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Purification of protein and recycling of polymers in a new aqueous two-phase system using two thermoseparating polymers

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Abstract

In this study we present a new aqueous two-phase system where both polymers are thermoseparating. In this system it is possible to recycle both polymers by temperature induced phase separation, which is an improvement of the aqueous two-phase system previously reported where one of the polymers was thermoseparating and the other polymer was dextran or a starch derivative. The polymers used in this work are EO50PO50, a random copolymer of 50% ethylene oxide (EO) and 50% propylene oxide (PO), and a hydrophobically modified random copolymer of EO and PO with aliphatic $C_{14}H_{29}$-groups coupled to each end of the polymer (HM–EOPO). In water solution both polymers will phase separate above a critical temperature (cloud point for EO50PO50 50°C, HM–EOPO, 14°C) and this will for both polymers lead to formation of an upper water phase and a lower polymer enriched phase. When EO50PO50 and HM–EOPO are mixed in water, the solution will separate in two phases above a certain concentration i.e. an aqueous two-phase system is formed analogous to poly(ethylene glycol) (PEG)/dextran system. The partitioning of three proteins, bovine serum albumin, lysozyme and apolipoprotein A-1, has been studied in the EO50PO50/HM–EOPO system and how the partitioning is affected by salt additions. Protein partitioning is affected by salts in similar way as in traditional PEG/dextran system. Recombinant apolipoprotein A-1 has been purified from a cell free *E. coli* fermentation solution. Protein concentrations of 20 and 63 mg/ml were used, and the target protein could be concentrated in the HM–EOPO phase with purification factors of 6.6 and 7.3 giving the yields 66 and 45%, respectively. Recycling of both copolymers by thermoseparation was investigated. In protein free systems 73 and 97.5% of the EO50PO50 and HM–EOPO polymer could be recycled respectively. Both polymers were recycled after aqueous two-phase extraction of apolipoprotein A-1 from a cell free *E. coli* fermentation solution. Apolipoprotein A-1 was extracted to the HM–EOPO phase with contaminating proteins in the EO50PO50 phase. The yield (78%) and purification factor (5.5) of apolipoprotein A-1 was constant during three polymer recyclings. This new phase system based on two thermoseparating polymers is of great interest in large scale extractions where polymer recycling is of increasing importance.

Keywords: Aqueous two-phase systems; Partitioning; Proteins; Polymers

1. Introduction

Aqueous two-phase systems are increasingly used for separation of biomolecules, cells and cell particles. The systems are composed of two incompatible polymers, e.g. dextran and poly(ethylene glycol)

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(PEG), or of one polymer (PEG) in high concentration of salt. These systems are suitable for biological samples because each phase contains 70–90% water, which means that biomolecules will not be denatured [1]. In lab-scale separations the PEG/dextran system has widespread use especially for membrane preparations [2]. For large scale extractions in industry the PEG salt systems have been used due to inexpensive phase forming chemicals [3,4]. The partitioning of proteins in aqueous two-phase systems depends strongly on protein hydrophobicity [5], charge and size [1,6]. The partitioning can be influenced by changing polymers, polymer molecular mass [7], the pH or by addition of salts in the system [8–10]. Advantages with aqueous two-phase extraction lie in volume reduction, high capacity and fast separations. The technique can be used early in purification processes on solutions containing cells or cell debris. Compared to other separation techniques (e.g. chromatography) it is relatively straightforward to scale up this purification method [4].

Recently the use of thermoseparating polymers in aqueous two-phase systems has been introduced. This makes it possible to perform temperature induced phase separation whereby a target protein can be separated from the polymer [11] and the polymer can be recycled [12]. Thermoseparating ethylene oxide–propylene oxide (EOPO) random copolymers have been used as top phase forming polymers instead of PEG in systems with dextran or a starch derivative as bottom phase polymer. In these systems a target protein is first partitioned to the EOPO phase. This phase is then isolated and the temperature is raised above the EOPO copolymer cloud point which leads to formation of an aqueous polymer phase and a water/buffer phase. The target protein is obtained in the water/buffer phase and the EOPO copolymer can be recycled [11,13,14].

In this paper we report a novel aqueous two-phase system where both of the copolymers are thermoseparating. Both phase forming polymers can be recycled. The principles are shown in Fig. 1. The possibility for recycling of both polymers means that the costs for aqueous two-phase extraction in a biotechnical process can be significantly reduced.

The polymers used are EO50PO50, a random copolymer of 50% (w/w) ethylene oxide (EO) and 50% (w/w) propylene oxide (PO), and HM–EOPO, a random copolymer of EO and PO with aliphatic $C_{14}H_{29}$ groups coupled to each end of the copolymer. Both of these polymers are thermoseparating i.e. in a water solution above a certain temperature (cloud point) a two-phase system is formed, the top phase will be almost depleted from copolymer and the bottom phase will be enriched in copolymer. The cloud point for the polymers decreases with increased propylene oxide concentration [15] and can also be lowered by addition of salt [11]. The lowest cloud point of a polymer is referred to as the lower critical solution temperature (LCST).

The LCST for EO50PO50 is 50°C at 10% (w/w) which is the same as the LCST for UCON 50HB5100 [16] and for HM–EOPO it is 14°C at 3% (w/w) concentration. The cloud point temperature for HM–EOPO is strongly dependent on the polymer concentration and it will decrease with lower copolymer concentration. The HM–EOPO polymer is strongly associating and will form micellar structures already at low concentrations, due to the aliphatic tails on the polymer [17]. We have recently investigated the aqueous two-phase system formed at temperatures above the cloud point of HM–EOPO [17]. The water/HM–EOPO system can be used for protein purification, in particular for extraction of more hydrophobic proteins to the HM–EOPO phase [17].

In the novel system presented here the top phase forming polymer is EO50PO50 and the bottom phase forming polymer is HM–EOPO (Fig. 1). We have selected three model proteins bovin serum albumin (BSA), lysozyme and apolipoprotein A-1 (Apo A-1) to partition in the EO50PO50/HM–EOPO system. The apolipoprotein A-1 is recombinantly produced in *Escherichia coli (E. coli)*. Apo A-1 studied in this case is a natural variant of the normal protein with a mutation where the arginine 173 has been substituted with a cystein. This mutation gives rise to a disulfide linked dimer. The protein is rich in α-helix structures. The α-helices are amphiphilic, creating an amphiphilic protein where one surface is hydrophobic and the other is hydrophilic [18]. The amphiphilic properties of Apo A-1 leads to the formation of micellar structures with other proteins and lipids in the solution [19,20].

