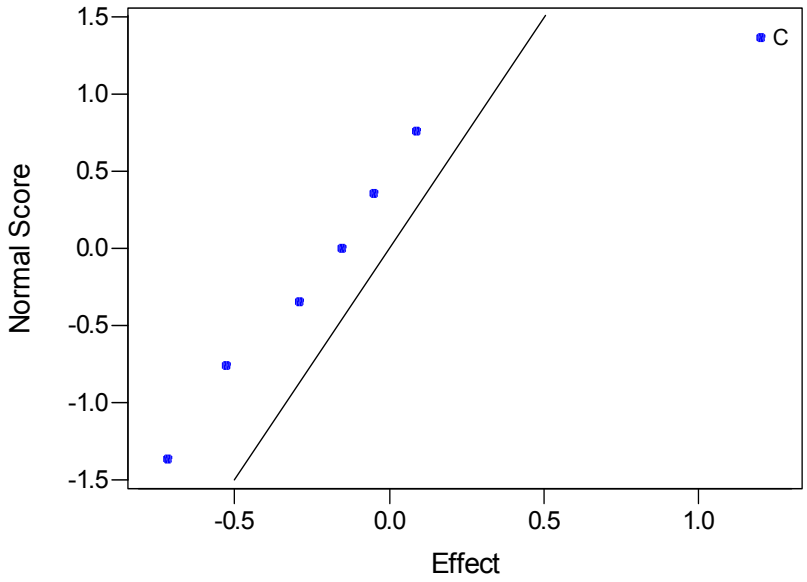

Fig A3.2 Normal probability plot of the effects (response = K partition; alpha = 0.10)

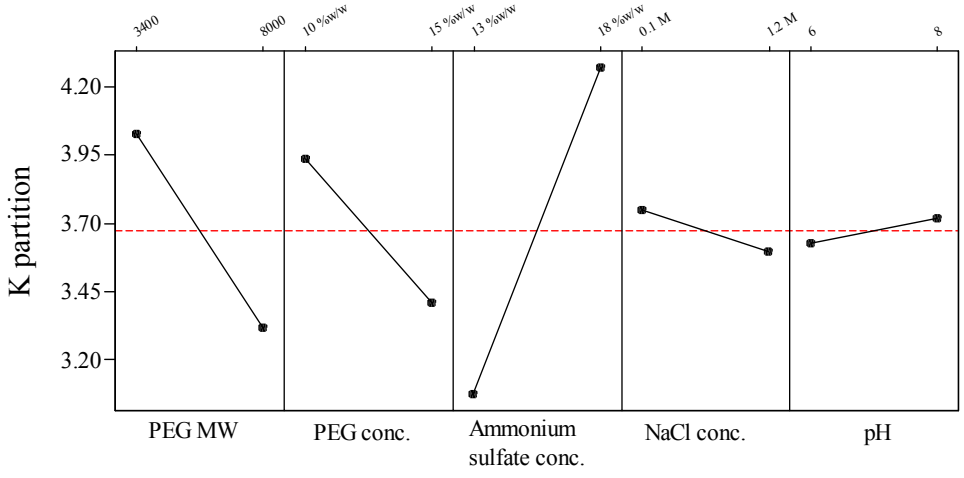

Fig A3.3. Main effects plot for K partition.

