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Purification of recombinant apolipoprotein A-1_{Milano} expressed in *Escherichia coli* using aqueous two-phase extraction followed by temperature-induced phase separation

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Abstract

A method for purification of recombinant apolipoprotein A1 in aqueous two-phase systems has been studied. A mutant of apolipoprotein A-I, the Milano variant, was expressed in *E. coli*. Phase systems containing ethylene oxide (EO)–propylene oxide (PO) random copolymers have been used. These polymers are thermoseparating and have the ability to separate into one water-rich and one polymer-rich phase when heated above a critical temperature i.e. the cloud point. The filtrate from an *E. coli* fermentation was added to a primary aqueous two-phase system composed of an EO–PO copolymer and Reppal, which is an inexpensive hydroxypropyl starch. Apolipoprotein A-I was partitioned to the top EO–PO copolymer phase and contaminating proteins to the bottom starch phase. The phase diagrams for Reppal PES 100-$EO_{50}PO_{50}$ (Ucon) and Reppal PES 100-$EO_{30}PO_{70}$ were determined. The effect on partitioning, when changing parameters such as polymer concentration, type of polymer, protein concentration, pH, salt concentration and volume ratio, were studied. Studies on *E. coli* DNA partitioning showed that DNA could be partitioned strongly to the bottom phase. An optimal system was scaled up from 5 g to 5 kg with similar degrees of purification, i.e. 2.5 and 2.7 and yields of 79% and 82% respectively. Furthermore temperature-induced phase formation was used for separation of apolipoprotein A-I from the copolymer by raising the temperature above the copolymer cloud point; thus, recovering protein in a 'clean' water phase.

Keywords: Aqueous two-phase systems; Thermo separating polymer; Apolipoprotein A-1; Recombinant proteins

1. Introduction

Aqueous two-phase systems have widespread use in biochemistry and biotechnology for purification of biological materials [1,2]. The systems are composed of two incompatible polymers, e.g. dextran and poly(ethylene glycol) (PEG), or a polymer e.g. PEG and a high concentration of salt e.g. phosphate. These systems are suitable for biological samples as the phases contain 70–90% water, thus, decreasing the possibility of denaturation of labile biomolecules [3]. The partitioning of proteins in aqueous two-phase systems depends mainly on the physicochemical properties e.g. protein hydrophobicity, charge and size [1]. The partitioning can be influenced by changing polymers, polymer molecular mass, the pH or by addition of salts to the system [4]. Advantages of aqueous two-phase extraction lie in volume reduction, high capacity and rapid separations. The technique can be used early in the purification on

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process streams containing whole cells or cell debris. Compared to other separation techniques (e.g. chromatography) two-phase extraction is relatively straightforward to scale-up [5]. Aqueous two-phase systems for lab-scale separations are most often composed of PEG and dextran. Dextran is a relatively expensive polymer and for large-scale purification, e.g. industrial-scale enzyme extraction, PEG/salt systems are more commonly used [6]. However, the PEG/salt systems have the disadvantage of low solubility for amphiphilic proteins which have a high tendency to aggregate in water solution. Recently the use of thermoseparating polymers in aqueous two-phase systems has been introduced. When such polymers are heated above a critical temperature i.e. the cloud point, the solubility of the polymer will decrease and a system composed of a water and a polymer phase is formed. This makes it possible to perform a temperature-induced phase separation whereby a target protein can be separated from the polymer and recovered in a 'clean' water phase [7].

In this study the purification of apolipoprotein $A1_{Milano}$ (Apo $A\text{-}1_M$) from a filtrate of *E. coli* proteins, using aqueous two-phase systems and temperature-induced phase separation is presented. Apo $A\text{-}1_M$ is a natural variant of apolipoprotein A1 with a mutation where arginine 173 has been substituted with cysteine [8]. This mutation gives rise to disulphide linked dimers. The molecular mass of the monomer is approximately 26 kDa and the p*I* is 5.6–5.7 [9]. The monomer of Apo $A\text{-}1_M$ has a dense structure [10] and contains six major α-helix segments. The α-helices are amphipathic, creating a protein where one surface is hydrophobic and the other is hydrophilic, and the protein has a strong tendency for aggregation between the hydrophobic surfaces [11]. There is also, therefore, a tendency for apolipoproteins to form micellar structures with other proteins and lipids in the solution [12]. Apolipoproteins are currently purified at laboratory scale with the use of centrifugation and ion-exchange chromatography or affinity columns and gel filtration [12]. The present work was carried out as a basis for the development of a large scale aqueous two-phase extraction process for Apo $A\text{-}1_M$.

In this study we have used polymer/polymer two-phase systems with the top phase enriched with a thermoseparating polymer and the bottom phase enriched with hydroxypropyl starch, i.e. Reppal PES 100, Reppal PES 200, which are low-cost substitutes for dextran [13,14]. The thermoseparating top-phase polymers used are random copolymers of ethylene oxide (EO) and propylene oxide (PO), with different % weight ratios of EO:PO, i.e. 50% EO/50% PO (Ucon) or 30% EO/70% PO (EO30PO70). The hydrophobicity of the copolymer increases with increasing propylene oxide content and the cloud point is decreased with an increase in hydrophobicity. The cloud point temperature further depends on the concentration of the copolymer [15].

The aim of the purification process was to partition Apo $A\text{-}1_M$ to the top phase in a primary-phase system containing the relatively more hydrophobic EO–PO copolymer and Reppal. After separating the phases, the top phase is isolated in a separate vessel, and phase separation is induced by heating above the copolymer cloud point [7,16,17], 50°C for Ucon and 40°C for EO30PO70 for a 10% solution. A new phase separation is obtained where a polymer-rich phase and a water-rich phase are formed. The temperature at which the polymers separate out of the solution can be lowered by addition of salts [16]. Target proteins are almost exclusively partitioned to the water-rich phase and the top-phase polymer can be recycled.

Experiments with a factorial design were used to highlight important variables for partition of Apo $A\text{-}1_M$ to the top phase. Different polymer combinations, polymer concentrations, volume ratios between top and bottom phases and the effect of the hydrophobic salt, triethylammonium phosphate, Et_3NHPO_4, were studied. The effect of the addition of urea was also studied in some of the two-phase systems. After the important variables had been determined, a suitable system with regard to purity and yield was selected. The possibility of scale up for use at process scale was also an important criterion in the selection of the system.

2. Experimental

2.1. Chemicals

Hydroxypropyl starch polymers Reppal PES 100 (M_r 100 000) and Reppal PES 200 (M_r 200 000)

were supplied from Carbamyl AB (Kristianstad, Sweden). The EO–PO random copolymer Ucon 50-HB-5100 (EO50PO50) (M_r 4000) was supplied from Union Carbide (New York, NY, USA) and EO30PO70 (M_r 3200) from Shearwater Polymers, (Huntsville, AL, USA). The antibodies for the ELISA assay were a gift from Pharmacia and Upjohn (Stockholm, Sweden). *E. coli* DNA was bought from Sigma Chemical (St. Louis, MO, USA). All other chemicals were of analytical reagent grade.

2.2. Apolipoprotein A-1_M

Recombinant apolipoprotein A-1_M was expressed in *E. coli* fermentations. Apo A-1_M is exported to the periplasmic space and is subsequently obtained in the fermentation broth. The cells were removed by flocculation followed by centrifugation. The supernatant containing Apo A-1_M was concentrated 10 times by ultrafiltration using a membrane with a 10 000 M_r cut off. The concentration of Apo A-1_M after ultrafiltration was 2.8 mg/ml and the total protein concentration was 30 mg/ml. The buffer in this starting material was 20 m*M* Tris–HCl pH 8, 150 m*M* NaCl, 10 m*M* EDTA and 0.1% Tween 80.

2.3. Phase diagrams

The borderline between one and two-phases is called the binodial curve. The polymer concentration of the two-phases in equilibrium with each other are described by tie-lines in the phase diagram. The concentration of Reppal PES 100 was determined with polarimetry using a digital polarimeter (Model AA-10) from Optical Activity (London, UK). By making a polarimetric standard curve for Reppal PES 100, the specific rotation could be determined, 194.6 degree ml g^{-1} dm^{-1}. The refractive index of Reppal PES 100, Ucon and EO30PO70 was determined at 20°C with a refractometer from Carl Zeiss (Oberkochen/Württ., Germany). Different concentrations of Reppal PES 100 and Ucon or EO30PO70 were mixed and the resultant systems were separated into two-phases by centrifugation for 10 min at 1360 *g*. Samples from the top and bottom phases were diluted and measured with polarimetry and refractive index. The Reppal PES 100 concentration in both phases was determined by polarimetry. Ucon and EO30PO70 concentrations were determined by refractometry by subtracting the refractive index contribution of Reppal PES 100. Some points, near the critical point, were determined by titrating the two-phase system with water until a one-phase system was reached. The phase diagrams for the Ucon 50-HB-5100/Reppal PES200 and EO30PO70/Reppal PES200 systems can be found in Refs. [18,19], respectively.

2.4. Two-phase systems

All polymer concentrations were calculated as % weight/weight (w/w). The two-phase systems were prepared by dissolving the pure polymers in the protein solution obtained after cell removal and ultrafiltration. Systems with a final weight of 5 g were prepared. All experiments were performed in duplicate and the experimental data are average values. The 5-g systems were mixed with the help of a magnetic stirrer and the 5-kg system was mixed with a motor driven propeller. The separation into two-phases was assisted by centrifugation at 1360 *g* for 10 min. The partitioning of molecules in two-phase systems is described by the partition coefficient *K*. It is defined as the concentration in the top phase, C_T, of the molecule of interest divided by the concentration in the bottom phase, C_B: $K=C_T/C_B$.

2.5. Temperature-induced phase separation

The top phase of the primary phase system, containing the EO–PO copolymer, was removed and isolated in a separate vessel. The temperature of this phase was increased above the copolymers cloud point. For the Ucon top phases, a temperature of 60°C was used and for EO30PO70, 50°C was used to achieve separation. No additional salts were added to the aqueous two-phase systems in this separation step.

2.6. Factorial experimental design

All phase system variables (e.g. polymer concentration, protein concentration) were tested at two different levels (shown with + and − in Tables 1 and 2). In the factorial experimental design, the selected variables were shifted simultaneously and

Table 1
Factorial design 1 for purification of Apo A-1_M

Experiment	Total protein conc. (mg/ml phase syst)	Polymer conc. (% R–% U)	Salt conc. (m*M*)	pH
1	9 (−)	12–7 (−)	10 (−)	8.0 (+)
2	13 (+)	12–7 (−)	10 (−)	8.0 (+)
3	9 (−)	10–6 (+)	10 (−)	7.3 (−)
4	13 (+)	10–6 (+)	10 (−)	7.3 (−)
5	9 (−)	12–7 (−)	20 (+)	7.3 (−)
6	13 (+)	12–7 (−)	20 (+)	7.3 (−)
7	9 (−)	10–6 (+)	20 (+)	8.0 (+)
8	13 (+)	10–6 (+)	20 (+)	8.0 (+)

Phase system parameters: protein and polymer concentration, salt, pH. The + and − signs were used when the response variables were calculated. R = Reppal PES200 and U = Ucon 50HB 5100. The salt added was sodium phosphate.

none of the experiments in the series was identical to the other. The experiments were carried out in random order to minimise effects of systematic errors. For each experiment, several responses were calculated (e.g. yield, purification factor). The effect of each variable was determined as the mean value of the responses including the level for the variables [20]. The variables giving the strongest influence on the response were identified by having the calculated effect with the highest absolute value. The calculated effects were both positive and negative and a positive effect indicated that the analysed variables on the positive level had the strongest influence. To ascertain that the calculated responses were significant, normal distribution plots were constructed, (not shown).

