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Protein partitioning in weakly charged polymer–surfactant aqueous two-phase systems

Ulf Sivars[a], Karin Bergfeldt[b], Lennart Piculell[b], Folke Tjerneld[a,*]

[a]*Department of Biochemistry, Lund University, Chemical Center, P.O. Box 124, S-221 00 Lund, Sweden*
[b]*Department of Physical Chemistry 1, Lund University, Chemical Center, P.O. Box 124, S-221 00 Lund, Sweden*

Abstract

The study includes partitioning of proteins in aqueous two-phase systems consisting of the polymer dextran and the non-ionic surfactant $C_{12}E_5$ (pentaethylene glycol mono-*n*-dodecyl ether). In this system a micelle-enriched phase is in equilibrium with a polymer-enriched phase. Charges can be introduced into the micelles by the addition of charged surfactants. The charge of the mixed micelles is easily varied in sign and magnitude independently of pH, by the addition of different amounts of negatively charged surfactant, sodium dodecyl sulphate (SDS), or positively charged surfactant dodecyl trimethyl ammonium chloride (DoTAC). A series of water-soluble model proteins (BSA, β-lactoglobulin, myoglobin, cytochrome *c* and lysozyme), with different net charges at pH 7.1, have been partitioned in non-charged systems and in systems with charged mixed micelles or charged polymer (dextran sulphate). It is shown that partition coefficients for charged proteins in dextran–$C_{12}E_5$ systems can be strongly affected by addition of charged surfactants (SDS, DoTAC) or polymer (dextran sulphate) and that the effects are directly correlated to protein net charge.

Keywords: Aqueous two-phase systems; Partitioning; Proteins; Surfactants; Dextrans

1. Introduction

Aqueous two-phase systems were introduced as a separation method for biomolecules in the mid 1950's by Albertsson [1,2]. In general, the two-phase systems are formed in solutions of two incompatible polymers. The most commonly used systems are based on poly(ethylene glycol) (PEG) and dextran (Dx). The systems separate into two water-rich phases (80–95%, w/w), each enriched in one of the polymers. For recent reviews on aqueous two-phase partitioning, see references [3–5]. Phase separation has also been obtained in water solutions of PEG and salts [4,6]. In some applications, the polymers have been modified with covalently bound groups, for example hydrophobic ligands such as benzoyl or valeryl groups attached to dextran [7], biospecific ligands for affinity partitioning such as NADH, fatty acids and textile dyes, and also charged groups [8–12]. Polyelectrolytes have strong effects on partitioning of biological materials. One example is the partitioning of CO–haemoglobin in charged PEG systems consisting of trimethylamino PEG and Dx [10]. The distribution was strongly affected by the charged polyelectrolyte when the pH was changed. The partition coefficient for CO–haemoglobin could be shifted from 0.01 to 10 between pH 6 and 8 (isoelectric point 7).

Separation of membrane components has been done in aqueous two-phase systems in which de-

*Corresponding author.

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tergents have been included. Chlorophyll–protein complexes have been separated from thylakoid membranes by including Triton X-100 in the aqueous two-phase system, PEG–Dx [13]. Integral membrane proteins have been isolated by the use of thermal phase separation in non-ionic surfactant systems (cloud-point extraction technique) [14]. The cloud-point extraction technique in Triton X-114 systems has been applied both for identification [15] and isolation of membrane proteins [5,16]. Recently, membrane-bound cholesterol oxidase has been directly solubilized and extracted from *Nocardia rhodochorus* cells in aqueous two-phase systems containing the non-ionic polyoxyethylene surfactant $C_{12}E_5$ (pentaethylene glycol mono-*n*-dodecyl ether) [17]. The partition of hydrophilic proteins has been studied in non-ionic micellar solutions, both experimentally and theoretically [18].

The phase behaviour of aqueous two-phase system consisting of polymer and non-ionic surfactant has recently been examined [19–22]. The surfactant forms micelles and the phase behaviour of polymer–micelle systems resembles systems of two incompatible polymers, e.g., PEG–Dx. It has been proposed that the surfactant phase can be viewed as a second polymer phase [20]. Charges can be introduced in the system by the use of a charged polymer or by incorporating an ionic surfactant into the micelles. When non-titrating phase components are used in the polymer–micelle systems it is possible to construct a large number of aqueous two-phase systems, in which pH and charge density can be varied independently.