Protein partitioning has been studied both in the

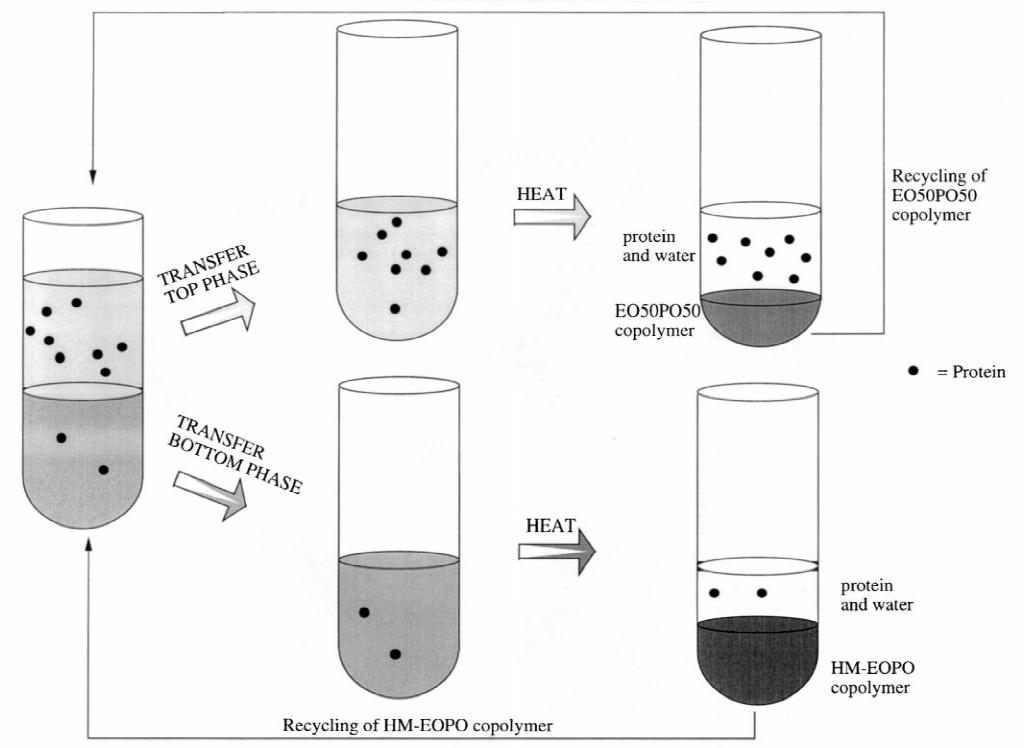

Fig. 1. Schematic picture of protein separation and recycling of polymer in an aqueous two-phase system with two thermoseparating polymers. First, an aqueous two-phase system composed of the polymers EO50PO50 and HM–EOPO is mixed. After phase separation the top EO50PO50 and bottom HM–EOPO phases are isolated and transferred to a water bath at a temperature above the cloud point of the polymers. This leads to formation of a water phase and a polymer enriched phase. After removal of the water phase the polymers can be recovered and reused in a new aqueous two-phase system.

polymer–polymer (EO50PO50/HM–EOPO) aqueous two-phase system and in the water–polymer systems obtained when performing the temperature induced phase separation on the separated phases, see Fig. 1. The partitioning of pure proteins are reported and the possibility to use salt to direct the partitioning. The amount of polymer that can be recycled in this type of system has been investigated. The results show that the HM–EOPO polymer can be recycled to higher extent than the EO50PO50 polymer. Protein purification from cell-free *E. coli* fermentation solution has been studied. The target protein apolipoprotein A-1 could be effectively purified with the new system and both EO50PO50 and HM–EOPO copolymers could be recycled during extractions of target protein from the fermentation solution. The EO50PO50/HM–EOPO aqueous two-phase system is thus a robust separation system, where the use of two thermoseparating polymers makes it possible to recycle both polymers after temperature induced phase separation and to obtain the target protein in a water phase.

2. Materials and methods

2.1. Chemicals

The top phase forming copolymer was Breox PAG 50A 1000 (EO50PO50) (M_r 3900) from International Speciality Chemicals (Southampton, UK). As bottom phase forming polymer the hydrophobically modified EOPO copolymer (HM–EOPO) (M_r 8000) from Akzo Nobel (Stenungsund, Sweden) was used, Fig. 2. This has an isophoronediisocyanate group in the centre and on both sides of this group random

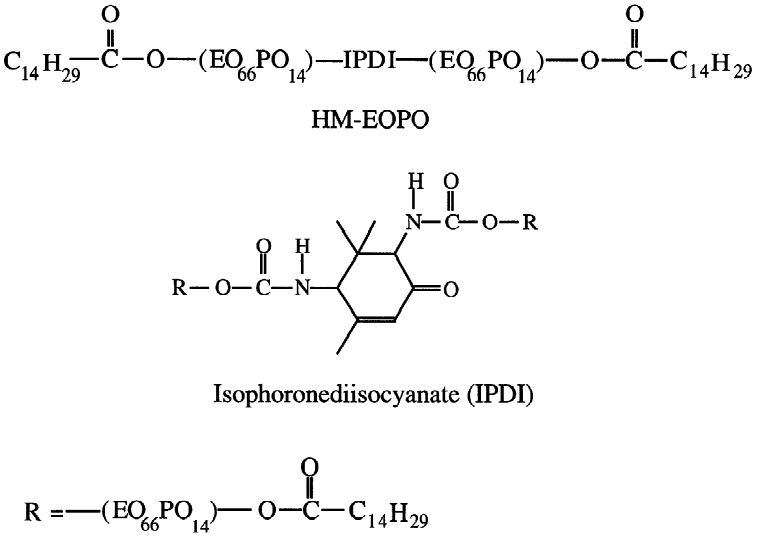

Fig. 2. Chemical structure of the HM–EOPO random copolymer. The EOPO chains in the polymer are composed of 66 EO and 14 PO units randomly ordered.

copolymer chains of ethylene oxide and propylene oxide are attached. Each EOPO chain is composed of 66 EO and 14 PO groups and on the ends of the polymer aliphatic $C_{14}H_{29}$ groups are attached. The salts used were of analytical reagent grade.

2.2. Proteins

Fatty acid free bovine serum albumin was purchased from Boehringer Mannheim (Mannheim, Germany), and hen egg white lysozyme from Sigma (St. Louis, MO, USA). The pure apolipoprotein A-1 produced in *E. coli* was a gift from Pharmacia and Upjohn (Stockholm, Sweden). This apolipoprotein A-1 was a recombinant variant, named Milano, which is a dimeric form in contrast to the normal monomeric form [13]. From the same company we were also given the cell free *E. coli* fermentation solution containing Apo A-1. The protein concentration in the extract was 70 mg/ml and 10% of the protein was Apo A-1. The buffer composition in this fermentation solution was 20 m*M* Tris HCl pH 8.0, 150 m*M* NaCl, 10 m*M* EDTA and 0.1% Tween 80.

2.3. Temperature versus concentration phase diagram

The temperature at which a solution of polymer in water starts to become turbid (visually) is the cloud point of the polymer. For the EO50PO50 copolymer this temperature was determined by preparing solutions with different concentrations of copolymer whereafter the tubes were placed in a thermostated water bath. The temperature was slowly increased, less than 0.5°C/min and the temperature where the solution started to become turbid was determined. For the HM–EOPO copolymer 3% (w/w) solutions were prepared and left to separate at different temperatures. The polymer concentrations in the water and polymer phases after thermoseparation was determined by refractive index measurements.

2.4. Polymer–polymer phase diagram

The concentration of polymers in the phases were determined by absorption and refractive index measurements. The HM–EOPO concentration was determined by using a Coomassie Brilliant Blue G250 analysis for protein determination, according to Bradford [21]. The absorption was measured at 595 and 465 nm and then subtracted from each other. The HM–EOPO standard curve was linear in the range 0–0.8% (w/w). The EO50PO50 concentration was determined using refractive index measurements. Refractive index standard curves were determined for both polymers at 4°C with a refracto-

meter from Carl Zeiss (Oberkochen/Württ., Germany). Aqueous two-phase systems with different polymer concentrations, EO50PO50 and HM–EOPO, were mixed. The systems were separated into two phases by centrifugation for 5 min at 1360 *g*. Samples from the top and bottom phases were diluted before measuring. The HM–EOPO concentration in both phases was determined with the Bradford assay. EO50PO50 concentrations were determined by refractometry by subtracting the refractive index contribution of the HM–EOPO copolymer. 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 diagram for the system EO50PO50/HM–EOPO/water was determined at 4°C to prevent thermoseparating of the HM–EOPO polymer.

2.5. Two-phase partitioning of pure proteins

All polymer concentrations were calculated as % (w/w). The primary polymer–polymer two-phase systems contained 5% EO50PO50 and 5% HM–EOPO. In partitioning studies of the model proteins 10 m*M* sodium phosphate buffer pH 7.0 was added to maintain constant pH. To investigate salt effects on the partitioning of the model proteins 100 m*M* of different salts were added to the systems. The protein concentration in the system was 1 mg/ml when the model proteins BSA, lysozyme and apolipoprotein A-1 were partitioned. The systems were weighed in as 8 g systems in test tubes. After careful mixing 7 g was transferred to a 10 ml pipette that was sealed at the end. The pipettes were used to measure the phase volumes accurately. The top phase was removed with a Pasteur pipette and a pure bottom phase was taken from the bottom hole of the pipette. All experiments were performed at least in duplicate.

The partitioning of molecules in two-phase systems is described by the partition coefficient *K*. It is defined as the concentration of the molecule in the top phase, C_T, divided by the concentration in the bottom phase, C_B: $K = C_T/C_B$. The protein partition coefficients were determined by measuring the absorbance at 280 nm. Due to the low cloud point of HM–EOPO (14°C) at low polymer concentrations, it was necessary to dilute the phases with methanol to prevent clouding. Standard curves for each of the proteins in 50% (v/v) methanol were determined. The spectrophotometer used was a UV-2101 PC from Shimadzu (Kyoto, Japan). Duplicates of all systems were prepared and mean values are given.

2.6. Purification from E. coli fermentation solution

The total protein concentration in the aqueous two-phase system when partitioning the cell free *E. coli* fermentation solution was either 20 or 63 mg/ml. The separation was enhanced by centrifugation at 800 *g*, 10 min. The partitioning was performed in normal graduated 15 ml glass tubes. All experiments were performed in duplicate. The systems were mixed and separated at room temperature (21°C).

The purification of Apo A-1 from the fermentation extract was analysed by adding samples from the phases on a sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) system, 18% Tris–glycine gel (Novex, San Diego, CA, USA). The gels were stained with Coomassie R250 and scanned with a densitometer (Personal Densitometer SI, Molecular Dynamics, Sunnyvale, CA, USA). Thus the percent Apo A-1 of the total protein concentration could be determined [12]. This value was used to calculate the purification factor: percent Apo A-1 in sample/percent Apo A-1 in starting material. The top height in the densitogram was used to calculate the yield. Adjustments for the volume change and the dilution of the samples were taken into account, in the calculation of the yield of target protein in the top phase:

$$\text{Yield} = (\text{top height} \times V^t \times \text{dilution})_{\text{sample}} / (\text{top height} \times V \times \text{dilution})_{\text{starting material}}$$

where V^t is the volume of the top phase and V is the volume of the starting material added to the two-phase system.

2.7. Temperature induced phase separation

A EO50PO50/HM–EOPO primary aqueous two-phase system was mixed and left to separate. The top (EO50PO50) phase was separated from the bottom (HM–EOPO) phase and each phase transferred into separate pipettes. The thermoseparation was performed in 10 ml pipettes that were sealed at both ends and placed in a water bath. Both phases were

thermoseparated by increasing the temperature to 55°C for 30 min. The protein concentration in the top and bottom phases was determined with spectrophotometer at 280 nm, and the partition coefficient was calculated.

2.8. Recycling of copolymer

The recycling studies were performed in graduated glass tubes, 15 ml, with known mass. Systems of 10 g were mixed and the separation into two-phases was enhanced by centrifugation, 1000 *g*, 10 min. The top phase was removed and the mass of the phase determined. The original tube containing only the bottom phase was weighed and the mass of the bottom phase was determined by subtracting the mass of the tube. The polymer concentrations in the top and bottom phases were determined in the same way as for the determination of the phase diagram. By knowing the mass of the phase and the polymer concentration in the phase the recovery of polymer was calculated. In all the thermoseparated systems studied the polymer phase was obtained in the bottom phase.

2.9. Purification with polymer recycling

Recycling of polymer was studied during purification of apolipoprotein A-1 from cell free *E. coli* fermentation solution. The amount of recovered polymers was determined after the thermoseparation step. New polymer was added to make up the original concentrations whereafter new proteins were partitioned in the system. The total protein concentration in the phase system was 5 mg/ml and the thermoseparation was performed at 55°C for 30 min. The experiments were performed in graduated glass tubes.

2.10. Salt partitioning

The salt partitioning was measured in systems containing 100 m*M* salt by using a conductometer. The phases were diluted 100 times with Milli-Q water (Millipore) before measuring the conductivity to ensure proper dissociation of the ions. Standard curves for each of the salts were previously determined. The conductometer used was a Metrohm 644 (Herisan, Switzerland). Duplicates of all systems were prepared and mean values are given. The samples were separated over night and measured at room temperature 21°C.

3. Results and discussion

3.1. Thermal phase separation

Among small molecules thermoseparation is seldom observed but for polymers it is more common [22]. The theoretical aspects of the thermoseparation phenomenon for polymers in water solution has been studied [16,23,24].

In the last years random copolymers of EO and PO have been used in aqueous two-phase systems because of their thermoseparating properties [11]. The use of EOPO copolymer instead of PEG in aqueous two-phase system, will result in two positive effects. The first is that the copolymer can be recovered after thermal phase separation and reused in a new aqueous two-phase system and the second effect is that the proteins can be recovered in a water phase. Cloud point curves for the polymers are shown in Fig. 3. EO50PO50 has a relatively high cloud point (LCST), 50°C, at 10% concentration. HM–EOPO has a much lower LCST, 14°C at a 3% HM–EOPO concentration. The concentration dependence on the cloud point temperature was stronger for the HM–EOPO compared to the EO50PO50 polymer. As is clearly seen in the cloud point diagram (Fig. 3) the copolymer phase for HM–EOPO has a much higher water content compared with the EO50PO50 copolymer rich phase. Thus the phase composition for the HM–EOPO/water system is affected much more than for the EO50PO50/water system when changing the temperature for thermoseparation. The EO50PO50 phase will always have a high polymer concentration after the thermoseparation. Thus, in a EO50PO50 water system proteins are strongly partitioned to the water phase because of excluded volume effects [11,25]. The polymer concentration is much lower in the polymer phase in the HM–EOPO/water system compared with the EO50PO50 water system. The relatively low concentration of HM–EOPO in the polymer rich phase, as seen in Fig. 3, means that this system

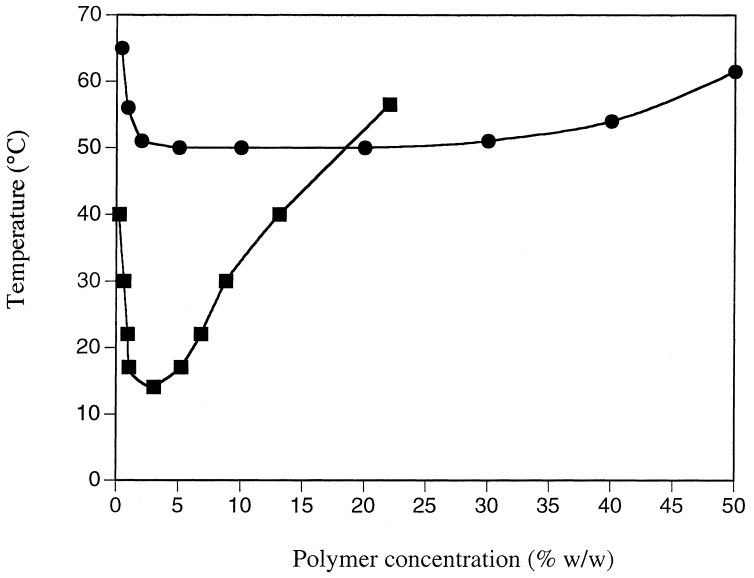

Fig. 3. Experimentally determined temperature versus concentration phase diagram for the water/EO50PO50 (●) and water/HM–EOPO (■) systems. The region within the coexistence curve is the two-phase region, representing conditions at which the polymer solution separates into two macroscopic phases. The region outside the coexistence curve is the one-phase region, representing conditions at which the polymer solution exists as a single homogeneous phase.

resembles more the non-ionic surfactant/water systems [26–28] than the thermoseparating aqueous systems of water and EOPO random copolymers. This effect may be due to the qualitative resemblance in molecular structure of HM–EOPO and non-ionic surfactants containing ethylene oxide and alkyl groups, e.g. $C_{12}E_5$. The alkyl end groups of HM–EOPO give the polymer surfactant properties and the polymer will associate and form micellar-like aggregates. The bifunctionality of the polymer may also lead to bridging between micelles [17,29]. The water/HM–EOPO system displays a much greater viscosity than the water/EO50PO50 system which supports the view of extended associations of polymer molecules [30].

3.2. Polymer–polymer aqueous two-phase system

The phase diagram for the EO50PO50/HM–EOPO/water system is shown in Fig. 4. The concave curve is the binodial or coexistence curve that forms the borderline between the one-phase and two-phase region. The tie-lines show polymer concentrations in the top and bottom phases for four different polymer compositions. The phase diagram was prepared at 4°C which is below the cloud point of the HM–EOPO polymer. This means that the phase separation is driven by an effective repulsion between the HM–EOPO and EO50PO50 polymers. The smaller EO50PO50 copolymer (M_r 3900) forms the top phase and the HM–EOPO copolymer (M_r 8000) forms the bottom phase. Phase separation in EO50PO50/HM–EOPO system is obtained at relatively low polymer concentration (below 4% total polymer) when comparing with normal EO50PO50/starch or EO50PO50/dextran systems (above 10% total polymer) [11,13]. This can be explained by formation of micellar like aggregates of the HM–EOPO polymer in water solution and the micelles are not compatible with the EOPO copolymer. Thus a polymer/micelle phase separation is obtained, which is analogous to the phase separation observed in detergent/polymer mixtures [31]. The partitioning and purification of protein was performed at 21°C. The phase systems obtained at 21°C had only slightly altered volume ratio compared with the systems obtained at 4°C. The HM–EOPO phase will be slightly larger at lower temperatures.

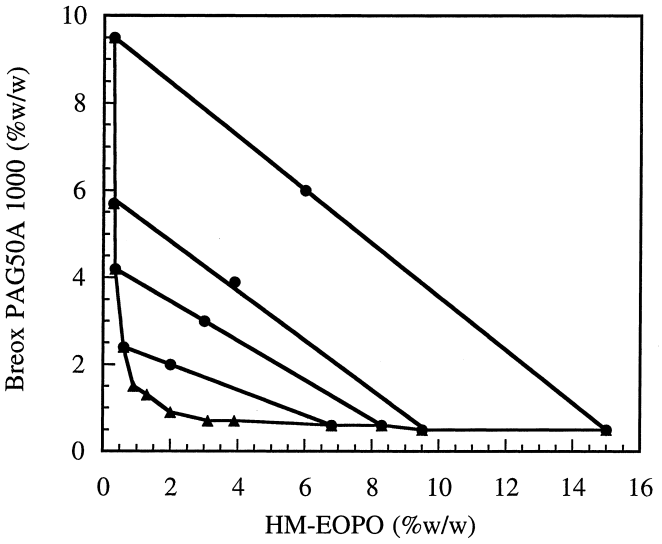

Fig. 4. Experimentally determined phase diagram for the EO50PO50/HM–EOPO/water aqueous two-phase system. The region within the coexistence curve is the two-phase region, representing conditions at which the polymer solutions separate into two macroscopic phases. The composition of the two macroscopic phases in equilibrium is described by the tie lines which connect two points on the coexistence curve. The phase diagram was determined at 4°C.

Incompatible polymers separate at very low concentrations if one or both of the polymers are large. Since both of the polymers are small the low critical point of the phase diagram can be understood if the effective polymer size of HM–EOPO is much larger due to the association of polymers. An interesting observation is that when mixing the pure EO50PO50 and HM–EOPO polymers a one-phase system was formed. The micelle formation of HM–EOPO is prevented when dissolving the polymer in a 100% EOPO copolymer and thus the polymers are soluble in each other. But when water is added the HM–EOPO will start to form micelles and the polymers are no longer compatible. Thus two aqueous phases are formed, one EO50PO50 rich and one HM–EOPO rich phase.

3.3. Salt partitioning in the EO50PO50/HM–EOPO system

Salts are frequently used in aqueous two-phase systems to direct partitioning of target molecules between the phases [6,8]. As different ions have different affinity for the two phases there will be a driving force toward uneven partitioning of the ions between the phases, although electroneutrality must be retained in each phase. The electrochemical driving force in partitioning has been explained by the formation of an electrostatic potential difference over the interface [6,8–10,25,32]. This potential difference is created by the different affinities of the ions for the two phases. The electrostatic potential difference will affect the partitioning of proteins or other charged molecules present in the phase system.

The salts that were used in protein partitioning, sodium phosphate, sodium chloride, sodium perchlorate and triethylammonium phosphate, were partitioned without protein in the EO50PO50/HM–EOPO two-phase system. The partitioning was performed at room temperature, 21°C. The partition coefficients for the salts are shown in Table 1. For all salts the partition coefficients were one or higher, which means that the partitioning of the salts was favoured to the top, EO50PO50, phase. Sodium phosphate was partitioned strongest to the top EO50PO50 phase with $K=1.42$. When compared with the other salts, sodium perchlorate was partitioned strongest towards the bottom HM–EOPO phase ($K=1.01$). It has been established that the partitioning of ions in aqueous two-phase systems follows the Hofmeister or lyohopic series with the more chaotropic (hydrophobic) ions partitioning

Table 1
The partition coefficient (K) for salts in systems containing 5% EO50PO50, 5% HM–EOPO and 100 mM salt. The partitioning was performed at 21°C. The top phase was enriched in EO50PO50 and the bottom phase was enriched in HM–EOPO. Partition coefficients higher than one show partitioning to the top EO50PO50 phase

Salt	Partition coefficient (K)
Et_3NH–phosphate (pH 7.0)	1.14±0.05
Sodium phosphate (pH 7.0)	1.42±0.05
NaCl	1.16±0.05
$NaClO_4$	1.01±0.05

towards the phase containing the more hydrophobic polymer [8,33,34]. Perchlorate is the most chaotropic ion of the anions studied and thus $NaClO_4$ is expected to partition stronger than the other salts to the phase that is most hydrophobic. The increased partitioning of salt to the top phase in the series of sodium salts of phosphate, chloride and perchlorate indicates that the HM–EOPO phase is relatively more hydrophobic when comparing with the EO50PO50 phase in this system. This can also be seen by comparing the partitioning of the hydrophobic triethylammonium cation with sodium. In a water/HM–EOPO system the partition coefficient of the salts follows the same order as in the EOPO/HM–EOPO system with the more hydrophobic ions partitioning to the HM–EOPO phase [17].

3.4. Protein partitioning in the EO50PO50/HM–EOPO system

The two polymers used in this studied are very similar. It is mainly the aliphatic groups at the ends of HM–EOPO and the EO–PO composition that differ between the polymers. Therefore we have investigated if the salt effects on protein partitioning in this aqueous two-phase system follow the normal salt effects seen in the traditional PEG/dextran and EOPO/starch systems. Three different model proteins, bovine serum albumin, lysozyme and apolipoprotein A-1 were partitioned at room temperature, 21°C, in a 5% EO50PO50–5% HM–EOPO system at pH 7.0. The model proteins had different charge and hydrophobicity. At the pH studied BSA and apolipoprotein A-1 were negatively charged and lysozyme was positively charged. Apolipoprotein A-1 is relatively more hydrophobic than the other two proteins because of its amphiphilic structure.

3.4.1. Bovine serum albumin

BSA is a negatively charged protein at pH 7.0, approximately −18 [35]. When changing the cation from triethylammonium to sodium a small decrease in the partitioning was noticed, Table 2 and Fig. 5. By changing the anions from $HPO_4^{2-}/H_2PO_4^-$ (i.e. phosphate at pH 7.0) to Cl^- and ClO_4^- the partition coefficient was increased. This partition behaviour can be correlated to the partitioning of the salts. When the hydrophobic ion, ClO_4^-, is added to the system the negatively charged BSA will be partitioned stronger to the top EO50PO50 phase compared with systems containing phosphate and chloride, because the ClO_4^- ion has stronger affinity to the HM–EOPO phase compared with the other anions.

3.4.2. Lysozyme

This protein is positively charged, +7, at pH 7.0, [36]. The change of cation from triethylammonium to sodium did not have any significant effect on the lysozyme partitioning, Table 2 and Fig. 5. In the series of anions $HPO_4^{2-}/H_2PO_4^-$, Cl^- and ClO_4^- the opposite partitioning behaviour to BSA was observed for lysozyme which was expected because of opposite net charge. ClO_4^- had the strongest effect in directing lysozyme to the bottom HM–EOPO phase.

The salt effects on BSA and lysozyme seen in our EOPO/HM–EOPO system follow the same trend as reported for EOPO/dextran [37] and EOPO/hydroxypropyl starch [33] systems. But in our case the more hydrophobic phase is the bottom phase, HM–EOPO phase. By introducing salt to the EOPO/HM–EOPO system we can influence the partitioning of proteins between the phases. However, hydrophobic interaction with HM–EOPO will have stronger effect on the partitioning, as can be seen for an amphiphilic protein.

3.4.3. Apolipoprotein A-1

The net charge of Apo A-1 was calculated at pH 7.0, [38] using the amino acid sequence for the protein reported by Cameselle et al. [39]. The calculations gave a negative net charge of −10 for the normal monomer, but in the mutant protein one

Table 2
The partition coefficients for the model proteins BSA, lysozyme and apolipoprotein A-1, both in the primary EO50PO50/HM–EOPO system and in the water–polymer systems after temperature induced phase separation. The system composition was 5% EO50PO50, 5% HM–EOPO, 100 m*M* salt and 1 mg/ml protein. The pH was kept at 7.0 with 10 m*M* sodium phosphate buffer. The EO50PO50/HM–EOPO system was separated at 21°C and the protein concentration in the top, EO50PO50, phase and bottom, HM–EOPO, phase was determined. The phases were isolated in separate vessels and transferred to a water bath where the thermoseparation was performed at 55°C for 30 min. The protein concentrations in the top, water, and bottom, polymer, phases after thermoseparation were determined

Salt	Partition coefficient (K)		
	EO50PO50/ HM–EOPO system (21°C)	Thermosep. of EO50PO50 phase (55°C)	Thermosep. of HM–EOPO phase (55°C)
		BSA	
10 m*M* sodium phosphate buffer	1.08	>100	1.55
+ 100 m*M* Et_3NH–phosphate	1.64	>100	0.87
+ 100 m*M* NaCl	2.01	>100	1.14
+ 100 m*M* $NaClO_4$	3.53	>100	0.83
		Lysozyme	
10 m*M* sodium phosphate buffer	1.75	>100	2.40
+ 100 m*M* Et_3NH–phosphate	1.41	>100	2.38
+ 100 m*M* NaCl	1.02	>100	1.94
+ 100 m*M* $NaClO_4$	0.41	>100	0.29
		Apo A-1	
10 m*M* sodium phosphate buffer	0.31	>100	2.74
+ 100 m*M* Et_3NH–phosphate	0.32	>100	1.34
+ 100 m*M* NaCl	0.24	>100	1.52
+ 100 m*M* $NaClO_4$	0.28	>100	2.61

arginine has been changed to cystein which gives a charge of −9. Thus the dimer of Apo A-1 will have a charge of −18. Both the charge and size, M_r 52 000, of the Apo A-1 dimer is comparable with BSA, −18 and M_r 69 000 respectively [40]. Apo A-1 is an amphiphilic protein which readily forms aggregates with other proteins and lipids. In vivo the protein forms micellar structures with lipids in lipoprotein complex [19].

Apo A-1 was strongly partitioned to the HM–EOPO phase in the EO50PO50/HM–EOPO system, Table 2 and Fig. 5. For this protein no significant salt effects were observed and K values were between 0.2–0.4. One explanation to why Apo A-1 is not affected by the salts added to the system is that the protein associates with micelles of HM–EOPO. The aliphatic groups at the ends of the HM–EOPO polymer has the ability to interact and form micellar-like aggregates which can associate with Apo A-1 [17]. This micelle–protein interaction will then be much stronger than the effects of the ions added to the system. Apo A-1 has previously been shown to have affinity for micelles of Triton and Tween included in EO50PO50/hydroxypropyl starch system [12], and to HM–EOPO micelles in water/HM–EOPO systems [17].

Some partition experiments of BSA and Apo A-1 were performed at a lower temperature, 4°C, but with the same polymer concentration as the experiments performed at 21°C. Due to the temperature change the tie line length will differ in the 4°C experiments compared to the 21°C experiments, 14.0% and 17.8% respectively. At 4°C BSA was partitioned less extreme to the top phase compared to the experiments performed at higher temperature (Table 3). This is probably due to the change in tie line length. It is a well known fact that the partitioning of a component will be more extreme at longer tie line lengths [6]. Similar salt effects were observed at both temperatures. For Apo A-1 the partitioning to the bottom phase was increased upon decrease in temperature (Table 3). This can be explained by increased entropy contribution to the partitioning at the higher temperature which in general will favour a more even distribution between the phases. The enthalpic effects, e.g interactions micelle–protein,

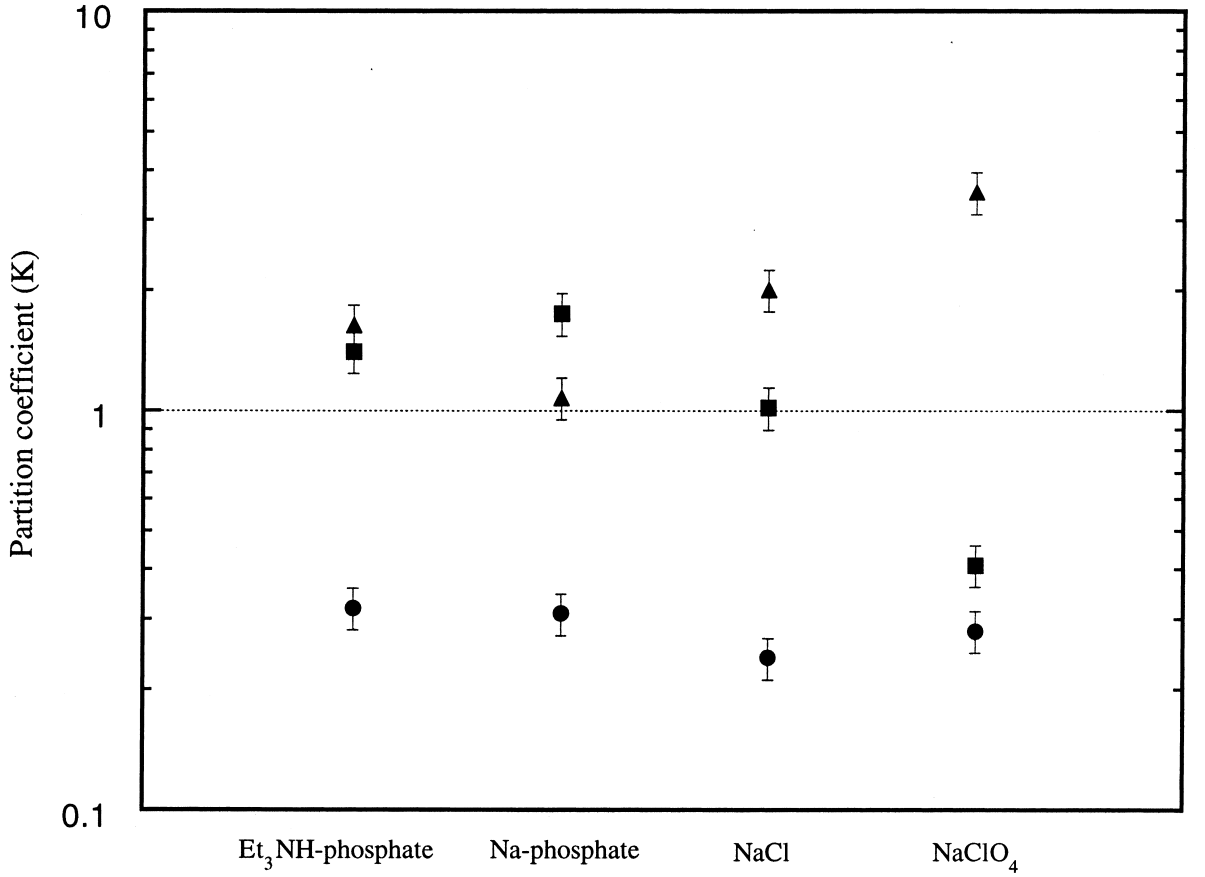

Fig. 5. Partition coefficients for BSA (▲), lysozyme (■) and apolipoprotein A-1 (●) in aqueous two-phase systems composed of 5% EO50PO50 and 5% HM–EOPO. Each system contained 1 mg/ml protein, 10 m*M* sodium phosphate buffer pH 7.0 and 100 m*M* of an additional salt to examine salt effects. The salts used were triethyl ammonium phosphate, sodium phosphate, sodium chloride and sodium perchlorate. The protein was partitioned at 21°C. Partition coefficients above one indicate a partitioning to the top EO50PO50 phase.

Table 3
The partition coefficient of BSA and apolipoprotein A-1 at 4 and 21°C in systems containing 5% EO50PO50, 5% HM–EOPO, 100 m*M* salt and 1 mg/ml protein. The pH was kept at pH 7.0 with 10 m*M* sodium phosphate buffer. EO50PO50 is enriched in the top phase and HM–EOPO in the bottom phase. The tie line length at 4 and 21°C are 14.0% and 17.8% respectively

Salt	BSA		Apo	
	4°C	21°C	4°C	21°C
Sodium phosphate	0.91±0.10	1.08±0.10	0.16±0.05	0.31±0.05
NaCl	1.62±0.10	2.01±0.10	0.15±0.05	0.24±0.05
NaClO4	3.00±0.10	3.52±0.10	0.16±0.05	0.28±0.05
Et_3NH–phosphate	2.65±0.10	1.64±0.10	0.19±0.05	0.33±0.05

are more dominating at lower temperature and thus the partitioning of Apo A-1 will be more extreme to the HM–EOPO phase at lower temperature.

3.5. Protein partitioning in temperature induced phase separation

The EO50PO50/HM–EOPO aqueous two-phase system is composed of two thermoseparating polymers. In a purification process proteins can in a first step be partitioned in the primary EO50PO50/HM–EOPO system, see Fig. 1. After temperature induced phase separation of both polymer phases the proteins should be recovered in the water phase and the polymers recycled for use in a new EO50PO50/HM–EOPO system. Therefore it was necessary to study protein partitioning in the water/EO50PO50 and water/HM–EOPO systems formed after the thermoseparation.

After phase separation in the primary system, the EO50PO50 and HM–EOPO phases were isolated in separate vessels and the temperature was increased over the cloud point of the polymers, 55°C for 30 min. Two new two-phase systems were formed, both with one phase enriched in polymer, the bottom phase, and one water phase, the top phase. The protein concentration was measured in the water and polymer phases, and the partition coefficients were calculated.

Top phase (EO50PO50) after thermoseparation: All studied proteins were exclusively partitioned to the water phase (Table 2) with K values over 100. These results are similar to what was obtained earlier with EOPO copolymers [11–13]. The thermoseparated polymer rich phase (EO50PO50) contains approximately 50% polymer. It is entropically unfavourable for proteins to partition to the concentrated polymer phase compared to a water phase due to an excluded volume effect which strongly directs proteins to the water phase [25]. Extreme partition coefficients are difficult to measure accurately and the K values are given as greater than 100 when no protein was detected in the copolymer phase. Similar partitioning behaviour was observed for the three proteins BSA, lysozyme and apolipoprotein A-1, in the thermoseparated water–EO50PO50 system (Table 2).

Bottom phase (HM–EOPO) after thermoseparation: In contrast to EO50PO50, the HM–EOPO polymer gives rise to a polymer phase which is much less concentrated after thermoseparation. This is clearly seen in the cloud point diagram in Fig. 3. This can be due to that the EO-chains of the polymer avoids the aliphatic groups and thus prevents a too highly concentrated polymer phase to be formed. This will strongly reduce the excluded volume effect and thus the protein will only weakly favour the water phase. The protein partition coefficients (Table 2) show that proteins were more evenly partitioned in the water/HM–EOPO system. The HM–EOPO recovered after thermoseparation will thus contain some protein contamination, in contrast to the recovered EO50PO50. The partitioning of proteins in the thermoseparated water/HM–EOPO system can in many cases be affected by salts in the same way as in the system composed of two polymers [17]. For lysozyme and Apo A-1 the partitioning followed the normal salt effects in aqueous two-phase systems rather well. For the positively charged lysozyme the positively charged hydrophobic ion, Et_3NH^+, gave a K value of 2.38. This ion partitions to the polymer phase and thus directs lysozyme to the water phase. The opposite effect was seen for lysozyme with the negatively charged ion, ClO_4^-, which gave a K value of 0.29. The hydrophobic (chaotropic) ClO_4^- directed the positively charged lysozyme into the HM–EOPO phase ($K = 0.29$).

3.6. Purification of Apo A-1 from a cell-free E. coli fermentation solution

As shown above it is possible to partition pure proteins in the EO50PO50/HM–EOPO system and to direct the partitioning by addition of salts using similar mechanisms as in e.g. PEG–dextran systems. The EO50PO50/HM–EOPO aqueous two-phase system was studied for purification of a target protein from a fermentation extract. Apo A-1 was selected as target protein because of the relatively extreme partitioning to the HM–EOPO phase for this protein. A cell-free *E. coli* fermentation solution containing Apo A-1 was used as starting material. Two different total protein concentrations, 20 mg/ml and 63 mg/ml, were used. The primary aqueous two-phase system was 5% EO50PO50–5% HM–EOPO. The results are shown in Table 4.

Table 4
Purification of apolipoprotein A-1 from cell-free *E. coli* fermentation solution (extract). The extract was partitioned in a system containing 5% EO50PO50 and 5% HM–EOPO. The total protein concentration was 20 and 63 mg/ml respectively, and the buffer composition was 18 m*M* Tris–HCl pH 7.5, 135 m*M* NaCl, 9 m*M* EDTA and 0.09% Tween 80. The partitioning was performed at 21°C. The purification factors and yields were calculated from SDS–PAGE scanning results. The EO50PO50 and HM–EOPO phases were analysed before thermoseparation. In the system with 20 mg/ml of protein EO50PO50 was enriched in the top phase and HM–EOPO in the bottom phase and in the system with 63 mg/ml of protein HM–EOPO was enriched in the top phase and EO50PO50 in the bottom phase

	$K_{protein}$	K_{Apo}	Volume (ml)	Purification factor	Yield (%)
E. coli extract 20 mg/ml	–	–	6.48	(1)	(100)
EO50PO50 phase	–	–	4.00	1.0	38
HM–EOPO phase	–	–	3.00	6.6	62
Partition coefficient (EO50PO50/HM–EOPO)	2.2	0.45	–	–	–
E. coli extract 63 mg/ml	–	–	6.48	(1)	(100)
HM–EOPO phase	–	–	0.70	7.2	45
EO50PO50 phase	–	–	2.30	0.7	22
Partition coefficient (HM–EOPO/EO50PO50)	0.49	4.3	–	–	–

When the cell-free *E. coli* fermentation solution was partitioned in the EO50PO50/HM–EOPO system Apo A-1 could be extracted into the HM–EOPO phase (Fig. 6). The purification factors in the 20 mg/ml and 63 mg/ml systems were 6.6 and 7.2, respectively, see Table 4. One positive effect of having high protein concentration, 63 mg/ml, in the system was that Apo A-1 could be concentrated in a small phase. A more than 9 fold volume reduction (from 6.48 ml to 0.7 ml) was obtained from the volume of cell-free *E. coli* fermentation solution (63 mg/ml) added to the system. Furthermore, the system has a high protein capacity. More than 50 mg/ml total protein can be included, although at these high concentrations some protein precipitation occurs. We have observed that with high protein concentration, e.g. 63 mg/ml, the phases will shift place, i.e. phase inversion due to change of phase density. HM–EOPO which normally is the bottom phase forming polymer will now be found in the top phase; protein precipitation was observed in the bottom of the tube and on top of this the EO50PO50 phase was localised. The yield of Apo A-1 in the HM–EOPO phase after extraction was 45% at the high protein concentration. Due to the protein precipitation the yield of Apo A-1 was low and the mass balance of Apo A-1 did not close. The yield of Apo A-1 could be increased to 62% by reducing the amount extract added to the system to 20 mg/ml. In this system with lower protein concentration no phase inversion was observed. A problem with high protein concentrations and precipitation in the system is that the yield is decreased. Thus, it can in some cases be advantageous to use lower protein concentrations and thus sacrificing the concentration effect but instead obtain high yields and purification. These results show that the aqueous two-phase system composed by EO50PO50 and HM–EOPO can be used for the initial purification of a target protein, and the purification can be combined with volume reduction.

3.7. Recycling of copolymers

In the EO50PO50/HM–EOPO systems both of the polymers can be recovered by thermoseparation. Thus the possibility to recycle and recover the polymers was investigated. This was first studied in pure systems without protein to simplify the measurement of polymer in the different phases. The aqueous two-phase system prepared had the polymer concentrations 5% EO50PO50 and 5% HM–EOPO.

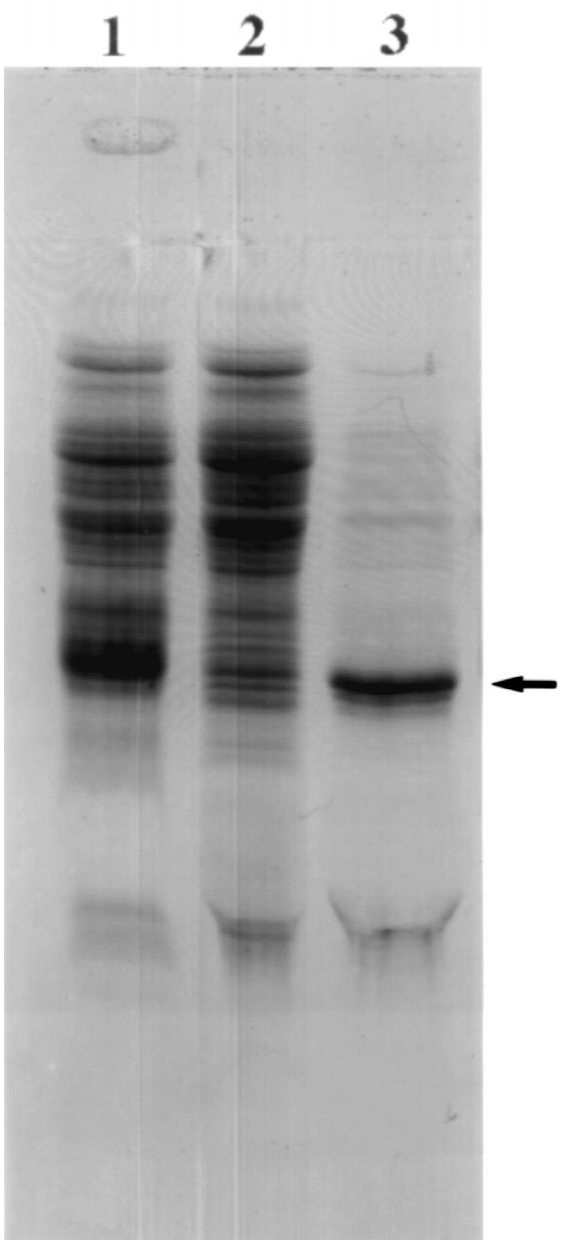

Fig. 6. Gel electrophoresis (18% SDS–PAGE) analysis of purification shown in Table 4. The gel was stained with Coomassie. In lane 1 the starting material, cell-free *E. coli* fermentation solution, is shown, lane 2 shows the bottom phase enriched in EO50PO50 copolymer and lane 3 show the top phase containing the HM–EOPO copolymer. The copolymer concentrations in the aqueous two-phase system were 5% EO50PO50 and 5% HM–EOPO. The protein concentration in the system was 63 mg/ml. As can be seen Apo A-1 was enriched in the HM–EOPO phase. (Apo A-1_M, →).

The primary EO50PO50/HM–EOPO systems were mixed and after phase separation the EO50PO50 and HM–EOPO phases were isolated in separate test tubes. The polymer concentrations in the phases of the primary system and in top (water) and bottom (polymer) phases after thermoseparation were determined. The concentrations of polymers were determined in the same way as in the phase diagram determinations. The polymer concentrations and recovery in the EO50PO50 and HM–EOPO phases and in the water and polymer phases after thermoseparation are shown in Table 5. The results show that in the primary system there was a relatively low concentration of HM–EOPO in the EO50PO50 phase and vice versa, i.e. 2% HM–EOPO in the EO50PO50 phase and 5% EO50PO50 in the HM–EOPO phase. The amount EO50PO50 recovered when thermoseparating the EO50PO50 phase was 72%, and only 1% of EO50PO50 was recovered in the HM–EOPO polymer phase. In the same way 95% of HM–EOPO was recovered in the polymer phase after thermoseparating the HM–EOPO phase and 2.5% of HM–EOPO was recovered after thermoseparation of the EO50PO50 phase. The overall recovery of EO50PO50 and HM–EOPO after temperature induced phase separation was 73% and 97.5%, respectively. Thus, HM–EOPO is a more effective polymer for recycling, with a recovery of nearly 98% in each cycle. The reason for this is the micelle formation of HM–EOPO polymers due to the hydrophobic end groups. This leads to high molecular weight aggregates which can be effectively recovered by thermoseparation. This can also be seen in Table 5 where the concentration of EO50PO50 is significantly higher than the HM–EOPO concentration in the water phase. The EO50PO50 concentration in the water phase (2.3%) for a sample separated 5°C above the cloud point is as expected from the cloud point diagram of the EO50PO50 polymer (see Fig. 3).

3.8. Purification combined with polymer recycling

Protein purification and recycling of copolymer during purification of target protein from a cell free *E. coli* fermentation solution was studied in the EO50PO50/HM–EOPO system. The copolymers were recycled and a new two-phase extraction was

Table 5
Polymer recovery by thermoseparation. Recovery and concentration of polymers were determined in a primary protein free EO50PO50/HM–EOPO system and in the phases after thermoseparation. The primary two-phase system contained 5% EO50PO50, 5% HM–EOPO and 10 m*M* sodium phosphate buffer pH 7.0 and was separated at 21°C. The phases from the primary two-phase system were isolated in separate vessels and transferred to a water bath where the thermoseparation was performed at 55°C for 30 min

	Recovery		Concentration	
	EO50PO50 (%)	HM–EOPO (%)	EO50PO50 (% w/w)	HM–EOPO (% w/w)
EO50PO50/HM–EOPO				
Top phase (EO50PO50)	98	5	13.9	0.3
Bottom phase (HM–EOPO)	2	95	0.2	11.8
Thermoseparation:				
Top phase				
Water phase	28	2	2.3	0.1
Polymer phase (EO50PO50)	72	2.5	52	2.1
Bottom phase				
Water phase	1	0	0.08	0.07
Polymer phase (HM–EOPO)	1	95	0.2	23.5
Total polymer recovered	73	97.5		

performed according to the principles in Fig. 1. The polymer concentration in the primary system was 5% EO50PO50 and 5% HM–EOPO, and the thermoseparation was performed in the same way as for the experiments shown in Table 5 (55°C for 30 min). The amount copolymer in the recycled phases was assumed to be the same as those determined in systems without proteins, see Table 5. To reestablish the initial amount of copolymer after recycling some addition of new copolymer had to be made. As seen in Table 5, 73% of the EO50PO50 copolymer could be recycled, and 27% new copolymer had to be added in each cycle. For HM–EOPO only 2.5% new copolymer had to be added as the amount of recycled HM–EOPO was 97.5%. The total protein concentration in the primary system was 5 mg/ml. A low protein concentration was chosen as this will not affect the phase system and thus the yield of recovered polymer after thermoseparation will be similar to the protein free system determined in Table 5. The results for three recycles of copolymer are collected in Table 6. The same volume ratio between top and bottom phase in the primary system was observed in the recycled systems compared with the initial system. The recovery (77–80%) and purification factor (5.5) of apolipoprotein A-1 were

Table 6
Recycling of polymers and purification of apolipoprotein A-1 from a cell-free *E. coli* fermentation solution performed in a primary system of 5% EO50PO50 and 5% HM–EOPO. The volume % of cell-free *E. coli* fermentation solution added to the system was 7.1%. The total protein concentration in the system was 5 mg/ml. The pH in the primary system was kept at 7.0 with 20 m*M* sodium phosphate buffer and the separation was performed at 21°C. The thermoseparation was performed at 55°C for 30 min. The volume ratio reported in this table refers to the volumes of the top and bottom phases in the primary system. The amount recovered copolymer in each thermoseparation step was assumed to be the same as reported in Table 5, i.e. 73% EO50PO50 and 97.5% HM–EOPO. The results are calculated from data obtained from scanning SDS–PAGE gels. The partition coefficient of total protein was determined by using a protein Coomassie assay. The phases analysed were water phases after thermoseparation

	$K_{protein}$	Purification factor$_{Apo}$	Yield$_{Apo}$ (%)	Volume ratio (top/bottom)
Initial system	1.1±0.1	5.3±0.5	78±5	1.3±0.1
First Recycle	1.0±0.1	5.5±0.5	78±5	1.2±0.1
Second Recycle	1.1±0.1	5.8±0.5	80±5	1.3±0.1
Third Recycle	1.0±0.1	5.4±0.5	77±5	1.3±0.1

very similar in the different systems. Also the partition coefficient for total protein was constant in the recycled systems. Samples, after thermoseparation, from top and bottom phases from the initial and the recycled systems were analysed by SDS–PAGE. No significant differences in protein concentrations or pattern were observed between the recycled systems, see Fig. 7. This shows that it is possible to recycle the copolymers at least three times and still obtain the same partitioning and phase behaviour of the system.

Not all protein could be removed from the HM–EOPO polymer phase. But by changing pH and if necessary performing an additional thermoseparation it will be possible to reduce the protein contamination of the HM–EOPO phase. There will always be a partitioning of proteins between the HM–EOPO and water phases, however, after removing the water phase one could perform a new extraction of protein from the HM–EOPO polymer phase by adding a new water/buffer phase. A disadvantage in this case will be that the concentration of the recovered protein will be reduced in the second extraction.

PEG/salt systems has traditionally been used for large scale extraction of proteins due to the low cost of the phase components. Drawbacks with this system are high salt concentration in both phases which reduces protein solubility and may cause

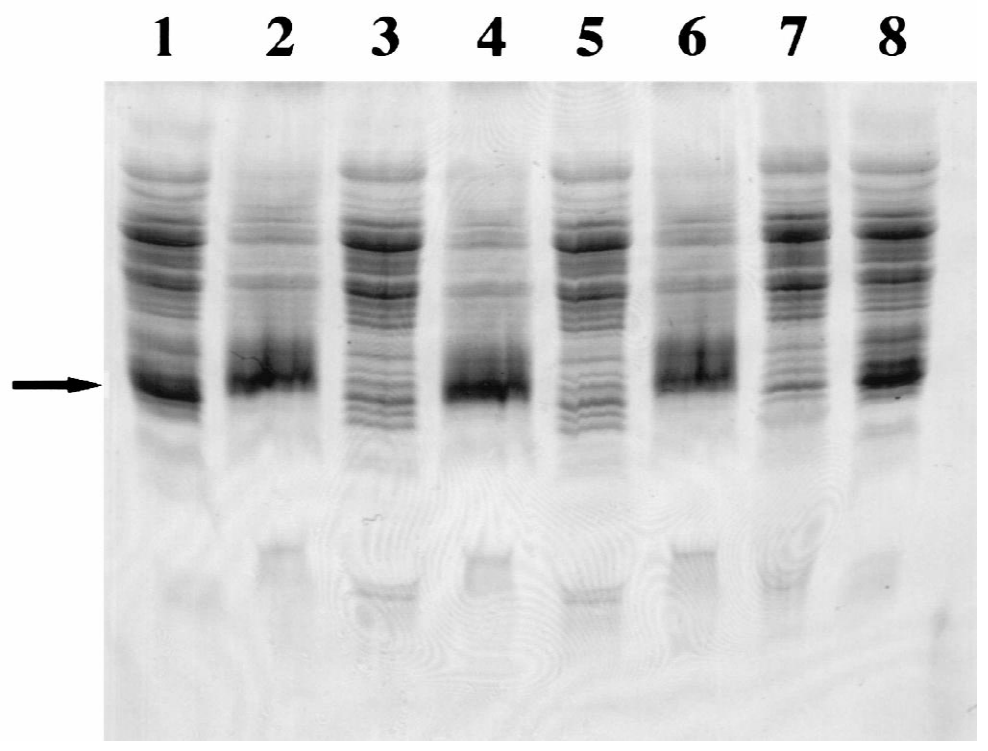

Fig. 7. Gel electrophoresis (18% SDS–PAGE) analysis of polymer recycling and protein purification shown in Table 6. The gel was stained with Coomassie. Samples form experiments with fresh copolymer and copolymer that has been recycled once and three times are shown. In lane 1 and 8 the starting material, cell-free *E. coli* fermentation solution, is shown, and in lane 2 and 3 the bottom and top phases from sample with fresh copolymer is shown. Lane 4 and 5 show the bottom and top phase after one step of recycling. Lane 6 and 7 show the bottom and top phase after three copolymer recycling steps. The top and bottom phases applied to the gel were water phases after thermoseparation. The system used was 5% EO50PO50, 5% HM–EOPO and 20 m*M* sodium phosphate pH 7.0. The protein concentration was 5 mg/ml and the thermoseparation was performed at 55°C for 30 min. (Apo A-1_M, →).

environmental problems. Recycling of phase components has been demonstrated in PEG/salt systems [41]. Hustedt showed that it is possible to recover the PEG polymer by back-extracting the protein partitioned to the PEG phase to a new salt phase. In this way it was possible to recover 90% of the PEG polymer [41]. The target protein will be recovered in a salt rich phase after back extraction from the PEG phase, this can in some cases give problems in the following purification steps. To reduce the salt concentration in the phase containing the target molecule, a ultrafiltration step is often used [41]. Thus, it is advantageous if the target protein can be back extracted into a phase at low salt concentration. An aqueous two-phase system for large scale use based on two polymers was earlier developed which could be operated at low salt concentrations. This was composed of EOPO copolymer and hydroxypropyl starch [11–13]. It was thus possible to recover the EOPO copolymer after thermoseparation, and contaminants were removed in the bottom phase containing low cost starch derivative. The system was shown to have high capacity and good purification properties. The disadvantage is that it is not possible to recover the starch polymer. In the new aqueous two-phase system with the two thermoseparating polymers EOPO and HM–EOPO the target protein can be partitioned to either phase and recovered in a water phase after thermoseparation. Furthermore, both of the polymers are recyclable. A technically even simpler system is the one-polymer system based on the HM–EOPO polymer that can be used for protein purification [17]. In this system the proteins are partitioned between the water and HM–EOPO phase. However, in a system with two thermoseparating polymers more possibilities are available for directing protein partitioning between the phases. Since both phases contain polymers there is less 'entropic' difference between the two phases in this system compared with a water/polymer system where there is always an entropic driving force for proteins to partition to the water phase [25]. For large scale extractions in bioprocesses it is advantageous to have access to a number of phase systems with different capabilities. The system composed of two thermoseparating polymers is an alternative for large scale protein extraction. In such a system the target protein and the contaminants can be directed to different phases, and both phase-forming polymers can be recovered by thermoseparation.

4. Conclusions

Mixtures of EO50PO50 and HM–EOPO copolymers in water form aqueous two-phase systems. Both polymers are thermoseparating and it is possible to phase separate both copolymers by increasing the temperature over the copolymer cloud point. Compared to PEG/dextran systems relatively low concentrations of copolymers are required to form two phases (e.g. 2%, w/w, of each copolymer). The aqueous two-phase system composed of EO50PO50/HM–EOPO can be used for separation of proteins. Partitioning of BSA and lysozyme can be directed by salt addition in similar way as in PEG/dextran and EOPO/starch systems. The amphiphilic apolipoprotein A-1 interacts with micelles formed by the HM–EOPO copolymer and partitions strongly to the HM–EOPO phase. After thermoseparation of the top, EO50PO50, and bottom, HM–EOPO, phases it is possible to recover the proteins in water solution and to recycle the copolymers. After thermoseparation 97.5% of HM–EOPO and 73% of the EO50PO50 copolymer could be recovered. Apolipoprotein A-1 could be extracted to the HM–EOPO phase from a cell free *E. coli* fermentation solution. Systems with recycled copolymers showed similar purification factors (5.5) and yields (78%) during three polymer recyclings. This aqueous two-phase system is both cost efficient and environmentally benign since both polymers can be recycled. The system is an alternative to the PEG/salt system in large scale extractions of proteins.

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