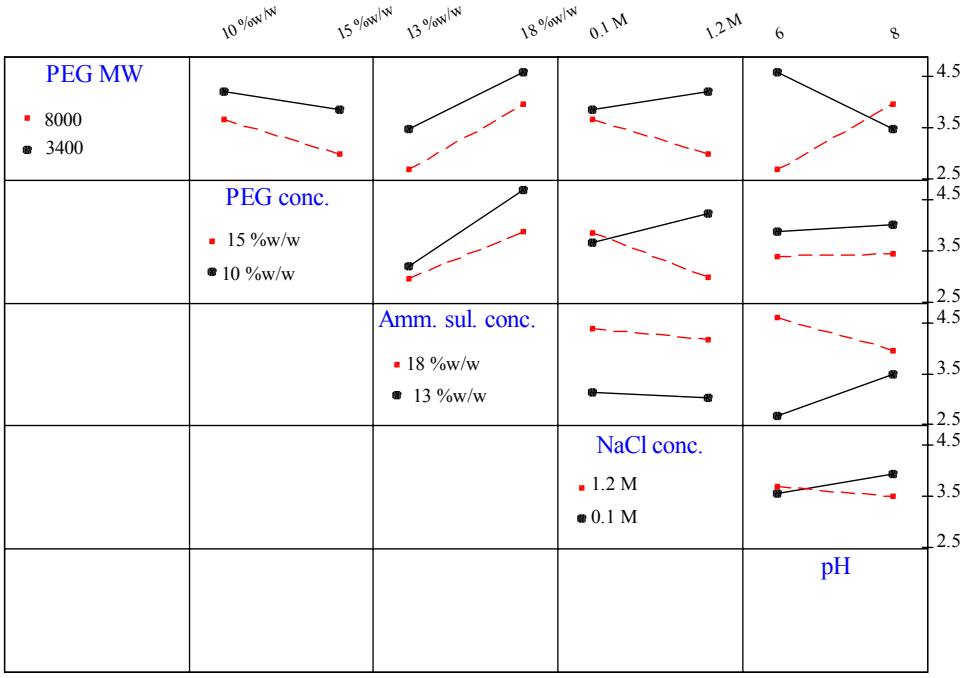

Fig A3.4. Interaction plot for K partition.

Significant Factors

Only C, ammonium sulfate concentration, was statistically significant.

This system was not further investigated to study the partitioning of lysozyme as the partition coefficient for tobacco protein was higher when compared to the previous two systems studied.

APPENDIX 4

PEG/Potassium phosphate/Lysozyme system

This system was used to study the partition behavior of pure lysozyme. The concentration of protein in the top and bottom phases was determined using the lysozyme activity assay and the partition coefficient calculated was used as response to analyze the fractional factorial experiment. A ¼ fractional factorial experiment with the same factors and levels as used for tobacco protein was used to study the effect on the partition coefficient.

Table A4.1. Factors and levels for fractional factorial study of native tobacco partitioning.

Label	Factor	Low level	High level
A	PEG molecular weight	3400	8000
B	PEG concentration (% w/w)	10	15
C	Salt concentration (% w/w)	13	18
D	NaCl concentration (M)	0.1	1.2
E	pH	6	8

Factorial Design

Factors: 5 Base Design: 5,8 Resolution: III

Runs: 8 Replicate: 1 Fraction: ¼

Blocks: none Center points: 0

Table A4.2. Experimental conditions and observed Partition Coefficient.

Run Order	PEG MW	PEG conc. %w/w	Potassium phosphate conc. %w/w	NaCl conc. M	pH	K partition
1	3400	10	13	1.2	8	38.56
2	8000	10	13	0.1	6	0.77
3	3400	15	13	0.1	8	14.18
4	8000	15	13	1.2	6	23.07
5	3400	10	18	1.2	6	17.70
6	8000	10	18	0.1	8	24.83
7	3400	15	18	0.1	6	9.77
8	8000	15	18	1.2	8	19.35

Design Generators: D = AB E = AC

Alias Structure

I + ABD + ACE + BCDE

A + BD + CE + ABCDE
B + AD + CDE + ABCE
C + AE + BDE + ABCD
D + AB + BCE + ACDE
E + AC + BCD + ABDE
BC + DE + ABE + ACD
BE + CD + ABC + ADE

Table A4.3. Estimated effects and coefficients for K (coded units).

Term	Effect	Coefficient
Constant		18.53
A	-3.048	-1.52
B	-3.872	-1.94
C	-1.233	-0.62
D	12.282	6.14
E	11.403	5.70
B*C	-2.832	-1.42
B*E	-11.058	-5.53

Table A4.4. Analysis of variance for K (coded units).

Source	DF	Seq. SS	Adj. SS	Adj. MS	F	P
Main Effects	5	613.4	613.4	122.7	*	*
2-Way Interactions	2	260.6	260.6	130.3	*	*
Residual Error	0	0.0000	0.0000	0.00000		
Total	7	873.9				

Table A4.5. Estimated Coefficients for K using data in uncoded units.

Term	Coefficient
Constant	-17.60
A	-7.00 E-4
B	36.77
C	-0.62
D	6.14
E	5.70
B*C	-1.42
B*E	-5.53

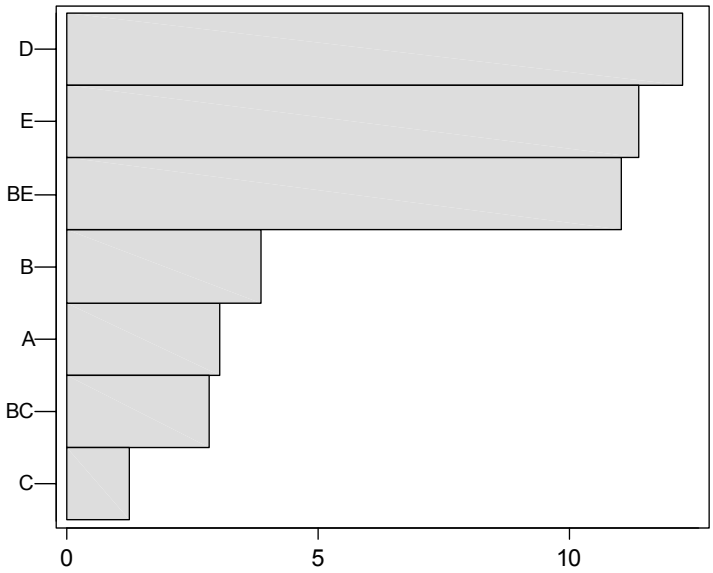

Fig A4.1. Pareto chart of the effects (response = K partition; alpha = 0.10).

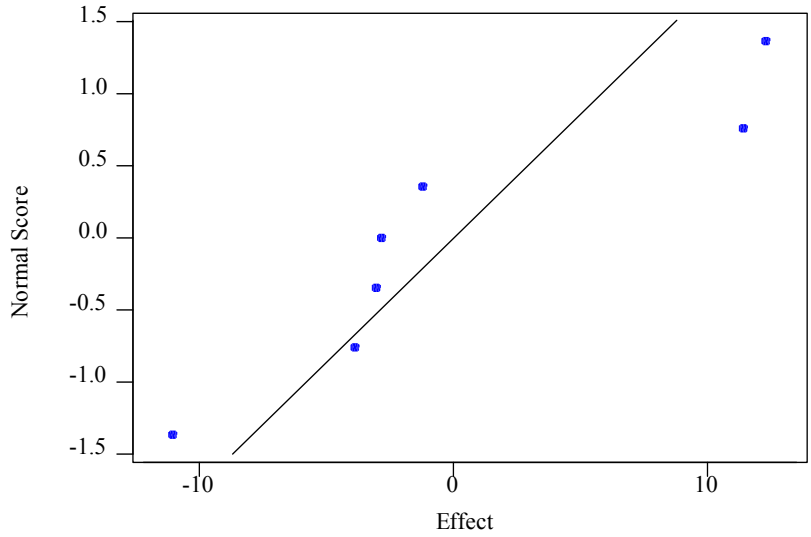

Fig A4.2. Normal probability plot of the effects (response = K partition; alpha = 0.10).

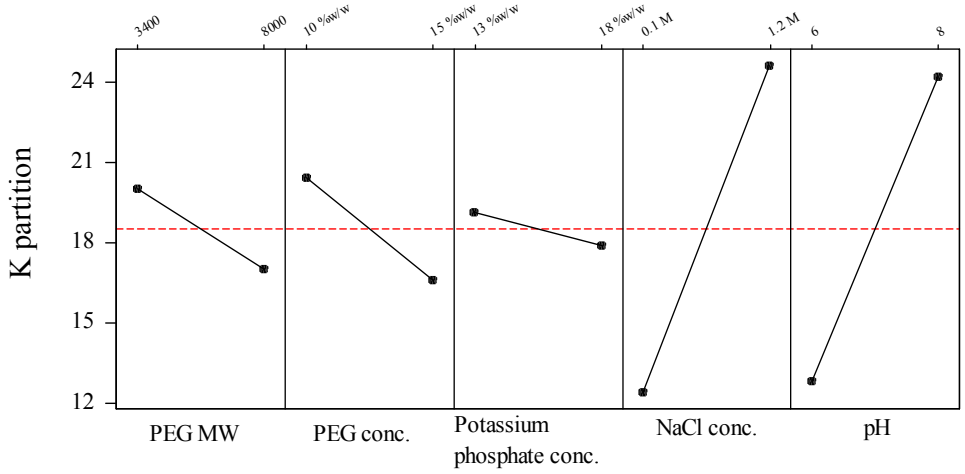

Fig. A4.3. Main effects plot for K partition.

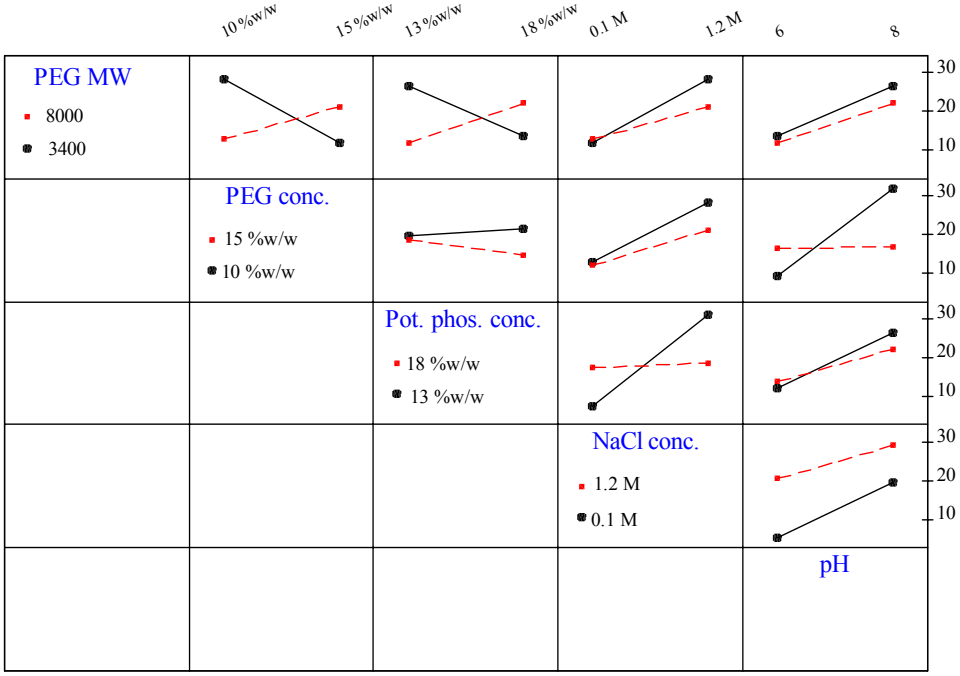

Fig A4.4. Interaction plot for K partition.

Significant factors

No factor was found to be statistically significant. The partition coefficient seems to increase with increase in sodium chloride concentration and pH, whereas it decreased with increase in the other three factors.

This system had comparable partition coefficients for tobacco protein when compared to the PEG/sodium sulfate system. Where as for lysozyme, the partition coefficient was less than that for PEG/sodium sulfate system. As a higher selectivity for

lysozyme could be obtained with the PEG/sodium sulfate system and hence this system was not investigated further.

APPENDIX 5

PEG/Sodium sulfate/Lysozyme system

This was the second system studied to determine the partition behavior of pure lysozyme. The concentration of protein in the top and bottom phases was determined using the lysozyme activity assay and the partition coefficient calculated was used as response to analyze the fractional factorial experiment. A ¼ fractional factorial experiment with the same factors and levels as used for tobacco protein was used to study the effect on the partition coefficient.

Table A5.1. Factors and levels for fractional factorial study of native tobacco partitioning.

Label	Factor	Low level	High level
A	PEG molecular weight	3400	8000
B	PEG concentration (% w/w)	10	15
C	Salt concentration (% w/w)	13	18
D	NaCl concentration (M)	0.1	1.2
E	PH	6	8

Factorial Design

Factors: 5 Base Design: 5,8 Resolution: III

Runs: 8 Replicate: 1 Fraction: ¼

Blocks: none Center points: 0

Table A5.2. Experimental conditions and observed Partition Coefficient.