2.7. Determination of protein and Apo A-1_M

The total protein content was determined according to Bradford [21], using Coomassie Brilliant Blue G. The absorption was measured at 595 and 465 nm and the absorption at 465 nm was then subtracted from the 595 nm absorption. Bovine serum albumin was used for standard. The spectrophotometer used was UV-2101 PC from Shimadzu (Kyoto, Japan).

Table 2
Factorial design 2 for purification of Apo A-1_M

Experiment	Bottom phase polymer (Reppal PES)	Top phase polymer	Top phase volume	Polymer conc. (%) (bottom–top)
1	100 (−)	Ucon (−)	small (−)	3.2–15.0 (−)
2	200 (+)	Ucon (−)	small (−)	3.6–14.0 (−)
3	100 (−)	EO30PO70 (+)	small (−)	4.5–16.0 (−)
4	200 (+)	EO30PO70 (+)	small (−)	4.5–16.0 (−)
5	100 (−)	Ucon (−)	large (+)	5.4–11.0 (−)
6	200 (+)	Ucon (−)	large (+)	6.0–10.0 (−)
7	100 (−)	EO30PO70 (+)	large (+)	7.0–11.0 (−)
8	200 (+)	EO30PO70 (+)	large (+)	6.3–12.0 (−)
9	100 (−)	Ucon (−)	small (−)	3.4 − 20.0 (+)
10	200 (+)	Ucon (−)	small (−)	4.0–20.0 (+)
11	100 (−)	EO30PO70 (+)	small (−)	4.5–19.0 (+)
12	200 (+)	EO30PO70 (+)	small (−)	4.5–22.0 (+)
13	100 (−)	Ucon (−)	large (+)	6.1–15.0 (+)
14	200 (+)	Ucon (−)	large (+)	7.3–14.0 (+)
15	100 (−)	EO30PO70 (+)	large (+)	8.5–11.0 (+)
16	200 (+)	EO30PO70 (+)	large (+)	10.1–12.0 (+)

Phase system parameters: type of polymer, polymer concentration and volume ratio of the phases. The response variable was the purification of Apo A-1_M. The positive and negative signs indicate how the parameters were changed. This sign was used when the response was calculated.

The concentration of Apo A-1_M was measured by an ELISA assay. A biotin conjugate was used and further coupled to an avidin that was alkaline-phosphatase linked. The alkaline phosphatase was used as a marker and the absorption was measured at 405 nm.

The yield was calculated as:

$$\text{Yield} = (C^t_{Apo} * V^t)/(C_{Apo} * V)$$

where C^t_{Apo} is the concentration of Apo A-1_M in the top phase and V^t is the volume of the top phase. C_{Apo} is the concentration of Apo A-1_M in the starting material and V is the volume of material added to the system. The purification factor of Apo A-1_M in the systems was calculated as:

$$\text{Purification factor} = (C^t_{Apo}/C^t)/(C_{Apo}/C)$$

where C^t is the protein concentration in the top phase and C is the protein concentration in the starting material.

Samples from top phases were also analysed with SDS–PAGE, 20% Phast gels (Pharmacia, Sweden), and stained with Coomassie R 350 (Phast Gel Blue R). After destaining, the gels were scanned with a densitometer (Personal Densitometer SI, Molecular Dynamics, Sunnyvale, CA, USA).

2.8. Urea partitioning

Urea was partitioned in a system containing 21% Reppal PES 200, 10% $EO_{30}PO_{70}$ and with the same buffer as in the starting material (10 m*M* Tris–HCl pH 8, 75 m*M* NaCl, 5 m*M* EDTA and 0.05% Tween 80). Three different concentrations of urea were used: 1, 2 and 3 *M*. Urea concentration in the aqueous phase was measured with ninhydrin [22]. The absorption of the samples was corrected by subtraction of the absorption from the blank system, containing no urea. The measurements were carried out using a UV–Vis scanning spectrophotometer, UV-2101 PC from Shimadzu (Kyoto, Japan). A urea solution was used for standard.

2.9. DNA partitioning

The partitioning of *E. coli* DNA was studied in systems containing 21% Reppal PES 200, 10% $EO_{30}PO_{70}$ and 3 *M* urea. The concentration of DNA in the system was 0.2 mg/ml. The buffer composition was the same as in the starting material (10 m*M* Tris–HCl pH 8.0, 75 m*M* NaCl, 5 m*M* EDTA and 0.05% Tween 80). The absorbance was measured at 260 and 320 nm, and the absorbance at 320 nm was then subtracted from the 260 nm absorbance. The absorbance of the blank system, without DNA, was subtracted from the samples.

3. Results and discussion

3.1. Phase diagrams

Two thermoseparating EO–PO random copolymers were investigated for Apo A-1_M purification, Ucon 50HB-5100 (EO50PO50) and EO30PO70, with cloud points of 50°C and 40°C, respectively. Phase diagrams for Ucon 50-HB-5100 and EO30PO70 with Reppal PES100 are shown in Figs. 1 and 2, respectively. EO30PO70 is relatively more hydrophobic, due to the higher propylene oxide content, when compared to Ucon 50-HB-5100 (EO50PO50) and can thus be expected to phase separate more effectively with the hydrophilic starch polymer Reppal. However, the two-phase region is slightly larger in the Ucon/Reppal PES100 system. This can be explained by higher molecular mass of Ucon 50-HB-5100 compared with EO30PO70, i.e., M_r 4000 and 3200, respectively [3]. The advantage of EO30PO70 compared to Ucon 50HB5100 is the lower cloud point, 40°C, which is more favourable to temperature sensitive proteins.

3.2. Factorial design experiments

The first step in the purification of Apo A-1_M was partitioning in a primary two-phase system containing the EO–PO copolymer and hydroxypropyl starch. The partitioning of proteins in aqueous two-phase systems is strongly affected by the composition of the phase system, where the most important variables are type of polymer, polymer concentration, salt composition and pH.

Two factorial design studies were carried out. The aim was to distinguish which of the phase-system variables had the strongest effect on the purification factor and yield of Apo A-1_M in the extraction

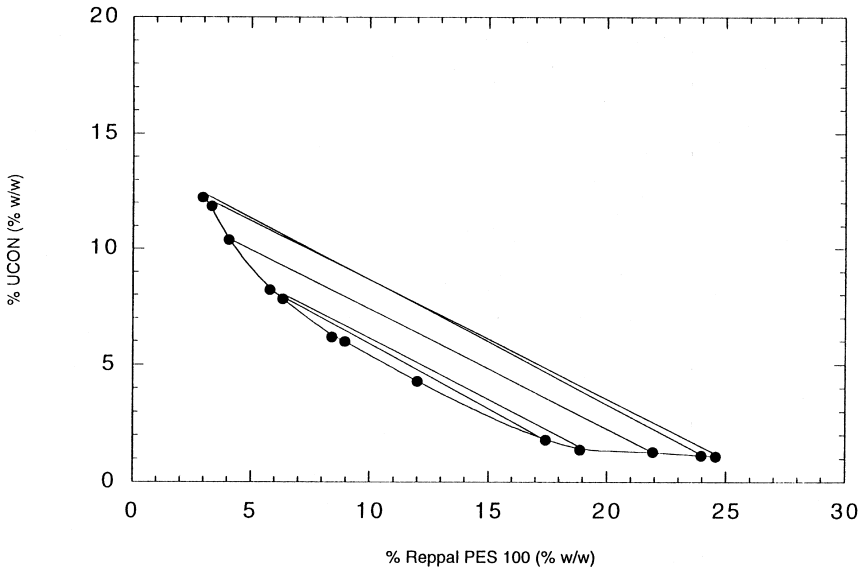

Fig. 1. Phase diagram of Reppal PES100-Ucon 50-HB-5100–water at 20°C.

aqueous two-phase system, and thus how these variables could effectively be manipulated to give an enhanced partitioning of Apo A-1_M to the top phase containing the thermoseparating EO–PO polymer. After extraction of Apo A-1_M into the EO–PO copolymer phase in the primary system, the target protein can be recovered by thermal separation above the cloud point of the copolymer. The overall purity and yield of Apo A-1_M is highly dependent on the design of the primary-phase system.

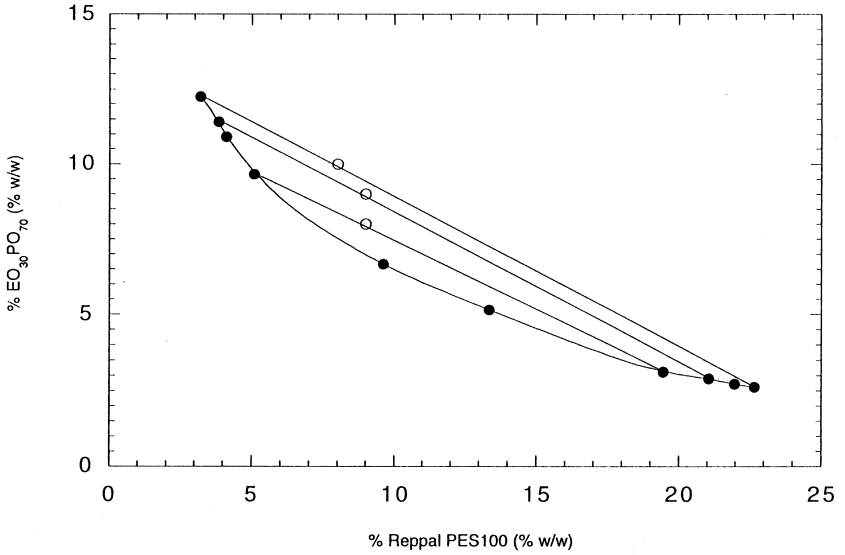

Fig. 2. Phase diagram of Reppal PES100-EO30PO70–water at 20°C.

3.3. Factorial design 1

3.3.1. Protein and polymer concentration, salt addition and pH

The aim of this experimental design was to study how the purification and yield of Apo A-1_M and the precipitation of *E. coli* proteins were affected by process variables i.e. total protein concentration, polymer concentration, salt addition and pH. Protein concentration was studied at 9 and 13 mg/ml. The concentrations were chosen so that the concentration was low enough to avoid precipitation but high enough to influence the phase system. The polymer concentration was studied at two different tie-line lengths with equal phase ratio, 12% Reppal PES200-7% Ucon 50HB5100 and 10% Reppal PES200-6% Ucon. The salt added was triethylammonium phosphate, a salt with a hydrophobic cation which was expected to increase the partitioning (K_{apo}) of Apo A-1_M to the EO–PO phase. The pH of the triethylammonium phosphate buffer was set to 7.3 and 8.0 and the salt concentration in the system was 10 or 20 m*M*. In Table 1 all eight experiments are listed. Four different responses were calculated: protein precipitation, K_{apo}, purification and yield, see Table 3.

The precipitation response variable was calculated after visual inspection. For the precipitation response, mean value 0.75, the effects were strong for the protein concentration, +0.25, and polymer concentration, −0.75, variables. The + and − signs show the level of the process variable with the strongest effect on the response e.g. the negative sign in front of the precipitation response for polymer concentration indicates that more precipitation is obtained when using high polymer concentration, see Tables 1 and 3. The response for protein concentration (+0.25) indicates that the precipitation of *E. coli* proteins was increased when the protein concentration was increased. The increased protein precipitation at higher polymer concentration is probably due to the fact that less protein can be dissolved in a more concentrated polymer phase. The partition coefficient for Apo A-1_M was significantly affected by the concentration of protein in the system and the pH. A high K_{apo} was reached when a high pH and low protein concentration was used. The purification was significantly improved by high polymer concentration and a low salt concentration. For proteins, it is well known that by partitioning at points further away from the binodial in the phase diagram, i.e. at higher polymer concentrations, it is possible to obtain more extreme *K*-values [23]. This explains the increased purification of Apo A-1_M when the polymer concentration was increased. For the yield response the significant variables were low protein and low-salt concentrations. That the yield was improved by low protein concentration is not surprising after the observation that the precipitation of *E. coli* proteins was increased by higher protein concentration.

3.4. Factorial design 2

3.4.1. Polymer composition and concentration

In this factorial experimental design the purification of Apo A-1_M was calculated as a response variable. The aim was to find out how the purifica-

Table 3
Results from the factorial design in Table 1

	Purification factor	Yield (%)	K_{apo}	Precipitation
Mean value	0.990	78.2	6.4	0.75
Protein conc.	+0.006	−6.8	−1.9	+0.25
Polymer conc.	−0.059	−0.7	−0.1	−0.75
Salt conc.	−0.031	−5.9	−0.5	0
pH	−0.009	+0.61	+1.4	0

The first row shows the mean value of the experiments. The rest of the table shows the responses for the different variables. The signs on the responses indicate if the parameter should be on the + or − level, see Table 1. The precipitation response variable was calculated after visual inspection. Systems with no precipitation were given 0, systems with precipitation at the interface were given 1, and systems with precipitation both at the interface and bottom phase were given 2.

tion was affected by the EO/PO ratio in the copolymer (EO50PO50 copolymer with EO30PO70), molecular mass of Reppal (PES 100 compared with PES 200) and the concentrations of phase forming polymers. Also studied was the effect of volume ratio, Table 2. The systems in this series were all at a high protein concentration, 23 mg/ml. Systems with comparable tie-line lengths in the four phase diagrams were compared. Two of the systems for each polymer combination were on the same tie-line but the volume of the two-phases were changed. The tie-line lengths used in these experiments were 15.8% and 25.5%. Theoretically, there should be no effect on protein partitioning by moving along a tie-line, i.e. by changing volume ratio [3]. In this experimental design the effects of different polymers, polymer concentration and volume ratio top/bottom phase could be calculated at the same time. The response variable, purification, for the four different parameters is shown in Table 4.

The results showed that the polymer concentration and the volume of the top phase were the most important phase-system parameters. A higher PO content in the copolymer was shown to have an improved effect on the purification. No significant difference was noticed when using the different bottom-phase polymers. This factorial design indicated that with a higher polymer concentration, the purification of Apo A-1_M was improved. The best results were obtained when high polymer concentration was combined with a small top phase and use of the EO30PO70 copolymer, Table 4. The fact that the volume ratio affected the purification was due to the high protein concentration in the systems. Protein precipitation at the interface was observed in systems with high polymer concentrations, and the precipitation increased when the top-phase volume was decreased. The results indicated that the bulk proteins were precipitated to a higher degree than Apo A-1_M, giving a higher purification of Apo A-1_M.

Table 4
The response of the phase system variables analysed in Table 2

Variable	Response (purification)
Bottom-phase polymer	+0.03±0.06
Top-phase polymer	+0.07±0.06
Volume of top phase	−0.08±0.06
Polymer concentration	+0.08±0.06

The response variable was calculated from the purification factors for Apo A-1_M in experiments 1–16.

3.5. Effects of polymer concentration

The factorial design experiments showed the importance of polymer concentration for purification

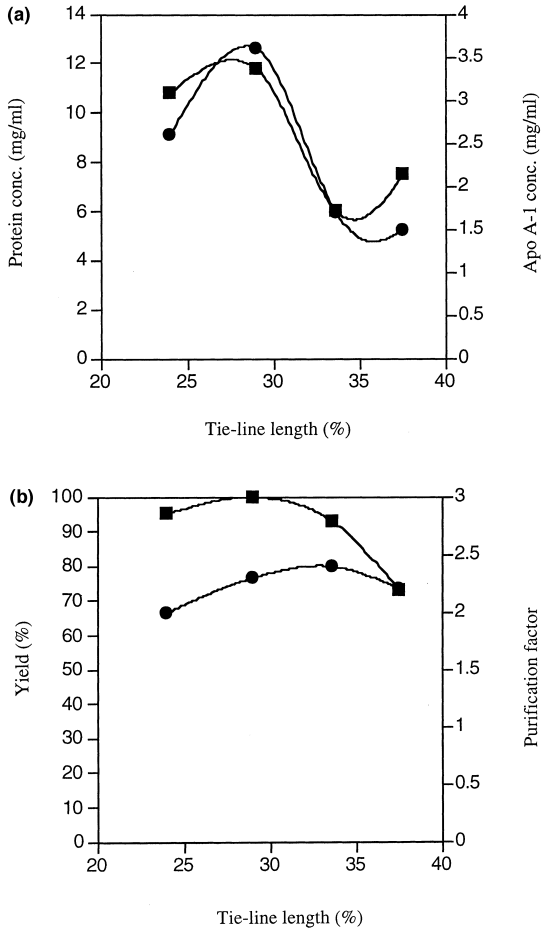

Fig. 3. Effects on purification and yield of Apo A-1_M in the primary top phase by increased polymer concentration measured as tie-line length. The starting material used had a pH 7.3. The polymers used were Reppal PES 200 and EO30PO70. The polymer concentrations for the four tie-lines were (Reppal PES 200/EO30PO70% w/w): 13.5/8.1, 14.7/9.0, 17.5/9.5 and 19.3/10.5. The data were calculated from ELISA results. (a) Protein concentration, (mg/ml, ■), and Apo A-1_M concentration, (mg/ml, ●), in the top phase in the primary system. (b) Purification factor, (■), and yield, (%) (●), of Apo A-1_M in the top phase in the primary system.

of Apo A-1_M in the primary-phase system. The effect of polymer concentration on the purification of Apo A-1_M was studied (Fig. 3b) by partitioning at different tie-line lengths in the Reppal/EO30PO70 system. The purification of Apo A-1_M was slightly increased with increasing tie-line length. At high polymer concentrations the solubility of the bulk proteins was decreased, (Fig. 3a), and an increased protein precipitation in the phase system was observed. The increase in purification of Apo A-1_M at long tie-line (34%) is due to precipitation of bulk proteins. At longer tie-line length, the yield decreased dramatically. The decrease in yield was probably due to the fact that Apo A-1_M is precipitated together with other proteins in the bottom phase. The experiments with increasing polymer concentration strengthen the results from the second factorial design experiment (Table 4) that the purification is increased with higher polymer concentration (Fig. 3b).

3.6. Addition of a hydrophobic counter ion

Triethylammonium phosphate is a salt with a hydrophobic cation. The salt has been shown to partition to the top phase, the more hydrophobic phase, in EO30PO70/Reppal systems [19]. This hydrophobic cation will enhance the partitioning of a hydrophobic and negatively charged protein to the EO30PO70 phase, [19]. Apo A-1_M has a p*I* of 5.6–5.7 [9] and will be negatively charged at the pH used in the system (7.5). The hydrophobic cation will thus enhance the partitioning of the negatively charged Apo A-1_M to the top phase in the primary system. The purification of Apo A-1_M was increased by increasing the triethylammonium phosphate concentration, see Fig. 4. The yield of Apo A-1_M was increased at low concentrations of the salt, 10–30 m*M*. When the concentration of the salt was 50 m*M* or higher, the yield decreased. This was probably due to Apo A-1_M precipitating at higher salt concentration. More precipitation of protein to the interface was observed when the salt concentration was increased. The increased purification of Apo A-1_M by addition of triethylammonium phosphate is in accordance with earlier results on protein partition with this salt [15,19].

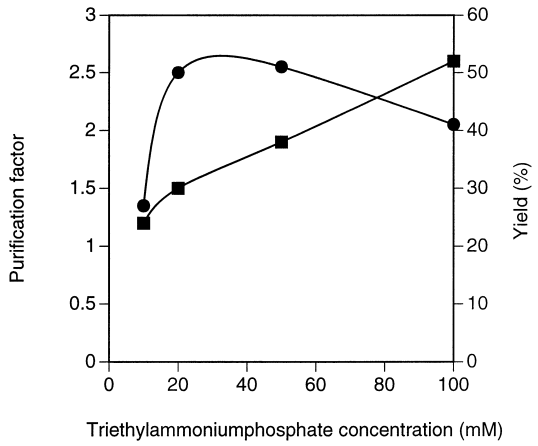

Fig. 4. Effect of triethylammonium phosphate on the degree of purification (■) and yield (%, ●) of Apo A-1_M in the primary top phase. The phase systems had the polymer concentrations 21% Reppal PES200–5% EO30PO70 (w/w). The data were calculated from ELISA results.

3.7. Addition of urea

Urea in high concentrations is often used to denature proteins [24]. Addition of urea leads to breaking of hydrogen bonding interactions. The effect of urea on Apo A-1_M purification was studied by varying the urea concentration from 1 *M* to 4.2 *M* in phase systems with high polymer concentration, 21% Reppal PES100-10% EO30PO70. When urea was added to the two-phase systems, a better purification and yield was obtained, (Fig. 5). This can be clearly seen in the SDS–PAGE analysis (Fig. 6). One explanation can be increased solubility of Apo A-1_M in the EO30PO70 phase at higher urea concentration. The solubility of Apo A-1_M in EO–PO copolymer solutions after urea addition was therefore investigated. Fig. 7 shows that the solubility of Apo A-1_M in Ucon (EO50PO50) solutions increased when urea was added. The exposure of hydrophobic surfaces on Apo A-1_M by partial unfolding in urea solution will lead to stronger interaction with the relatively hydrophobic EO–PO copolymer, thus leading to reduced aggregation of Apo A-1_M molecules and increased partitioning to the EO–PO phase. An additional effect of urea will be the breaking of aggregates between Apo A-1_M and other proteins in the solution. The results indicated that a

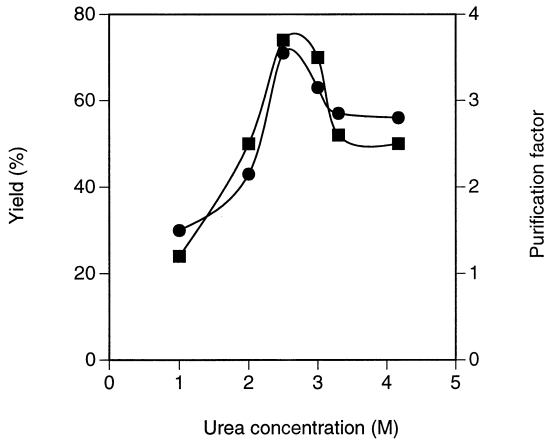

Fig. 5. Effect on the degree of purification (■) and yield (%, ●) of Apo A-1_M in the primary top phase when urea is added to the two-phase system. The phase systems had the polymer concentrations 21% Reppal PES200–10% EO30PO70 (w/w). The data was calculated from ELISA results.

concentration of 2.5 *M* urea was optimal (Figs. 5 and 6).

Urea partitioned nearly evenly between the two polymer phases of the primary system, $K=0.9$, see Table 5. The partitioning was the same independent of the urea concentration in the system, i.e 1–3 *M*. These results are expected as urea is a small uncharged molecule and such molecules partition evenly between the two-phases [1]. In the temperature-induced phase system the urea partitioned strongly to the water phase. This can be explained by the gain in entropy due to the urea interaction with the hydrogen-bonded water structure, which favours urea partitioning to the water-rich phase [25].

3.8. DNA partitioning

The removal of DNA is of importance in processes which involve recombinant organisms. Therefore, DNA partitioning was investigated. *E. coli* DNA was partitioned strongly to the bottom phase in the primary aqueous two-phase system (10% EO30PO70–21% Reppal PES 200) with Tris buffer, pH 8.0, and 3 *M* urea. The partition coefficient in the system was 0.01. Thus, DNA was effectively separated from the target protein in this aqueous two-phase extraction. The extreme partitioning of DNA agrees with results previously reported by Albertsson [1].

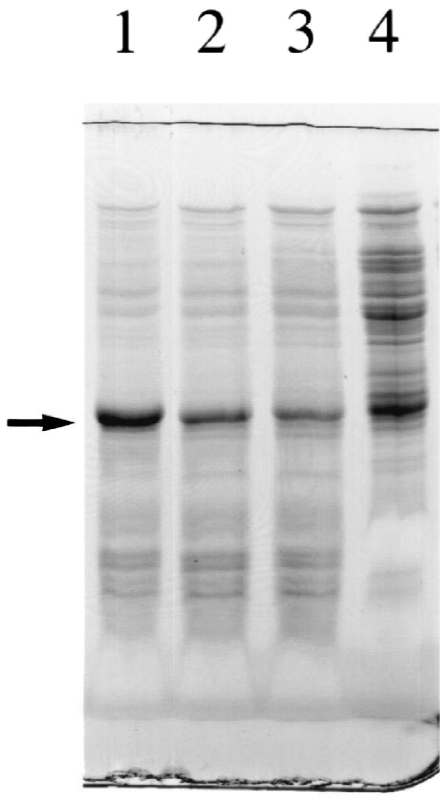

Fig. 6. Analysis by SDS–PAGE of samples from primary top phases with different concentration of urea. The phase systems had the polymer concentrations 21% Reppal PES200–10% EO30PO70 (w/w). To all lanes 3 mg of protein was added. The gel was stained with Coomassie. Lane 4 shows the starting material. Lanes 1,2 and 3 show protein after separation in systems containing 2.5, 2.0 and 1.0 *M* urea, respectively. (Apo A-1_M, →).

3.9. Temperature-induced phase separation

The system that gave the highest purification and yield in the primary partitioning step was selected for studies of temperature-induced phase separation. The more hydrophobic copolymer was chosen for the primary system with a relatively high polymer

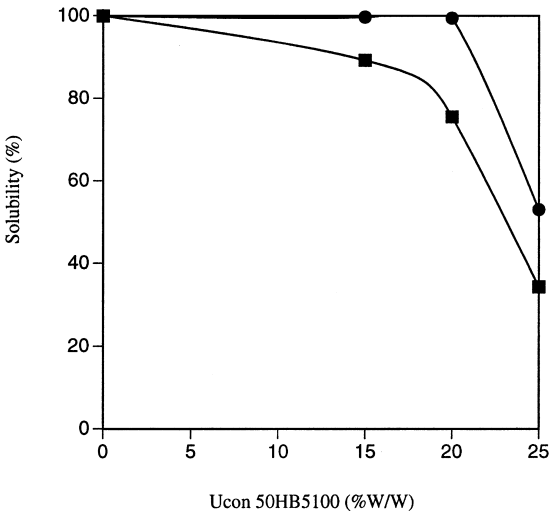

Fig. 7. Solubility of Apo A-1_M in Ucon solutions of different concentrations. Apo A-1_M dissolved in buffer, 20 m*M* Tris–HCl pH 8, (■). Effect on solubility when 3 *M* urea is added (●).

Table 5
Partitioning coefficients for urea in the aqueous two-phase system containing 21% Reppal PES 200 and 10% EO30PO70

	Partition coefficient (*K*)		
	1 *M* urea	2 *M* urea	3 *M* urea
Primary-phase system	0.9	0.9	0.9
Temperature-induced phase separation[a]	7.4	7.0	7.1

The buffer concentration in the system was 10 m*M* Tris–HCl pH 8, 75 m*M* NaCl, 5 m*M* EDTA and 0.05% Tween 80. The partition coefficients were calculated from spectrophotometric measurements using the ninhydrin reagent.

[a] The *K*-value is for partitioning between the water and polymer (EO30PO70 plus water) phases, (urea in water phase/urea in polymer phase), formed at 60°C.

concentration (17% Reppal PES100–12% EO30PO70) and 2.5 *M* urea. An *E.coli* fermentation solution, after cell removal and concentration, containing Apo A-1_M was added to the primary two-phase system. After phase separation in the primary system, the top phase was isolated in a separate vessel and temperature-induced phase separation at 50°C was performed. When the protein concentration in the top phase is very high there will be an increase in cloud point for the EO30PO70 copolymer, which is 40°C in water solution. Therefore the temperature for phase separation was increased to 50°C, to ensure adequate separation of the phases. In normal situations the EO30PO70 copolymer phase forms the bottom phase in the thermal separation step. The density of the polymer solution is higher than that of the water–protein solution. However, when urea is used in concentrations higher than 2 *M*, the polymer phase has a lower density than the water–urea–protein phase and forms the top phase.

The purification factor and yield for the primary top phase and the temperature-induced water phase are shown in Table 6. The yield was 82% with a purification factor of 3.0 after thermoseparation. No loss of Apo A-1_M was observed in the thermal separation step. A concentration of the proteins was obtained due to the volume reduction in the temperature-induced phase separation. In this step all proteins in the primary top phase are partitioned to the water phase, as earlier demonstrated [16,17]. The removal of contaminating proteins can be observed when comparing lane 1 and 2. When analysing the polymer phase on SDS–PAGE, with silver nitrate staining, no protein bands were observed in this phase (see Fig. 8, lane 3).

Table 6
Purification of Apo A-1_M using temperature-induced phase separation

Sample	Apo A-1_M conc. (mg/ml)	Protein conc. (mg/ml)	Yield (%)	Purification factor
Starting material	0.67	6.0	(100)	(1)
Primary top phase (20°C)	0.60	2.0	82	2.7
Water phase after thermoseparation (50°C)	0.75	2.2	82	3.0

The primary aqueous two-phase system contained 17% Reppal PES200, 12% EO30PO70 and 2.5 *M* urea. The data were calculated from ELISA results.

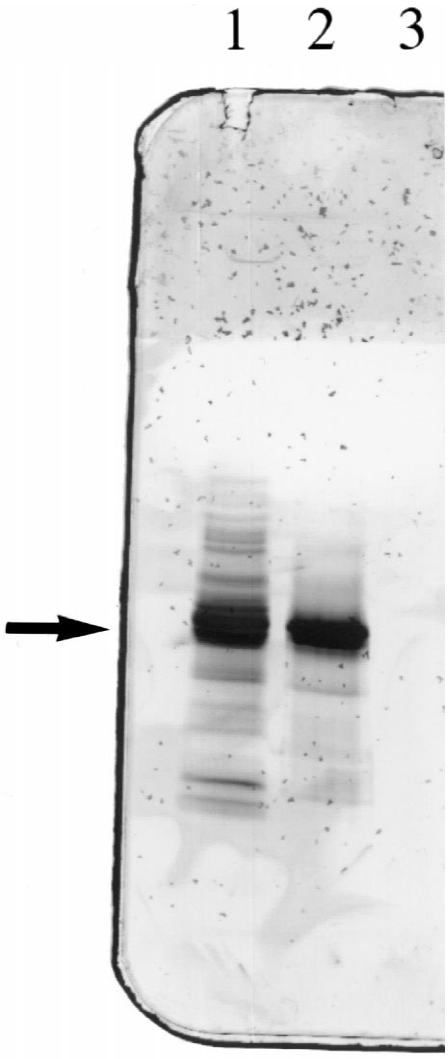

Fig. 8. Analysis by SDS–PAGE of samples from thermoseparated water phase and polymer phase. The phase systems had the composition of 17% Reppal PES200–12% EO30PO70 (w/w) and 2.5 *M* urea. To all lanes 3 mg of protein was added. The gel was stained with silver nitrate. Lane 1 shows the starting material, lane 2 the water phase after a temperature-induced phase separation and lane 3 the polymer phase after temperature-induced phase separation. (Apo A-1_M, →).

3.10. System scale-up

Aqueous two-phase systems can readily be scaled up [23]. The partitioning of protein is independent of the size of a system. As long as the phase concentrations in the systems are the same, the partitioning will also be the same. However, phase separation times will increase and may also show a dependence on the geometry of the separation vessel. The time of phase separation can be reduced by use of centrifugal separation [2]. The scale up of Apo A-1_M purification was studied. The phase system that was found optimal in the small scale experiment was used for a 5-kg phase system.

The large scale experiments were carried out directly after termination of an *E. coli* fermentation. After fermentation the cells were removed by flocculation and centrifugation. The phase components were added directly to the cell-free *E. coli* protein solution. The final concentrations were 17% Reppal PES 200, 12% EO30PO70, 2.5 *M* urea, 3.8 mg/ml total protein and 0.4 mg/ml Apo A-1_M. The polymers were dissolved by stirring and the phases were separated by centrifugation. The top EO30PO70 phase was pumped to a separate vessel and the temperature increased to 50°C. After 30 min the water phase containing Apo A-1_M could be removed. Data on purification and yield from the primary separation and the thermoseparation are shown in Table 7 and compared with data from a 5-g system. As is clear from this table, the scale up, by a factor of 1000, can be done with no loss of purification and yield. Another advantage with the two-phase extraction is the possibility of isolating the target protein in a smaller phase and thus obtain a volume reduction. This is shown (Table 7) in the volume reduction factor which compares the volume of starting material and water phase after thermal separation. A volume reduction of 30% was obtained both in small and large scale.

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Table 7
Effects on the purification and yield of Apo A-1_M when scaling up the two-phase system and the temperature-induced separation

	Purification factor	Yield (%)	Volume (l)	Volume reduction
5 g system				
Starting material	1	100	0.0029	–
Top phase from primary system	2.5	79		
Water phase after temperature-induced separation	2.5	77	0.0020	0.69
5000 g system				
Starting material	1	100	2.5	–
Top phase from primary system	2.7	82		
Water phase after temperature-induced separation	2.7	81	1.80	0.72

The phase system composition was 17% Reppal PES200, 12% EO30PO70 and 2.5 *M* urea. The starting material was an *E. coli* protein solution. The data were calculated from ELISA determinations.

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