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Polymer recycling in aqueous two-phase extractions using thermoseparating ethylene oxide–propylene oxide copolymers

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Abstract

This is a study on the recovery and recycling of copolymer in aqueous two-phase systems containing random copolymers of ethylene oxide (EO) and propylene oxide (PO). The random copolymers separate from water solution when heated above the lower critical solution temperature (LCST). The primary phase systems were composed of EOPO copolymer and hydroxypropyl or hydroxyethyl starch. After phase separation the upper EOPO phase was removed and subjected to temperature induced phase separation. Copolymers with different EO/PO compositions have been investigated, EO50PO50 [50% EO and 50% PO (w/w)], EO30PO70 and EO20PO80. The temperature required for thermoseparation decreases when the PO content of the copolymer is increased. The effect on the recovery of copolymer after addition of salts, a second polymer or protein was investigated. The added components increased the recovery of copolymer after thermoseparation, e.g., increased the amount copolymer separated from the water phase after thermoseparation. Recycling of copolymer and measurements of polymer concentrations in the primary top and bottom phases after repeated recycling steps was performed. The fluctuation in polymer concentration of the phases was very small after recycling up to four times. Partitioning of the proteins BSA and lysozyme was studied in primary phase systems after recycling of copolymer. The partition coefficients of total protein and lysozyme was not significantly changed during recycling of copolymer. More than 90% of the copolymer could be recovered in the thermoseparation step by optimising the temperature and time for thermoseparation. In repeated phase partitionings in EOPO–starch systems the EO50PO50 copolymer could be recovered to 77% including losses in primary system and thermoseparation, which is equivalent to a total copolymer reuse of 4.3 times.

Keywords: Partitioning; Aqueous two-phase systems; Polymer recycling; Ethylene oxide–propylene oxide; Thermoseparating polymers

1. Introduction

Aqueous two-phase systems offer a mild method to separate biomaterials [1]. One reason for this is the high water concentration in both phases. The traditional aqueous two-phase systems have been the PEG–dextran and PEG–salt systems [PEG=poly(ethylene glycol)] [1]. The former system is formed due to polymer–polymer incompatibility and the latter is formed by salting out of the polymer with phosphate or sulphate salts. A typical PEG–dextran system contains 10–20% polymer in both phases. A typical PEG–salt system contains 20–30% PEG plus salt in the top phase, 10–15% phosphate or sulphate salt in the bottom phase.

The PEG–salt system has been extensively used for large-scale extractions mainly of industrial en-

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zymes [2–8]. These systems have been shown to be cost efficient for primary protein recovery [9]. A novel aqueous two-phase system for large-scale use has been introduced where the systems are formed by starch derivatives, hydroxypropyl starch, and random copolymers of ethylene oxide (EO) and propylene oxide (PO) [10–12]. The starch derivatives are low-cost substitutes for dextran and the EOPO copolymers are recyclable. The EOPO copolymers are thermoseparating, i.e., over a critical temperature (the cloud point) the polymer separates from the water solution and a new aqueous two-phase system is formed where a water phase is in equilibrium with a polymer phase. Many thermoseparating polymers contain ethylene oxide groups. PEG is a thermoseparating polymer [13] but its cloud point is too high (above 100°C) for use in a thermoseparating process for the separation of biomaterials. Several studies have been performed on the thermoseparation of polyethylene oxide polymers [13–15] and EOPO copolymers [16]. Random copolymers of ethylene oxide and propylene oxide have lower cloud points than PEG. Ucon 50 HB-5100 (Ucon) and Breox 50A 1000 (Breox) are random copolymers of 50% EO and 50% PO. These polymers have a cloud point of 50°C, and they have been used in separation systems for protein purification [10–12]. In the first stage the target protein is partitioned to the EOPO-rich phase in the primary EOPO–starch aqueous two-phase system (see Fig. 1). In the second stage the EOPO-rich phase is removed and the temperature is increased above the cloud point of the EOPO copolymer. A new two-phase system is formed with an EOPO-rich bottom phase and a top phase which is enriched in water. In this water–polymer two-phase system the proteins are quantitatively partitioned to the water phase [11,17]. The thermoseparating copolymer forms the bottom phase in the thermoseparation step and the copolymer concentration in the bottom phase after thermoseparation is above 40% (w/w). The lower mixing entropy in this polymer rich phase compared to the water rich phase can explain why proteins are strongly excluded from the polymer rich phase [18]. The protein-free EOPO copolymer can be recycled to the primary phase system for a new extraction.

Due to the low cost of the starch polymer and the possibility to recycle the EOPO copolymer this type of aqueous two-phase system is an attractive alternative to the PEG–salt system in large-scale extractions. One advantage with the thermoseparating system is that low salt concentrations can be used and thus there is low risk for salting out and precipitation of protein. Another advantage is that

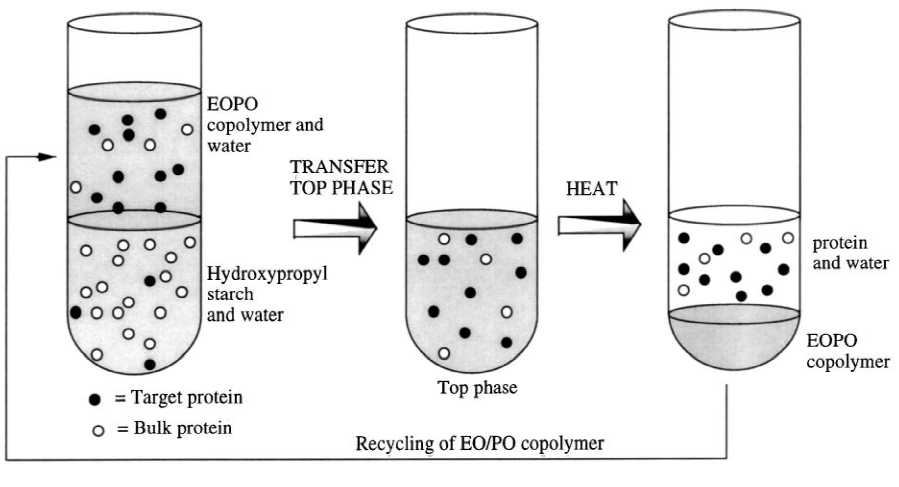

Fig. 1. Purification of a target protein in an aqueous two-phase system with recycling of EOPO copolymer by thermoseparation. Black dots symbolise the target protein and white dots the bulk proteins. The target protein is recovered in the top EOPO phase in the primary phase system, whereafter the top phase is isolated and heated over the cloud point of the copolymer. After thermoseparation the target protein is recovered in the water rich top phase and the bottom copolymer enriched phase is free of protein. The copolymer phase is recycled and used in a new extraction.

the target protein is recovered in a water solution after the thermoseparation of EOPO copolymer which facilitates the further downstream processing.

The focus in this paper is on recovery and recycling of EOPO copolymer by thermoseparation. There have been a number of articles published describing the use of EOPO copolymers in aqueous two-phase systems but few have discussed the practical aspects of the thermoseparation. We have now studied different alternatives to influence the thermoseparation to obtain optimal copolymer recovery. This includes the time and temperature that is required for separation and how different types of added components such as salts, proteins and a second polymer influence the thermoseparation. The thermoseparating polymers used are all random copolymers of ethylene oxide and propylene oxide and the role of EO/PO composition was studied. As bottom phase forming polymers different derivatives of starch were included in the primary system: hydroxypropyl starches (Reppal PES) and hydroxyethyl starch (Solfarex A85). The polymers used in the primary two-phase system will influence the thermoseparation of the EOPO phase. Our results show that the copolymer recovery is more dependent on the temperature than the time for thermoseparation. With sufficient heat transfer 5 min for thermoseparation is enough for effective copolymer recovery. Higher PO content in the copolymer gives lower cloud point and higher molecular mass of the copolymer leads to a more efficient thermoseparation and recovery. Addition of salt, protein or a second polymer also increases the recovery of copolymer at the thermoseparation of the EOPO phase. The recycling of EOPO copolymer could be performed without affecting the protein partitioning in the primary phase system.

2. Materials and methods

2.1. Chemicals

The thermoseparating polymers studied were: Breox PAG 50A 1000 (EO50PO50) (M_r 3900) from International Speciality Chemicals (Southampton, UK), EO30PO70 (M_r 5400) and EO20PO80 (M_r 3400) from Shearwater Polymers (Huntsville, AL, USA). As bottom phase forming polymer the starch derivatives Reppal PES 100 (M_w 165 000, M_w/M_n = 2.15), a hydroxypropyl starch from Carbamyl (Kristianstad, Sweden), and Solfarex A85, a technical grade hydroxyethyl starch from AVEBE (Veendam, The Netherlands), were used. All salts and reagents used were of analytical-reagent grade.

2.2. Protein

The proteins used were fatty acid-free bovine serum albumin (BSA) obtained from Boehringer Mannheim (Mannheim, Germany) and hen egg lysozyme obtained from Sigma (St. Louis, MO, USA).

2.3. Two-phase systems

All polymer concentrations were calculated as % (w/w). The starch polymers were dissolved in water and added from stock solutions, 28%. The copolymers were added as pure substances. Buffers and salts were added from 1 *M* stock solutions. Aqueous two-phase systems were prepared with a final mass of 10 g. All samples were prepared in 15-ml graduated glass tubes of 110 mm×15 mm I.D. The systems were separated at room temperature and the phase separation was enhanced by centrifugation, 5 min at 1800 *g*. The phases were separated and the top EOPO phase was placed in a water bath to perform the temperature-induced phase separation. For study of copolymer recovery also pure EOPO–water systems were used. Solutions of 10% (w/w) EOPO copolymer in water were prepared and the different components (salt, protein, starch polymer) were added.

2.4. Temperature-induced phase separation

Top copolymer phases from the primary phase system or aqueous EOPO solutions were placed in a thermostated water bath for the thermoseparation. Graduated 15-ml glass tubes of 110×15 mm I.D. were used. To improve the phase separation the tubes were centrifuged for 30 s at 1000 *g* after the heating. The cloud points for the different copolymers were determined by slowly increasing the temperature on 10% solutions of the copolymer. The

temperature at which the copolymer solution started to become turbid was taken as the cloud point of the copolymer. The coexistence curves were obtained by determining the copolymer concentration in the water-rich and copolymer-rich phases after thermoseparation for 30 min.

2.5. Determination of phase composition

The refractive index standard curves for the polymers, salts and proteins were determined at 20°C with a refractometer from Carl Zeiss (Oberkochen/Württ., Germany). The refractive index of the top and bottom phase was determined and the contribution from starch polymer, salt and protein was subtracted from the total refractive index. The resulting refractive index is due to the contribution from the copolymer which thus gives the concentration of EOPO copolymer. The starch polymer concentration was determined by measuring the optical rotation using a digital polarimeter (Model AA-10) from Optical Activity (London, UK). Standard curves for the starch polymers were prepared and the concentration of the starch polymer in the different phases was determined. The salt concentration was determined by measuring the conductivity, using a Metrohm 644 conductometer (Herisan, Switzerland).

2.6. Protein partitioning

The partitioning of molecules in two-phase systems is described by the partition coefficient K. It is defined as the concentration of the target molecule in the top phase, C_T, divided by the concentration in the bottom phase, C_B: $K=C_T/C_B$. The total protein concentration was determined by measuring the absorbance at 280 nm. The spectrophotometer used was UV-2101 PC from Shimadzu (Kyoto, Japan). Lysozyme concentration was determined by measuring the enzyme activity with *Micrococcus lysodeikticus* cells as substrate [19].

2.7. Determination of copolymer recovery

The polymer recovery (R_{pol}) after thermoseparation have in these studies been defined as: $R_{pol}=m_{thermosep.}/m_{start}$ where $m_{thermosep.}$ is the mass of EOPO copolymer recovered in the lower polymer enriched phase after thermoseparation and m_{start} is the total mass of EOPO copolymer in the top EOPO phase of the primary phase system or in a water–EOPO solution.

2.8. Recycling of copolymer

The concentration of copolymer was determined in the phases after thermoseparation. This was performed in a reference primary phase system without protein. The primary top phase was placed in a water bath 15°C over the cloud point of the copolymer for 5 min. The copolymer concentration was calculated by measuring the refractive index. These data were used as reference for calculating the recovery of copolymer during the recycling experiments.

In the recycling experiments the primary phase system was weighed, mixed and the phases separated. The top phase was isolated and weighed into a new tube with known mass. The thermoseparation was performed at 15°C over the cloud point of the copolymer for 5 min, whereafter the tube was centrifuged for 30 s at 1000 *g*. The water phase was pipetted from the copolymer phase and the tube with copolymer was weighed. The recovery of the copolymer could be calculated by knowing the mass of the copolymer phase and by using data from the reference system on the concentration of copolymer in this phase. To the tube containing recycled copolymer new fresh bottom phase polymer was added, plus some addition of copolymer to make up to the initial concentration, and other phase components, i.e., salt and protein.

3. Results and discussion

In the last years there have been a number of studies on the use of EOPO–starch aqueous two-phase systems to purify proteins [10–12]. In this type of aqueous two-phase system the aim is to recover the target protein in the top EOPO phase and the contaminating proteins should be partitioned to the bottom starch phase (Fig. 1). After extraction in the primary phase system the top EOPO enriched phase is removed to a separate vessel and heated over a critical temperature. In this way the main part

of the EOPO copolymer can be recovered and reused in a new aqueous two-phase extraction.

The recovery of the copolymer by thermoseparation will be affected by the heat transfer capacity and the geometry of the vessel. The sample in a narrow tubing will be more effectively heated than sample in a wide tube. The problem of losses of sample in the interface upon separating the phases is also affected by the geometry of the vessel. In a small tube the interfacial area is much smaller and thus less sample will be lost in the interface compared with a wider tubing. The recovery of copolymer in the thermoseparating aqueous two-phase system (Fig. 1) has been studied as an effect of the components added to the system as well as temperature and time of phase separation. One type of sample tube was used throughout which gave the same vessel geometry in all the experiments.

3.1. Phase diagrams for two-polymer systems

A phase diagram for the aqueous two-phase system with the polymers EO20PO80 and hydroxypropyl starch (Reppal PES 200) is shown in Fig. 2. Reppal PES 200 (M_w 210 000) is an identical hydroxypropyl starch to Reppal PES 100 (M_w 165 000) with a slightly higher molecular mass.

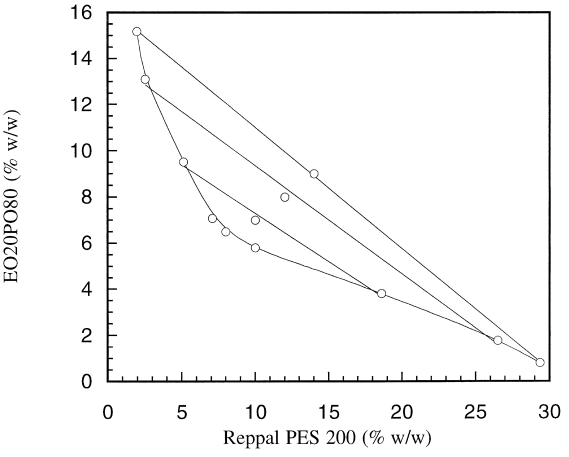

Fig. 2. Phase diagram for EO20PO80–Reppal PES 200 in water solution at 20°C. The region above the binodal curve is the two-phase region. The tie lines connecting two points in the binodal curve describes the concentration of polymer in the top and bottom phase, respectively.

Phase diagrams for the EO50PO50–Reppal PES 100 and PES 200 [12], EO30PO70–Reppal PES 100 [11] and EO50PO50–hydroxyethyl starch (Solfarex A85) [12] systems have previously been published. As seen in Fig. 2 the segregation between the two polymers is not complete, i.e., in the top phase there will be bottom phase polymer present and vice versa for the other phase. Thus, some EOPO copolymer will be lost in the primary bottom phase and the total recovery of EOPO copolymer after thermoseparation when calculated from the primary phase system can never be 100%. However, with polymers of higher molecular mass the segregation of the polymers will be more complete which will increase EOPO recovery [1].

3.2. Copolymer coexistence curves

The three copolymers studied have different EO/PO compositions and different molecular masses. EO20PO80 has the lowest cloud point (lower critical solution temperature, LCST), 30°C, and EO30PO70 and EO50PO50 have LCSTs at 40 and 50°C, respectively. In Fig. 3 the coexistence curves for the three

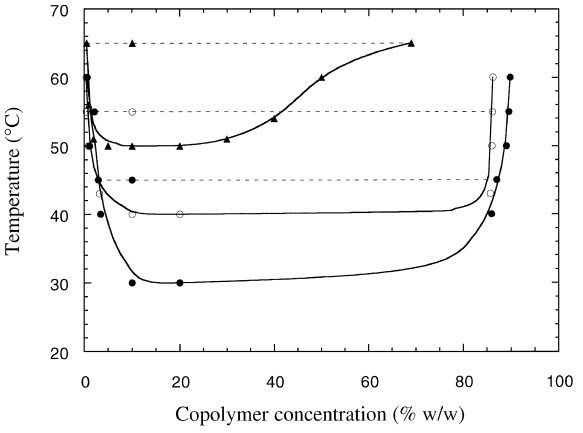

Fig. 3. Coexistence curves for the random EOPO copolymers investigated, EO20PO80 M_r 3400 (●), EO30PO70 M_r 5400 (○) and EO50PO50 M_r 3900 (▲). The experimental point in the thermoseparation experiments at 10% copolymer concentration and 15°C over the LCST has been indicated on the tie lines. The curves were determined for pure copolymer in water solution. Above the coexistence curve the solution will separate into one water phase with low copolymer concentration and one aqueous phase enriched in copolymer. Below the coexistence curve the copolymer solution will exist in one homogeneous phase.

different EOPO copolymers are shown. The EO20PO80 and EO30PO70 polymers have very similar coexistence curves. It is only the lower critical solution temperature that differs. After thermoseparation at 15°C over the LCST both polymers give water phases with low polymer concentration and polymer phases which are more than 75% concentrated in copolymer. The water phase of the EO50PO50 system has a higher polymer concentration compared with the two other EOPO copolymers and the copolymer phase after thermoseparation is much less concentrated, 45% copolymer. This can be explained by the fact that the EO50PO50 copolymer has the lowest hydrophobicity of the studied copolymers, i.e., the lowest content of PO, and thus the lowest tendency to separate from water solution.

3.3. Polymer recovery after thermoseparation

Recovery in the thermoseparation step was studied using pure water–EOPO copolymer mixtures. The total recovery of copolymer after thermoseparation for 5 min is shown in Table 1. To be able to compare the results for the EOPO copolymers a temperature was selected at 15°C above respective cloud point. In this way the recovery of the copolymer could be correlated to the phase behaviour of the copolymers after thermoseparation. The recovery of copolymer is determined by the coexistence curves shown in Fig. 3. From these curves the concentrations in the copolymer and water phases and the volume ratio of the phases can be determined. Thus, the recovery of copolymer was measured and compared with the calculated recoveries using the phase diagram (Table 1). For EO20PO80 and EO30PO70 the calculated and measured recoveries are similar. In the EO50PO50 case the measured copolymer recovery was significantly lower than the calculated value, 69 and 84%, respectively. This can be due to that 5 min at 65°C is not a sufficient time for a total thermoseparation of the EO50PO50 polymer. The copolymer with the highest molecular mass, EO30PO70 M_r 5400, gave the highest copolymer recovery after thermoseparation, 96%. A general effect for thermoseparating polymers is that phase separation of polymer from water is more efficient for larger polymers [13].

3.4. Effect of temperature and time for phase separation

The recovery of copolymer will be affected by the temperature and time for the thermoseparation. To determine these effects 10% (w/w) EOPO copolymer solutions in water were prepared and the recovery of copolymer after thermoseparation was examined (Table 2). It is important to determine these effects as a judicious choice of the thermoseparation parameters will improve the copolymer recovery and the separation of protein from copolymer.

Thermoseparation at different temperatures showed that the recovery increased with the temperature, Table 2. This effect is not linear and with EO30PO70 and EO20PO80 at temperatures more than 15°C over the lowest cloud point (LCST) there was no further increase in the recovery of copolymer from, 96 and 90%, respectively. From the coexistence curves (Fig. 3) it can be seen that the polymer concentration in the copolymer phase does not change significantly at temperatures more than 15°C over the LCST. The copolymer concentration in the water phase approaches zero which will have an effect on the recovery (Fig. 3). From the phase

Table 1
Cloud points and recovery for the EO20PO80, EO30PO70 and EO50PO50 copolymers[a]

	Molecular mass	Cloud point (°C)	Measured polymer recovery (%)	Calculated polymer recovery (%)
EO20PO80	3400	30	90±3	90
EO30PO70	5400	40	96±3	96
EO50PO50	3900	50	69±3	84

[a] The cloud points and copolymer recoveries were determined for 10% (w/w) water solutions of the copolymers. The recovery was determined after thermoseparation for 5 min at 15°C above the cloud point of each of the copolymer. The calculated polymer recoveries were calculated from the volume ratio and polymer concentrations obtained from the tie lines for the respective copolymers (Fig. 3).