Run Order	PEG MW	PEG conc. %w/w	Sodium sulfate conc. %w/w	NaCl conc. M	pH	K partition
1	3400	10	13	1.2	8	124.00
2	8000	10	13	0.1	6	1.00
3	3400	15	13	0.1	8	18.27
4	8000	15	13	1.2	6	134.33
5	3400	10	18	1.2	6	52.10
6	8000	10	18	0.1	8	6.06
7	3400	15	18	0.1	6	12.50
8	8000	15	18	1.2	8	34.83

Design Generators: D = AB E = AC

Alias Structure

I + ABD + ACE + BCDE

A + BD + CE + ABCDE
B + AD + CDE + ABCE
C + AE + BDE + ABCD
D + AB + BCE + ACDE
E + AC + BCD + ABDE
BC + DE + ABE + ACD
BE + CD + ABC + ADE

Table A5.3. Estimated effects and coefficients for K (coded units).

Term	Effect	Coefficient
Constant		47.89
A	-7.66	-3.83
B	4.19	2.10
C	-43.03	-21.51
D	76.86	38.43
E	-4.19	-2.10
B*C	-9.61	-4.80
B*E	-42.67	-21.34

Table A5.4. Analysis of variance for K (coded units).

Source	DF	Seq. SS	Adj. SS	Adj. MS	F	P
Main Effects	5	15705	15705	3141	*	*
2-Way Interactions	2	3826	3826	1913	*	*
Residual Error	0	0	0	0		
Total	7	19531				

Table A5.5. Estimated Coefficients for K using data in uncoded units.

Term	Coefficient
Constant	72.06
A	-1.70 E-3
B	151.45
C	-21.51
D	38.43
E	-2.10
B*C	-4.80
B*E	-21.34

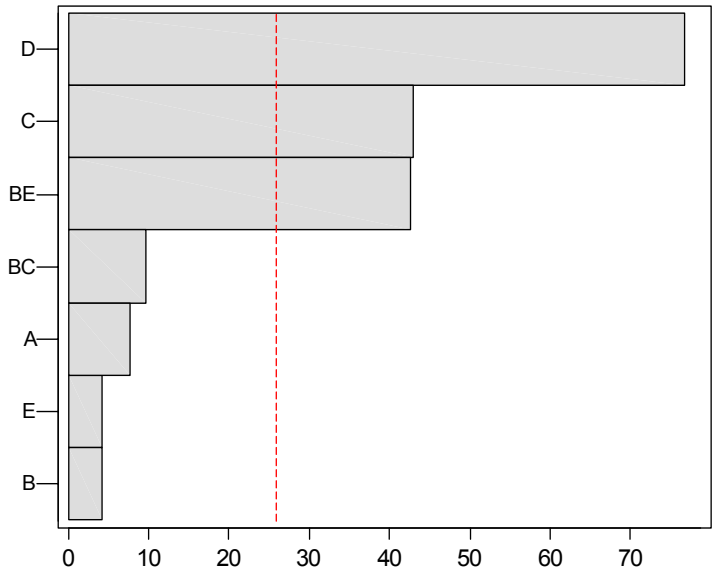

Fig A5.1. Pareto chart of the effects (response = K partition; alpha = 0.10).

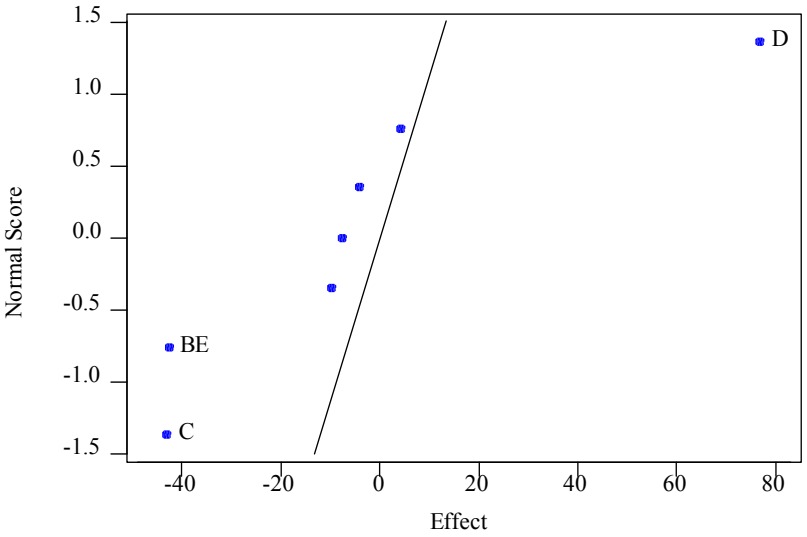

Fig.A5.2. Normal probability plot of the effects (response = K partition; alpha = 0.10).

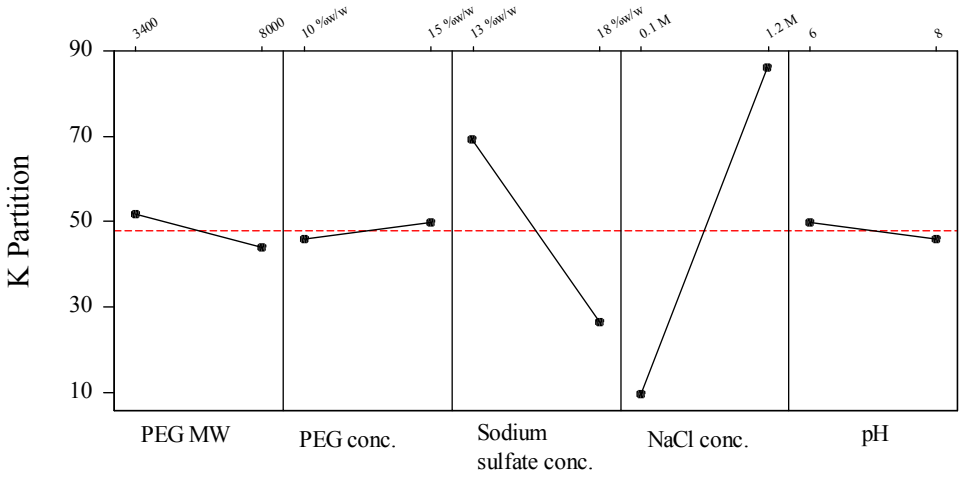

Fig.A5.3. Main effects plot for K partition.

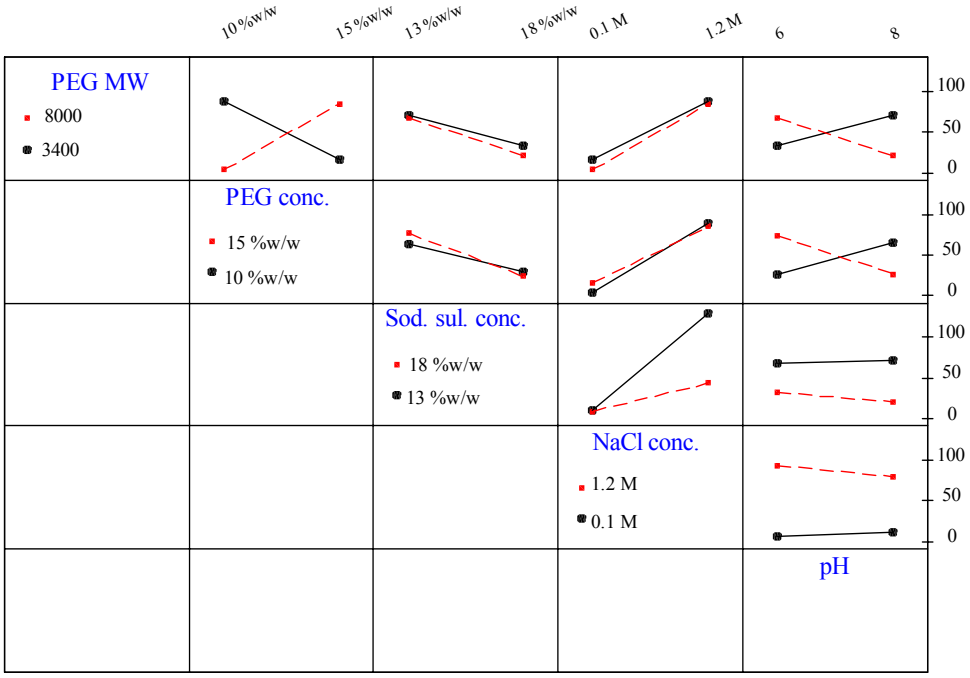

Fig.A5.4. Interaction plot for K partition.

Significant factors

C – sodium sulfate concentration.

D – sodium chloride concentration.

BE interaction aliased with CD interaction.

Among the three systems studied, this system showed the best combination of low partition coefficient for tobacco protein and high partition coefficient for lysozyme. It was further studied by spiking lysozyme into the tobacco extract by performing a

response surface study with C - sodium sulfate concentration and D - sodium chloride concentration as the variable factors.

APPENDIX 6

Central composite design – Response surface analysis

When trying to optimize a response (selectivity), a sequence of two-level factorial designs can be used to identify important factors. Two level designs can be used to model only linear effects. Once near the optimal conditions, quadratic effects need to be modeled. This involves including center points in the analysis, which will allow testing for curvature in the model. This set of experiments required the variation of sodium sulfate and sodium chloride concentration while maintaining all other factors fixed. The condition under which maximum selectivity of lysozyme over tobacco protein could be obtained was determined.

PEG/Sodium sulfate/Tobacco extract /Lysozyme system

Table A6.1. Fixed Factors.

Label	Factor	Level
A	PEG Molecular Weight	3400
B	PEG concentration	10 %w/w
E	pH of phase forming salt	7

Table A6.2. Varying Factors.

Label	Factor	Low level	High level
C	Salt concentration (% w/w)	9	15
D	NaCl concentration (M)	0.4	1.4

Central Composite Design

Factors: 2 Blocks: none Center points: 5

Runs: 13 Alpha: 1.414

Table A6.3. Experimental conditions and observed Selectivity.

Run Order	Sodium sulfate conc. %w/w	NaCl conc. M	Selectivity
1	9	0.4	24
2	15	0.4	162
3	9	1.4	35
4	15	1.4	55
5	7.75	0.9	19
6	16.2	0.9	31
7	12	0.19	21
8	12	1.60	29
9	12	0.9	79
10	12	0.9	67
11	12	0.9	67
12	12	0.9	36
13	12	0.9	23

Response Surface Regression: K partition versus Sodium sulfate conc., NaCl conc.

Table A6.4 Estimated Regression Coefficients for K partition.

Term	Coefficient	SE Coefficient	T	P
Constant	54.40	16.92	3.215	0.015
C	21.93	13.38	1.639	0.145
D	-10.59	13.38	-0.791	0.455
C*C	-3.58	14.35	-0.250	0.810
D*D	-3.75	14.35	-0.261	0.801
C*D	-29.44	18.92	-1.556	0.164

S = 37.84 R-Sq = 45.5% R-Sq(adj) = 6.6%

Table A6.5. Analysis of variance for K (coded units).

Source	DF	Seq. SS	Adj. SS	Adj. MS	F	P
Regression	5	8375.1	8375.1	1675.02	1.17	0.409
Linear	2	4743.5	4743.5	2371.77	1.66	0.258
Square	2	165.3	165.3	82.66	0.06	0.944
Interaction	1	3466.3	3466.3	3466.27	2.42	0.164
Residual Error	7	10020.9	10020.9	1431.56		
Lack-of-Fit	3	7773.7	7773.7	2591.24	4.61	0.087
Pure Error	4	2247.2	2247.2	561.80		
Total	12	18396.0				

Unusual Observations for K partition

Observation	K partition	Fit	SE Fit	Residual	St Residual
2	162.000	109.025	29.912	52.975	2.29R
6	31.500	78.252	29.912	-46.752	-2.02R

R denotes an observation with a large standardized residual.

Table A6.6. Estimated Regression Coefficients for K partition using data in uncoded units.