The phase diagrams of weakly charged polymer–surfactant aqueous two-phase systems have recently been studied [22]. The systems studied were mixtures of $C_{12}E_5$ and Dx. Charges were introduced into the system by using the polyelectrolyte sodium dextran sulphate (DxS) and/or by addition of the anionic surfactant sodium dodecyl sulphate (SDS) forming mixed micelles with $C_{12}E_5$. It was shown that these mixtures tend to demix segregatively into two coexisting phases, both with a water content of up to 78–92% (w/w). By increasing the charge density on either the micelles or on the polymer, the system became more miscible. When charged mixed micelles and polyelectrolyte were mixed in different proportions the system reached a demixing maximum at a certain composition, which segregated in a similar way to the uncharged system.

This study examines protein partitioning in polymer–surfactant aqueous two-phase mixtures, both in uncharged and weakly charged systems. The study includes a series of five well-characterized water-soluble model proteins with different net charges at a specific pH. We have investigated how the partitioning is affected by changing the composition of the polymer–surfactant systems, by the use of different mixtures of charged mixed micelles and/or polyelectrolytes. In addition to mixed micelles with SDS, the study also includes systems containing mixed micelles of $C_{12}E_5$ and a positively charged surfactant, dodecyl trimethyl ammonium chloride (DoTAC). We have also investigated the partitioning at the proteins' isoelectric points and the effects on protein partitioning of adding inorganic salt 0.1 *M* NaCl to the systems. The yield of the proteins and the activity of lysozyme in the different systems was studied.

2. Experimental

2.1. Chemicals

Dextran T500 ($M_r \approx 500\,000$), was purchased from Pharmacia (Uppsala, Sweden). Sodium dextransulphate (DxS), with a degree of sulphate substitution (DS) of 0.025 (mole sulphate per mole monomer sugar), was obtained from TdB Consultancy (Uppsala, Sweden). The precursor was originally from Pharmacia (Uppsala, Sweden) ($M_r \approx 70\,000$). The polymers were purified by ultrafiltration and freeze-dried. $C_{12}E_5$ was obtained from Nikko Chemicals (Tokyo, Japan) and had a cloud-point of about 30°C and a critical micellar concentration (CMC) of 0.065 m*M* (0.003% (w/w). The surfactant was used without further purification. SDS was purchased from Merck (Darmstadt, Germany). DoTAC, was obtained from Tokyo Kasei (Tokyo, Japan). All other chemicals were of analytical grade. Bicinchoninic acid (BCA) reagents A and B for protein determination were obtained from Pierce (Rockford, IL, USA). Dried *Micrococcus lysodeik-*

ticus cells were purchased from Sigma (St. Louis, MO, USA). All water used was of Millipore quality.

2.2. Proteins

The following proteins were obtained from Sigma: bovine serum albumin (essentially fatty acid free); β-lactoglobulin A from bovine milk; myoglobin from horse heart and cytochrome *c* from horse heart. Lysozyme (EC 3.2.1.17) from hen egg white was obtained from Boehringer (Mannheim, Germany). The properties of the proteins are shown in Table 1.

2.3. Two-phase systems for partitioning of proteins

All phase diagrams were obtained from Bergfeldt and Piculell [22], except for the system containing DoTAC. The phase diagrams in Ref. [22] were all made with Dx T70 at 10°C with no addition of salt. All experiments in this study were performed with Dx T500 at 3–4°C with addition of a 5 m*M* buffer giving the correct pH in all systems. The DxS used was the same as in [22]. The phase boundary was investigated in the different polymer–surfactant systems to see the effect of these changes (for the method see Section 2.5). A polymer with higher molecular mass (Dx T500 compared with Dx T70), slightly decreased the miscibility in the uncharged Dx–$C_{12}E_5$ system. The binodial curve varies between different systems and also in the same system with salt type and concentration. The salt effect was strongest for systems with charged micelles–uncharged polymer or vice versa. The uncharged system (Dx–$C_{12}E_5$) and systems where both phase components were negatively charged [DxS–($C_{12}E_5$ + 1.5% SDS)] were almost unaffected by changes in the salt composition. This phase behaviour resembles the salt dependence that has been found in two-phase systems containing polyelectrolytes [4,23]. To get systems with comparable compositions, in order to compare partition coefficients, we kept the concentration difference of the phase components between the two coexisting phases approximately constant in the different systems. In practice, this was performed by mixing systems at approximately the same distance from the binodial (Chapter 3 in [5]). The partition coefficient should only be marginally affected by the small variation in the concentration difference between the different systems (see Table 2). The volume ratios between the top and bottom phases were between 0.25–0.58 in the systems.

Table 1
Protein properties

Protein	Molecular mass (Da)	p*I*	Net charge at pH 7.1
BSA	69 000 [30]	4.8 [32]	−18 [32]
β-Lactoglobulin	35 000 [30]	5.1 [30]	−5 [34]
Myoglobin	17 500 [30]	7.1 [30]	0 [30]
Cytochrome *c*	13 000 [31]	9.4 [30]	+6 [35]
Lysozyme	13 900 [31]	11.0 [33]	+7 [36]

Aqueous two-phase systems (2 g) were prepared from stock solutions of the polymers and surfactant in water (20%, w/w). Polymer and surfactant solutions were mixed with water, buffer, proteins (0.5 mg/ml or 0.05%, w/w) and in some cases salt (0.1 *M* NaCl) at room temperature. The total and phase compositions for the different systems are shown in Table 2. All concentrations used were calculated as weight percentages. The concentration of DoTAC was chosen so that it had the same mole fraction of ionic to non-ionic surfactant as in the corresponding Dx–($C_{12}E_5$ + SDS) system. The systems were left standing at 3–4°C in a cold room for at least 15 min, to reach the correct temperature, and then they were mixed again and equilibrated at the same temperature for at least 1 h. If the phases were still cloudy, the systems were centrifuged to clear the phases, at 1800 *g* for 3–4 min at 3–4°C. The top phase was the surfactant-rich phase and the bottom phase the Dx-rich phase. Note that both phases contain the non-ionic surfactant, $C_{12}E_5$ (see Table 2) and that the concentrations in both phases are above the CMC of $C_{12}E_5$.

2.4. Protein partition coefficient, yield and lysozyme activity determination

The top and bottom phases were isolated using a syringe and diluted five to twenty times. The phases were analyzed for their protein content using the BCA-method [24]. The samples were incubated at 60°C for 30 min in a thermostated water bath. The samples were then cooled to room temperature, by

Table 2
Total and phase compositions of studied polymer–surfactant aqueous two-phase systems

System	Buffer and salts[a]	Total system			Top phase			Bottom phase		
		Dx–DxS (%)	$C_{12}E_5$ (%)	H_2O (%)	Dx–DxS (%)	$C_{12}E_5$ (%)	H_2O (%)	Dx–DxS (%)	$C_{12}E_5$ (%)	H_2O (%)
Dx–$C_{12}E_5$	PB, AB, NaCl	3.8	4.9	91.3	0.4	15.7	83.9	5.5	1.7	92.8
DxS–$C_{12}E_5$	PB, AB	9.4	5.2	85.4	6.7	11.5	81.8	12.2	≈0	87.8
	NaCl	5.2	5.5	89.3						
DxS–($C_{12}E_5$ + 1.5% SDS)	PB, AB, NaCl	4.3	6.0	89.7	3.1	11.9	85.0	8.2	≈0	91.8
Dx–($C_{12}E_5$ + 0.5% SDS)	PB, AB, NaCl	4.3	4.2	91.5	0.3	15.2	84.4	6.1	1.5	92.4
Dx–($C_{12}E_5$ + 1.5% SDS)	PB	5.6	5.2	89.2	1.1	15.1	83.9	7.8	1.4	90.8
	AB	11.3	7.5	81.2						
	NaCl	3.9	3.6	92.5						
Dx–($C_{12}E_5$ + 0.45% DoTAC)	PB	4.3	4.2	91.5	0.4	15.5	84.1	6.1	1.4	92.5
Dx–($C_{12}E_5$ + 1.37% DoTAC)	PB	6.0	5.8	88.2	1.7	15.0	83.3	7.4	3.3	89.3

All values are given in weight percent, except the ionic surfactants which are in weight percent of non-ionic surfactant.
[a]Phase compositions were all determined in systems containing PB. