Purification of human antibodies by using liquid/liquid extraction

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Summary

Aqueous two-phase extraction is a well-established technique with wide spread use in laboratory separations and some impact in industrial scale separations. We have investigated the use of aqueous two phase systems for the purification of a human antibody expressed in corn. The antibody was expressed fully non-glycosylated. We show that by using a poly(ethylene)glycol/phosphate or poly(ethylene)glycol/citrate systems in combination with an interface precipitation step it is possible to recover 80 percent of the antibody with a 10-fold purity. The method is simple, very robust, and easy to scale up.

Introduction

Mixing two hydrophilic polymers or one hydrophilic polymer and a salt at well-defined concentrations forms two liquid phases at equilibrium (Forciniti, 1999b; Forciniti et al. 1991). These systems are called aqueous two-phase systems (ATPS). ATPS can be used for the purification of biological products because proteins and other biological materials distribute unequally between the two phases. Aqueous two-phase extraction is a well-established technique with wide spread use in laboratory separations and some impact in industrial scale separations. For either industrial or recombinant proteins ATPS offer notable advantages (Winter et al. 1999; Lorch, 1999). For industrial enzymes, the method's utility stems from the ease with which it can be adapted to continuous production and scaled up to meet industrial needs. For example, Genencor International (Winter et al. 1999) currently uses aqueous two-phase extraction coupled with ion exchange chromatography for the purification of chymosin. The most common system is made up by poly(ethylene)glycol (PEG) and dextran. However, PEG/salt systems are particularly attractive from an industrial view point because of their low cost. Common salts used include sodium or potassium phosphate, ammonium sulfate, sodium chloride and sodium citrate. The selectivity of the technique can be improved by adding affinity ligands, by operating it in liquid chromatography mode (one phase is immobilized and the other is used as the eluent (Walsdorf, 1990; Huang and Forciniti, 2002)), or by a combination of both. Whether the technique is used in batch or in chromatography modes, all the know-how that exists for liquid/liquid extraction using organic solvents and for chromatography is available.

The transgenic production of antibodies in plants needs to be followed by a robust separation process. Otherwise, the cost advantages associated with the production of the antibody in plants are lost. Because the use of plants for the production of human proteins is relatively knew, little is known about the downstream processing units that would present an economic advantage. Kusnadi *et al.* (Kusnadi et al. 1997) have recently pointed out that there is a lack of data in the downstream processing of proteins from transgenic plants. They also pointed out that most of the published work is at laboratory scale using techniques of questionable scaling up procedure. Our research helps to fill this gap.

We have developed a new process by which human antibodies expressed in corn are isolated to high purity and yield using aqueous two-phase extraction. Different modes of op-

eration were explored while searching for optimum conditions. We have found that one extraction step, where the target antibody concentrates in the bottom phase, followed by a second extraction step, where the target antibody precipitates at the interface, yields the best results. The optimum purification protocol consists of the following steps: 1) Extraction of the antibody (and contaminating proteins) from the corn meal using a NaCl solution. 2) Addition of poly(ethyleneglycol) of various molecular weights (10,000, 8,000, 3350, or 1450) and one or more of the following salts (sodium phosphate monohydrate, sodium phosphate dehydrate, sodium citrate, citric acid, sodium chloride, lithium chloride) to a concentration high enough to induce the formation of two liquid phases at equilibrium. 3) Removal and disposal of the upper phase (PEG-rich). 4) Addition of PEG to the bottom phase to create a second ATPS. 5) Recovering of the antibody from the new liquid/liquid interface. 6) Removal of the excess salt by diafiltration.

Materials

The antibodies were generously supplied by Monsanto Protein Technologies (St. Louis, MO). PEG of various molecular weights was purchased from Sigma (St. Louis, MO). All the other chemicals were of analytical grade.

Methods

Extraction of the Antibody. Extraction of the antibody from the corn meal was done with a solution of NaCl (150mM) for 8h. The suspension was centrifuged for 30 min at 10,000 rpm, filtered through a filter paper, and then filtered through a 0.45μm membrane. For some experiments the corn meal extract was concentrated 3X by ultrafiltration (5kd cut off) whereas for others the meal extract was not concentrated.

Partitioning Experiments.

a) Single extraction

The partitioning experiments were performed in poly(propylene) centrifuge tubes. The systems were prepared according to Forciniti (Forciniti, 1999a). In short, a known amount of the phase-forming species in solid form (PEG and potassium phosphate or PEG and sodium citrate), a salt in solid form or from a stock solution, and enough corn meal extract to complete 10 g were added to a tube. The polymer molecular weight was 1,450, 3,350, 8,000, or 10,000. The pH was adjusted with HCl or NaOH as needed. All experiments were done in duplicate and some were repeated three or more times. The content of the tubes were mixed in a rotary mixer for 20 minutes after initial manual mixing and then centrifuged at 2,000 rpm for 20 minutes. The volumes of the top and bottom phases were noted and samples were carefully removed from each phase with a pipette. The antibody concentration was determined by running the phases through a Protein A column calibrated with human IgG or with the same antibody previously purified. Protein concentration in each phase was determined by one or all of the following: Absorbance at 280 nm, the Bradford assay, or the BCA assay. The course of the separation was also monitored by gel permeation chromatography and by isoelectric focusing.

Purity was calculated as the ratio between the amount of antibody to total protein. Fold purification in stage i was calculated as the ratio between the purity of the antibody in stage i to the purity of the antibody in the meal extract.

Table 1 shows the systems' IDs, phase-forming species concentrations, and PEG molecular weight used in this work.

Table 1. Aqueous-Two Phase systems identification			
System ID	*[PEG] % w/w*	*[Salt % w/w]*	*PEG MW*
A	*16.1*	*10 (PK)*	*8000*
B	*10.0*	*15(PK)*	*3350*
C	*14.8*	*13.3*	*3350*
D	*19.25*	*10.0*	*3350*
E	*20.0*	*10.0*	*1450*
F	*18.0*	*12.0*	*1450*
G	*15.0*	*20.0(Citrate)*	*3350*
H	*14.8*	*18.25(Citrate)*	*3350*

b) Multiple extractions
The protocol followed in all multiple extraction experiments was as follows. The top phase was completely removed after the first extraction. Enough PEG and salt were added to create a new ATPS. The contents of the tube were well-mixed and centrifuged at 2,000 rpm for 20 minutes. This process was repeated for each new extraction step. Samples of top and bottom phases were removed after complete phase separation in each step and the concentration of antibody measured as described above.

c) Single extraction followed by precipitation
The protocol for single extractions followed by precipitation at the interface was as follows. Two-phase systems were created as in (a). The top phase and the interface were removed and discarded. More PEG was added to induce the formation of two phases separated by an interface where the antibody precipitated. The protein was carefully removed from the interface and its concentration determined as described in (a).

d) Gel Isoelectric Focusing. Isoelectric focusing was performed using a BioRad horizontal platform connected to a BioRad power source. The gels were focused until the voltage reached 1,500 volts and then focusing was continued at this voltage for approximately two hours. Different ampholytes under native or denatured (urea) conditions were used. The gels were stained with Coomassie Blue.

e) PAGE and SDS-PAGE. PAGE gels were run at constant voltage (55V). Samples were dissolved in 62.5 mM TRIS (pH 6.8), 25% glycerol and 0.01% bromophenol blue, the running buffer was 25 mM TRIS base and 192 mM glycine. SDS-PAGE gels were run at a constant voltage of 60V. The samples were dissolved in 62.5 mM TRIS (pH 6.8), 2% SDS, 25% glycerol, 0.01% bromophenol blue, 5% BME(2-mercaptoethanol), and boiled at 90 ^{0}C for 1 to 2 minutes and immediately cooled in ice water. The anode buffer was a 24.2 g/L TRIS (pH 8.9) solution whereas the cathode buffer was a 12.1 g/L TRIS base, 17.9 g/L tricine, and 1 g/L SDS. All gels were stained with Coomassie Blue.

f) Multiple Angle Laser Light Scattering. Samples of the antibody crude extract or from the phases of the ATPS were injected through a 50 μL or a 250 μL loop into a gel permeation column (Protein KW-803, Shodex). Two detectors were connected in series: 1) a Hitachi UV spectrophotometer and 2) a Wyatt multiple angle laser light scattering detector. The flow rate was either 1 ml/min or 0.5 ml/min.

The extinction coefficient of the antibody was calculated as ε = 1.15 cm^2/mg. The molecular weight of the antibody was determined by Debye plotting of the scattering data using an internal calibration constant for the UV detector.

Results.

Some coarse characterization of the impurities is necessary to rationally design the separation of any single protein from a complex mixture. Two physical properties of contaminant proteins that are particularly valuable for the design of a separation protocol are their molecular weight and isoelectric points. The molecular weights and isoelectric points of the impurities were determined by using MALS (Multi Angle Laser Scattering equipment), IEF (Isoelectric Focusing), SDS-PAGE, and PAGE. MALS chromatograms show a peak around 8ml that corresponds to 94% of the antibody. There is a small shoulder at lower retention times that corresponds to antibody dimers (this has been confirmed by dynamic light scattering). Most of the impurities are smaller than the antibody (broad multiple peaks at higher retention volumes). Only one impurity is larger than the antibody (peak around 5.5ml). Most proteins have an isoelectric point between pH 5 and 8 (but at least one protein has an isoelectric point higher than the antibody), which is lower than the one of the antibody (~9.2).

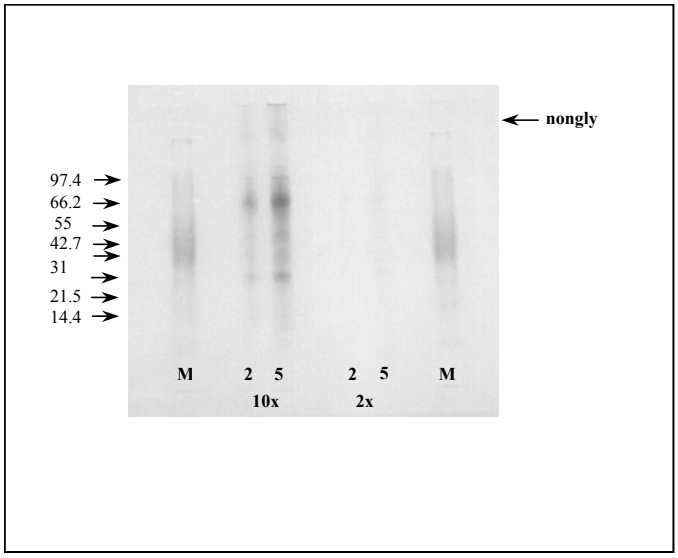

Figure 1. M: Marker; 2: 2 µl; 5: 5 µl 10x: 10 times concentrated sample 2x: 2 times concentrated sample

SDS-PAGE shows two major bands (around 30 and 48kd). The crude extract shows multiple bands corresponding to different protein sizes. PAGE of the crude extract shows that most of the contaminants are smaller than the antibody (Figure 1, confirming the results obtained by MALS). The differences in isoelectric point and size between the antibody and the impurities suggest that aqueous two-phase extraction (because of the sensitivity of the partition coefficient to pH and molecular weight of the substance being partitioned) could yield a product of high purity.

The experimental matrix explored in this work covered a range of PEG molecular weights, type of salt, pH, temperature, and the addition of additives. Single step extractions, multiple steps extraction, and one extraction combined with a precipitation step were explored. In the initial, exploratory, steps we used two PEG/Phosphate systems (System A: 16.1% PEG 8,000, 10% potassium phosphate and System B: 10% PEG 3,350, 15% potassium phosphate) at pHs 6, 7 and 8. The extract was concentrated 3-fold, mostly to facilitate the determination of the antibody. The concentration of antibody in the crude extract was 0.24 mg/ml and its purity 4.1%. Total protein was measured at 280 nm, by the Bradford test or the BCA assay whereas the amount of antibody was quantified by running the sam-

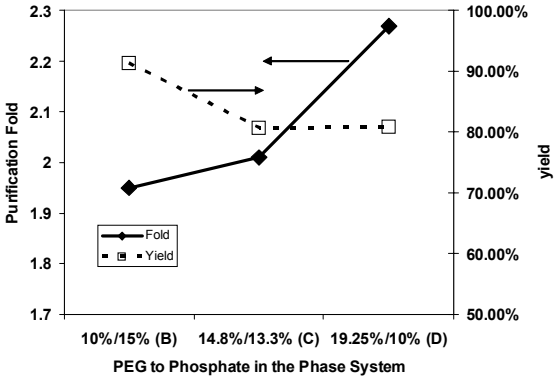

Figure 2. Effect of volume ratio between top and bottom phases on yield and purification fold. pH: 6. PEG molecular weight: 3350.

ples through a Protein A column. Because no antibody was detected in the top phase, partition coefficients were not calculated. The best conditions (high yield and high purity) were obtained at pH 6. Using low molecular weight PEG systems increases the recovery of the antibody but the purity of the preparation deteriorates. The increase recovery observed when using a lower molecular weight PEG is expected since the lost of antibody is attributed to precipitation at the interface, and this should be minimized by using a low molecular weight polymer. Because of its high recovery, we decided to explore further the effect of the volume ratio between top and bottom phases of the PEG-3350. System B's top phase represents approximately 25% of the total volume, System C's top and bottom phase are nearly the same size, and System's D top phase is about 75% of the total volume. All systems have the same tie line length. The results are summarized in Figure 1. Although in "true" partitioning (equilibrium distribution of a solute between two liquid phases) the volume ratio between the phases should not affect partitioning, the relative volume of both phases is expected to have an effect here because the antibody is partitioning between the bottom phase and the interface. It is clear that the smaller the volume of the bottom phase the higher the purity but the lower the yield. A small volume in the bottom phase means that the solubility of the protein may be exceeded and therefore, the antibody would precipitate at the interface (decreasing yield). Because most of the impurities are smaller than the antibodies (and therefore they are not expected to precipitate at the interface), the same argument cannot be used to explain the increase in purity. Why the impurities are partitioning more strongly into the top phase remains unknow. We can speculate that the higher concentration of antibody in the bottom phase is "pumping" the impurities into the top phase. A similar phenomenon was previously observed by us in the purification of crystallins from calf lenses (Bermudez and Forciniti, 2002).

Table 2. Effect of pH on purity and recovery for PEG/citrate systems

	A280nm	BCA	BCA
pH	4	5	6
Composition(PEG/salt)	15%/20%	15%/20%	15%/20%
Initial [Ab] (mg/ml)	0.108	0.108	0.108
Final [Ab] (mg/ml)	0.136	0.223	0.344
Initial purity	3.20%	7.30%	7.30%
Final purity	4.09%	29.79%	22.77%
Fold	1.28	4.08	3.12
Recovery (Yield)	77.72%	61.70%	63.57%

Because HPLC chromatograms show that most impurities are smaller than the antibody, attempts to improve the purification fold and yield were done by using PEG 1,450/ potassium phosphate systems. A lower molecular weight PEG should decrease the precipitation of the antibody at the interface increasing the purification fold and yield. The chosen pHs were 6,7, and 8 and the temperature was held at 25 ^{o}C. The overall PEG and phosphate compositions were 20 and 10 % for system E and 18 and 12 % for system F, respectively. Although the purity of the antibody isolated with a PEG 1,450/phosphate system was slightly better than when a PEG 3,350/phosphate system was used, the recovery of the antibody in the bottom phase was too low. Visual observation of the interface shows a decrease in the amount of antibody precipitated at the interface; unfortunately, this low molecular weight PEG allows the bulky antibody to partition into the top phase. As the pH increases from 6 to 8 both the fold in purity and the recovery decrease; a similar trend was observed with PEG 3,350

systems. As the pH approaches the isoelectric point of the antibody, the protein solubility decreases and thus it deposits at the liquid/liquid interface in the form of aggregates.