Term	Coefficient
Constant	-295.63
C	34.52
D	241.31
C*C	-0.40
D*D	-14.99
C*D	-19.63

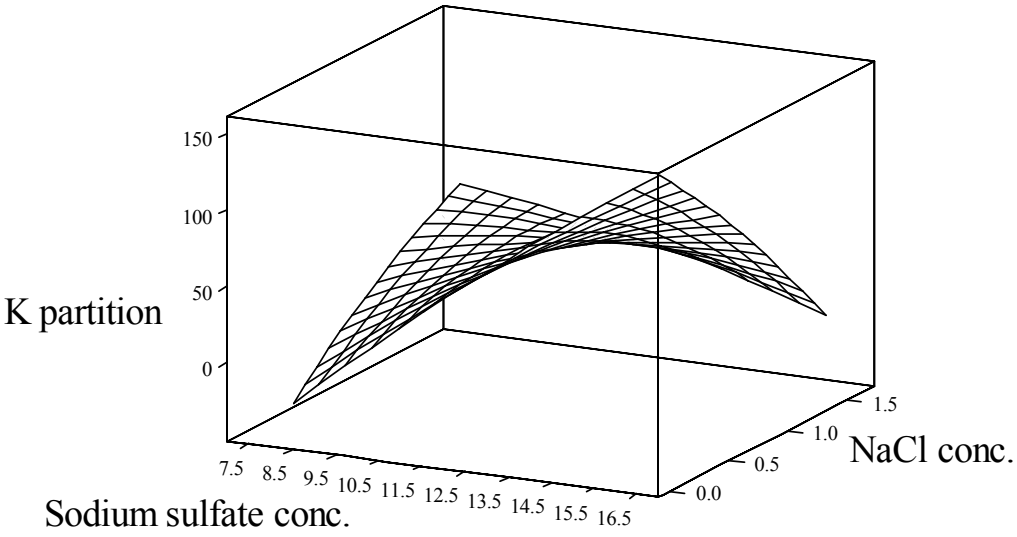

Fig A6.1. Response surface plot of K partition.

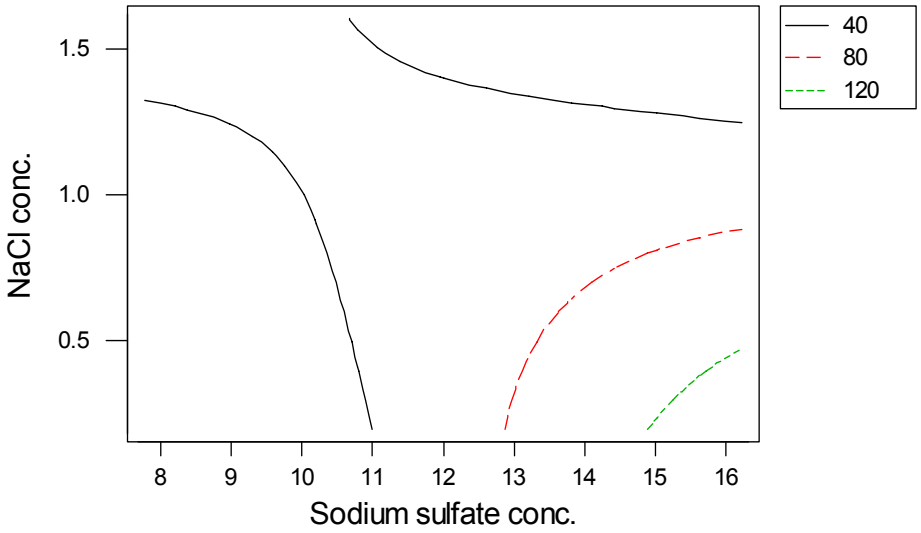

Fig A6.2. Contour plot of K partition.

Using the response optimizer function in MINITAB (version 13) for response surface analysis, the conditions and the maximum selectivity (Global Solution) of lysozyme that could be obtained for this system were determined to be 16.2 % w/w sodium sulfate concentration, 0.19 M NaCl concentration with selectivity of 57.7.

VITA

Deepa Balasubramaniam

Deepa Balasubramaniam was born on February 2nd, 1980 in Chennai, India. She graduated with a Bachelor of Technology degree in Chemical Engineering from Sri Venkateswara College of Engineering affiliated to the University of Madras, India in May 2001. In August 2001, she began her study for a Master of Science degree in Biological Systems Engineering at Virginia Tech. After completion Deepa plans to work on research and development of recombinant protein based therapeutics.