Table 2
Recovery of copolymer in the polymer enriched phase after thermoseparation, as a function of time and temperature[a]

Time (min)	Recovery (%)									
	EO50PO50		EO30PO70				EO20PO80			
	60°C	65°C	50°C	55°C	60°C	65°C	35°C	40°C	45°C	55°C
5	22.3	69.2	92.8	95.9	97.0	96.0	No separation	62.3	90.0	87.9
10	39.3	70.9	93.9	95.6	97.1	–	7.8	64.4	87.5	–
15	46.9	63.7	93.7	96.4	96.6	–	–	–	–	–
30	45.8	62.7	93.5	96.5	97.0	–	–	–	–	–

[a] Three different copolymers were investigated, EO50PO50, EO30PO70 and EO20PO80. The initial copolymer solutions were 10% (w/w) copolymer in water. Thermoseparation at the indicated temperatures resulted in formation of a water and a copolymer enriched phase. The recovery of copolymer was determined by weighing the copolymer phase and measuring the copolymer concentration.

diagram it can be seen that an increased molecular mass of the copolymer will give a water phase with lower concentration of copolymer. Thus, more of the copolymer can be recovered after thermoseparation if a copolymer of higher molecular mass is used.

The time for the thermoseparation also has a critical role in the recovery of copolymer. In Fig. 4 the recovery of EO30PO70 (Table 2) is shown as a function of time at three different temperatures. At 5 min the recovery was higher than 94% and after 10–15 min a threshold value was reached after which copolymer recovery no longer increased. The recovery of copolymer has a stronger dependency on temperature than on separation time after a minimum time of 5 min for the phase separation.

3.5. Bottom phase polymer: effect on EOPO recovery

The phase composition of a two-phase system with two polymers in water will depend on the chemical properties and molecular mass of the polymers [1]. Polymers of higher molecular mass separate from each other at lower polymer concentrations than the corresponding polymers of lower molecular mass. The segregation of the polymers is

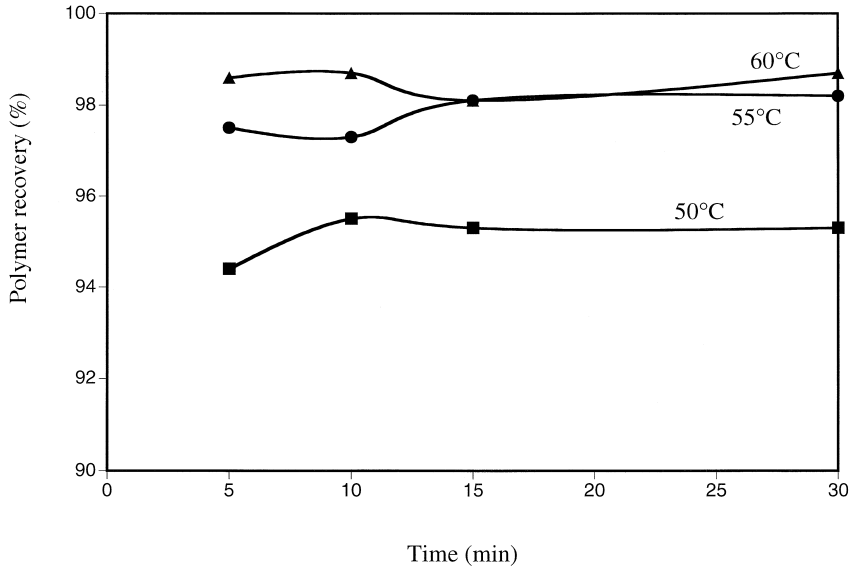

Fig. 4. The recovery of EO30PO70 copolymer after thermoseparation at 50°C (■), 55°C (●) and 60°C (▲) for different times, 5–30 min. The initial solutions were 10% (w/w) of copolymer dissolved in water.

Table 3
The polymer concentrations in the top phase in the primary phase system when different starch polymers were used as bottom phase forming polymer[a]

EOPO copolymer	Starch polymer
10.8% / EO50PO50	3.7% / Reppal PES100
15.7% / EO50PO50	1.4% / Solfarex A85
11.2% / EO30PO70	3.0% / Reppal PES100
13.2% / EO30PO70	1.1% / Solfarex A85

[a] The mixed systems had the total composition 13% starch polymer and 6% EOPO copolymer. The systems were phase separated at 21°C.

also more complete, i.e., there will be less of the bottom phase forming polymer in the top phase if polymers of high molecular mass are used. Thus, it is necessary to study how the choice of bottom phase polymer in the primary phase system affects the recovery of copolymer at thermoseparation. A higher molecular mass of the bottom phase forming polymer in the primary system may increase the recovery of EOPO polymer. Secondly it is important to study if and how the presence of a low concentration of polymer from the primary system affects the thermoseparation step.

Table 3 shows that the concentration of EOPO copolymer in the top phase in the primary system increases when Solfarex A85 is used instead of Reppal PES 100. Conversely, the concentration of starch polymer is decreased in the top phase when Solfarex A85 is used instead of Reppal PES 100. Both of these effects are due to the increased molecular mass of Solfarex compared with Reppal. In Table 4 the recovery of copolymer (EO50PO50) after thermoseparation is shown as a function of additions of salts and starch polymers. The recovery of copolymer was calculated after thermoseparation of 10% EOPO–water solution. For study of the effect of bottom phase polymer a concentration of 3% starch was selected as this is a typical concentration of starch obtained in the EOPO phase after the extraction in the primary phase system. The thermoseparation of pure EO50PO50 in water gave a copolymer recovery of 69% (Table 4). The addition of Reppal PES 100 or Solfarex A85, 3% (w/w), to the copolymer solution increased the copolymer recovery to 86 and 87%, respectively. The reason for improved recovery of copolymer after addition of the starch polymer may be due to the incompatibility between the polymers. The concentration of starch polymer is under the critical concentration required for obtaining two phases but the concentration is high enough for effectively repulsive interactions between the copolymer and the starch derivative. This may lead to more rapid phase formation of copolymer during the thermoseparation. The same effect was observed also for EO30PO70 and EO20PO80 but as the recovery is already high for these copolymers in pure water solution the effect of

Table 4
The effect on copolymer recovery after addition of different salts or polymers to the EO50PO50 solution[a]

Addition	Recovery of EO50PO50 copolymer (%)
–	69
10 m*M* sodium phosphate	91
100 m*M* sodium phosphate	94
10 m*M* sodium sulphate	92
100 m*M* sodium sulphate	95
10 m*M* sodium chloride	89
100 m*M* sodium chloride	90
3% Reppal PES 100	86
3% Reppal+10 m*M* sodium phosphate	92
3% Solfarex	86
3% Solfarex+10 m*M* sodium phosphate	90

[a] The recovery of EO50PO50 was determined after thermoseparation at 65°C for 5 min. The copolymer solution was 10% (w/w) EO50PO50 in water.

a second polymer becomes smaller than for the EO50PO50 copolymer.

3.6. Salt addition: effect on EOPO recovery

Normally when partitioning biomaterial in EOPO–starch systems different salts or buffer ions are used to maintain a constant pH. The addition of salt will affect the cloud point of the copolymer [10,20], thus the polymer recovery will be influenced. The effect on the thermoseparation by additions of common salts at different concentrations has been studied (Table 4). In the cases studied the salts decreased the solubility of the copolymer by lowering the cloud point, which was earlier reported by Harris et al. [10]. There was a significant increase from 69 to 90% in recovery of EO50PO50 when adding only 10 m*M* of the sodium salt of phosphate, sulphate and chloride. The other copolymers, EO20PO80 and EO30PO70, were also affected by addition of the salts but not as effectively (results not shown) because of the already high (>90%) recovery. When the salt concentration was increased to 100 m*M* there was only a slight improvement of the recovery compared to 10 m*M* salt, see Table 4. No significant differences in the polymer recovery were seen when the different salts were used. One explanation for the increased copolymer recovery by salt addition is that the hydrophilicity of the water phase increases when adding salt. Johansson et al. [21] have shown that salts partition stronger to the water phase in a thermoseparated water–EO50PO50 system. Thus the relatively hydrophobic copolymer will be repelled from the water phase and partition stronger to the copolymer phase. This explains why the effect when going from a water solution to 10 m*M* salt is more pronounced than the effect of increasing the salt concentration from 10 to 100 m*M*.

3.7. Protein addition: effect on EOPO recovery

The thermoseparating aqueous two-phase systems have been developed for purification of proteins [11,12]. Thus, there will be proteins present in the EOPO phase from the primary phase system when the thermoseparation step is performed. For this reason it is important to investigate how the addition of protein to an EOPO phase will affect the recovery of EOPO polymer in the thermoseparation step. Water–EOPO copolymer systems containing 10 mg BSA/ml, were prepared (see Table 5). The thermoseparation studies were performed for 5 min at a temperature 15°C above the cloud point of the copolymers. No strong effect on the recovery of EO20PO80 and EO30PO70 was observed (Table 5). This is probably due to the fact that the recovery of these polymers is already high, 90 and 95%, respectively. For the EO50PO50 copolymer the recovery was increased from 69% to 89% when adding protein. In the thermoseparating water–EOPO systems native proteins are generally partitioned exclusively to the water phase. Thus, in this type of system the partitioning is much more extreme than the partitioning observed in a two-polymer system or in a PEG–salt system. The copolymer phase contains typically 40–70% (w/w) copolymer after thermoseparation. It has previously been shown that when using this type of EOPO copolymers no protein will be detected in the copolymer phase after thermoseparation [10,11]. This is due to the entropy effect which makes it very unfavourable for a protein to partition to the concentrated copolymer phase [18]. Thus, the reason for the increased recovery of EO50PO50 can be explained by that BSA is partitioned 100% to the water phase and that BSA has a net negative charge of −18 at pH 7.0 [22]. The protein plus counter ions function as a salt and increase the hydrophilicity of the water phase. This leads to decreased cloud point for the copolymer and thus increased recovery of copolymer at the thermoseparation.

Table 5
The effect on the recovery of copolymer after addition of 10 mg/ml of BSA to the copolymer solution[a]

	% Recovered copolymer		
	EO20PO80	EO30PO70	EO50PO50
No protein	90.0±3	95.9±3	69.2±3
10 mg/ml BSA	85.6±3	95.6±3	88.8±3

[a] The thermoseparation was performed at 15°C above the cloud point for 5 min. The copolymer solutions were 10% (w/w) in water.

3.8. Recycling of thermoseparating polymer

The aim of using thermoseparating copolymers is to recover the target protein in a solution free from copolymer and recycle the copolymer. Thus, it is important to investigate the robustness of the two-phase system with recycled copolymer. The primary phase system studied was EO50PO50–hydroxypropyl starch (Reppal PES100) and the recycling of EOPO copolymer was performed as shown in Fig. 1. The concentration (w/w) of copolymer in the copolymer phase after thermoseparation was determined by refractive index measurements. By measuring the mass of the copolymer phase the recovery of copolymer could be calculated after each thermoseparation step. New fresh bottom phase polymer and some additions of copolymers, typically 15–25%, had to be done to the recycled copolymer phase to make up a new system. When recycling copolymer it was possible to retain the concentrations in the following systems at approximately the initial concentrations during three recyclings of EOPO copolymer, which can be seen in Table 6.

Table 6
The fluctuations in polymer concentration in the phases during recycling of copolymer[a]

	Concentration (%)	
	EO50PO50	Reppal PES100
First system		
Top phase	10.8	3.7
Bottom phase	1.1	22.2
First recycling		
Top phase	10.5	3.7
Bottom phase	1.0	22.7
Second recycling		
Top phase	10.7	3.6
Bottom phase	0.8	22.5
Third recycling		
Top phase	11.0	3.4
Bottom phase	0.8	22.2

[a] The concentrations shown are of primary phase systems before thermoseparation. Recycled copolymer after thermoseparation was used to prepare the primary phase systems except for the first system. The primary systems were mixed to obtain the polymer composition 13% (w/w) Reppal PES 100, 6% (w/w) EO50PO50. The primary phase systems were prepared and separated at 21°C and the thermoseparation was performed at 65°C for 5 min.

Recycling experiments with systems containing an artificial protein mixture of BSA and lysozyme were studied to investigate how much the fluctuation in polymer concentration in the primary system affected the partitioning of protein. Table 7 shows how the partition coefficients of total protein and lysozyme were affected during four recyclings of EOPO copolymer. The partition coefficients were relatively constant during the recyclings of EOPO copolymer. Only four recycling steps were studied but there is no reason to believe that drastic changes should be observed during further recyclings of EOPO copolymer. This shows that it is possible to recycle thermoseparating EOPO polymers without affecting the partitioning of proteins in the primary phase system. Thus, the recycling of copolymer facilitates the design of cost efficient and robust aqueous two-phase systems for large scale protein purification.

The partitioning of protein in aqueous two-phase systems is affected by salt addition [1,23]. The recycling experiments in systems containing protein (lysozyme and BSA) were performed in systems with different salt compositions (Table 7). Chaotropic ions will partition to the more hydrophobic copolymer phase and direct oppositely charged proteins to this phase. At pH 7.0 lysozyme is positively charged (+7) [24] and thus the most chaotropic of the investigated anions, ClO_4^-, will direct lysozyme to the copolymer phase [25,26]. Chloride has a position between perchlorate and phosphate in the Hofmeister series [27] and thus chloride has a much weaker effect on the partitioning than perchlorate.

Polymer recycling can be operated successfully even if different salts are added to the system (Table 7, see Table 4 for the effect of addition of different salts or polymers to EO50PO50 solution on recovery). The salts partition stronger to the water phase at thermoseparation [21]. The volume of the copolymer phase is small, and there will be a low amount of salt recycled in each step, which thus does not influence the protein partition coefficient.

Lysozyme was partitioned to the EOPO phase by addition of $NaClO_4$ at the same time as the total protein was partitioned to the bottom phase (Table 7). In the artificial protein mixture lysozyme can thus

Table 7
Copolymer recovery and partition coefficients (K) for total protein and lysozyme during copolymer recycling[a]

	Salt	Partition coefficients		Total copolymer recovery (%)
		Lysozyme	Total protein	
First extraction	–	0.43	0.41	–
	NaCl	0.80	0.68	–
	$NaClO_4$	5.86	0.73	–
First recycling	–	0.34	0.34	74
	NaCl	0.82	0.68	75
	$NaClO_4$	5.97	0.68	76
Second recycling	–	0.39	0.32	78
	NaCl	0.90	0.63	80
	$NaClO_4$	6.30	0.85	77
Third recycling	–	0.49	0.34	77
	NaCl	0.81	0.67	77
	$NaClO_4$	6.69	0.92	79
Fourth recycling	–	0.42	0.34	75
	NaCl	0.93	0.65	78
	$NaClO_4$	6.10	0.90	82

[a] The primary phase system was 13% Reppal PES100, 6% EO50PO50 system, 10 mM sodium phosphate, pH 7.0 and 100 mM salt. The protein concentration in the system was 5 mg/ml of BSA and 1 mg/ml of lysozyme. Thermoseparation was performed at 65°C for 5 min. Total copolymer recovery after thermoseparation was calculated for each recycling from the mass of copolymer in the primary phase system.

be seen as the target protein. By recycling of EOPO copolymer it was possible to perform five repeated extractions of lysozyme while retaining similar partition coefficients.

The total recovery of copolymer calculated from the primary system was approximately 77% in each recycling step with EO50PO50 and Reppal PES100 as phase forming polymers (Table 7). Most of the copolymer (≈13%) was lost in the primary phase system due to partitioning to the bottom phase. Additionally about 10% was lost in the thermoseparation step, i.e., equivalent to a recovery of EO50PO50 at 90% in this step. A total EOPO recovery of 77% in each cycle is equivalent to a 4.3 time reuse of copolymer in the two-phase separations. The recovery of copolymer can be increased by using a higher molecular mass for the bottom phase polymer. Also an increased molecular mass of the EOPO copolymer will increase the recovery of copolymer both in the primary phase system and in the thermoseparation step. The recovery of EO30PO70 (M_r 5400) was 96% in the thermoseparation step (Table 2) thus reducing the losses in this step to 4% in each recycling.

4. Conclusions

In aqueous two-phase extractions using EOPO–starch system it is possible to recycle the EOPO copolymer by thermoseparation. High-molecular-mass copolymers separate more efficiently than polymers of low molecular mass from the water phase in the thermoseparation step. A recovery of 96% could be obtained with EO30PO70 of M_r 5400. In the primary aqueous two-phase system the two polymers will separate more completely from each other if polymers of high molecular mass are used. Starch derivatives of high molecular mass reduce loss of copolymer to the bottom phase and increase overall recovery of EOPO copolymer in the thermoseparation step. In a protein extraction process the

top copolymer phase in the primary two-phase system will contain protein, salt and a low concentration of bottom phase polymer. These additions to the EOPO copolymer phase leads to increase in recovery of copolymer after thermoseparation. By optimising the thermoseparation step more than 90% of the copolymer can be recovered and recycled to the primary phase system. Repeated phase separation can be performed with recycled copolymer and the polymer concentrations can be maintained relatively constant in the new systems using recycled copolymer. The small fluctuations in polymer concentration in the primary top and bottom phases during copolymer recycling does not change the partitioning of protein. The EO50PO50 copolymer can be recycled to 77% in each cycle which is equivalent to a 4.3 times reuse of copolymer. The polymer costs for extractions with aqueous two-phase systems can in this way be lowered.

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References

[1] H. Walter, G. Johansson, Methods Enzymol. 228 (1994) 451–469.
[2] H. Hustedt, K.H. Kroner, M.-R. Kula, in: H. Walter, D.E. Brooks, D. Fischer (Eds.), Partitioning in Aqueous Two-Phase Systems – Theory, Methods, Uses and Applications To Biotechnology, Academic Press, New York, 1985, pp. 529–587.
[3] M.-R. Kula, K.H. Kroner, H. Hustedt, Adv. Biochem. Eng. 24 (1982) 73–118.
[4] M.-R. Kula, Bioseparation 1 (1990) 181–189.
[5] K.H. Kroner, H. Hustedt, M.-R. Kula, Process. Biochem. 19 (1984) 170–179.
[6] A. Veide, A.-L. Smeds, S.-O. Enfors, Biotechnol. Bioeng. 25 (1983) 1789–1800.
[7] A. Veide, T. Lindbäck, S.-O. Enfors, Enzyme Microb. Technol. 6 (1984) 325–330.
[8] M.J. Bolander, P.G.M. Hesselink, N. Papamichael, H. Hustedt, J. Biotechnol. 19 (1991) 19–34.
[9] K.H. Kroner, H. Hustedt, M.-R. Kula, Process. Biochem. 19 (1984) 170–179.
[10] P.A. Harris, G. Karlström, F. Tjerneld, Bioseparation 2 (1991) 237–246.
[11] J. Persson, L. Nyström, H. Ageland, F. Tjerneld, J. Chromatogr. B 711 (1998) 97–109.
[12] J. Persson, L. Nyström, H. Ageland, F. Tjerneld, Biotechnol. Bioeng. 65 (1999) 371–381.
[13] S. Saeki, N. Kuwahara, M. Nakata, M. Kaneko, Polymer 17 (1976) 685–689.
[14] R.E.J. Goldstein, J. Chem. Phys. 80 (1984) 5340–5341.
[15] G. Karlström, J. Phys. Chem. 89 (1985) 4962–4964.
[16] H.-O. Johansson, G. Karlström, F. Tjerneld, Macromolecules 26 (1993) 4478.
[17] P.A. Alred, A. Kozlowski, J.M. Harris, F. Tjerneld, J. Chromatogr. 659 (1994) 289–298.
[18] H.-O. Johansson, G. Karlström, F. Tjerneld, C.A. Haynes, J. Chromatogr. B 711 (1998) 3–17.
[19] L.A. Decker, Worthington Enzyme Manual, Worthington Biochemical Corp., Freehold, NJ, 1977, pp. 185–188.
[20] J. Persson, H.-O. Johansson, I. Yu. Galaev, B. Mattiassson, F. Tjerneld, Bioseparation (1999) in press.
[21] H.-O. Johansson, G. Karlström, B. Mattiasson, F. Tjerneld, Bioseparation 5 (1995) 269–279.
[22] T. Peters, Adv. Prot. Chem. 37 (1985) 161–245.
[23] G. Johansson, Acta Chem. Scand. B 28 (1974) 873–882.
[24] C. Tanford, M.L. Wagner, J. Am. Chem. Soc. 76 (1954) 3331–3336.
[25] K. Berggren, H.-O. Johansson, F. Tjerneld, J. Chromatogr. A 718 (1995) 67–79.
[26] H.-O. Johansson, G. Lundh, G. Karlström, F. Tjernels, Biochim. Biophys. Acta 1290 (1996) 289–298.
[27] R.K. Scopes, in: C.R. Canton (Ed.), Protein Purification – Principles and Practice, 2nd ed., Springer-Verlag, New York, 1988.