A PEG/salt system that it is particularly attractive is PEG/citrate because citrate is environmentally benign (as opposite to phosphate). Moreover, the use of citrate buffers allows us to expand the pH range towards the acidic end, which considering the physical chemistry properties of the antibody and the impurities should improve the purification fold. PEG/citrate systems were prepared at 25 ^{o}C and at pHs 4,5, and 6. The composition of the system was 15% PEG and 20% citrate (System G). The appropriate amounts of citric acid and trisodium citrate were added to adjust the pH. Table 2 summarizes our results.

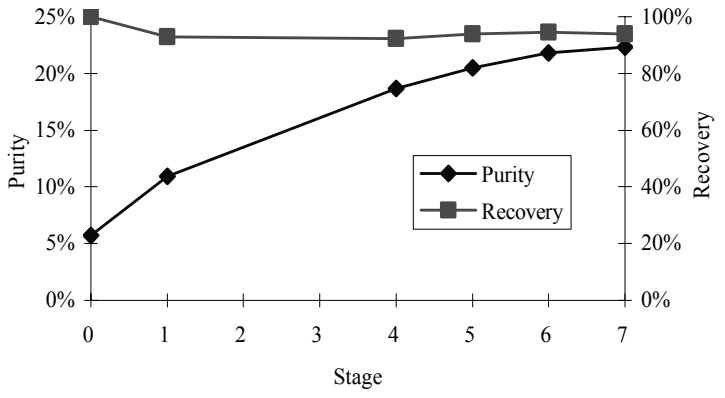

Figure 3. Purity and Recovery vs. the number of stages.

The proteins (including the antibody) precipitate in the bottom phase at pH 4 whereas the antibody was partially precipitated in the bottom phase and at the interface at pH 5. At pH 6, the antibody was completely precipitated at the interface. This combination of partitioning into the bottom phase and precipitation at the interface observed at pHs 5 and 6 will be explored later. A battery of experiments was performed using different total phase-forming species concentrations at pHs 5 and 6 and at 25 ^{o}C . The results show that an increase in tie-line length (an increase in the both the concentration of PEG and citrate) drastically decreases the yield because the solubility limit of the antibody is exceeded.

Multiple Extractions

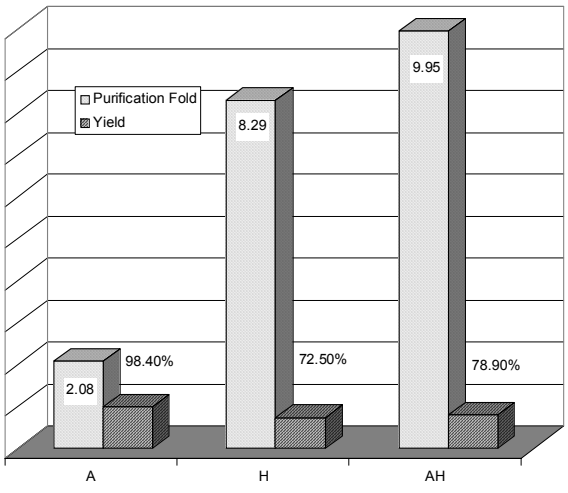

Figure 4. Purification folds and yields obtained by using two PEG/phosphate systems in series. The antibody is recovered in the bottom phase of the first system and at the liquid/liquid interface of the second. .

Basic engineering principles suggest that a series of extraction steps should be more effective than a single step. Figure 3 shows the results for a series of seven consecutive extractions with system A. By performing a serial extraction (7 stages) we were able to recover 94% of the antibody with 22% purity (~5 fold increase). This is more than twice the purity obtained in a single stage. The figure suggests that the purity reaches a plateau after seven stages.

Extraction Followed by Precipitation

Although we recovered nearly 100% of the antibody using a single extraction with System B (PEG (3,350)/potassium phosphate (10%/15%), pH 6), the

final purity was still low; i.e. lower than 50%. A small change in the concentration of the phase-forming species (System H (PEG-3,350/Potassium phosphate,14.8%/18.25%, pH 6) yielded a 41% pure antibody but the recovery is substantially lower. Therefore, we decided to use two extraction steps sequentially. For the first step we used System A (low purity and high yield) and for the second step we used System H (high purity low yield). Under these conditions the antibody is concentrated, as a precipitate, at the interface of the second ATPS. This two-steps extraction yields a product that is 50% pure with a 79% yield (Figure 4).

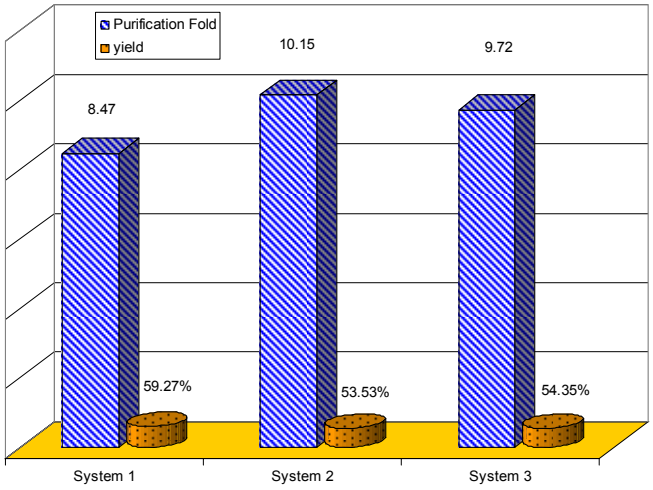

Figure 5. Purification factors and yields of a series of PEG/citrate systems

A similar approach was followed using PEG/citrate systems. The results presented in Figures 5 and 6 suggest that PEG/citrate systems at pH 5 of compositions 12%/17% and 13%/18% are good choices for the 1st stage of a two-phase extraction because high recovery of the target protein is more important than purity in the 1st stage. The PEG/citrate system at pH 5 of composition 16%/21% can be used for the 2nd stage. Similarly, a PEG/citrate composition of 9%/14% and one of 10%/15% can be the candidates for the 1st stage and one of 13%/18% or 14%/19% would be used for the 2nd stage. The results are summarized in Figure 10. The fold purification is around 10 but the yields are very low compared to the yields obtained, under similar conditions, by using PEG/phosphate systems. Further optimization of these systems by varying the concentrations of the phase forming species increases the yield but the values are still below those of PEG/phosphate systems (Figure 6). Still, the purity obtained with PEG/citrate systems is better (61%) than with PEG/phosphate systems. For System 2 at pH 5 and System 3 at pH 6 some antibody still remained in the bottom phase after the second extraction; this explains the lower yield.

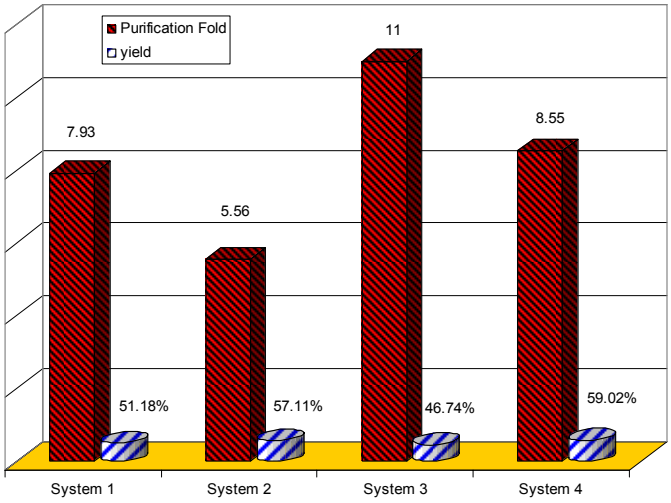

Figure 6. Purification factors and yields of a series of PEG/citrate systems

Conclusions.

We have shown that a transgenic antibody expressed in corn can be purified up to ten-fold with yields ranging from 60 to 80% by using a two steps separation scheme consisting of a first liquid-liquid extraction step followed by a precipitation step (at a liquid/liquid interface). The process is simple and it does not required specialized equipment. More importantly, the process is particularly suitable to handle large volumes of crude extract; a situation likely to be found in the production of antibodies by transgenic plants.

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Acknowledgments. This work was performed under a contract with Monsanto Proteins Technology; St. Louis, MO, USA.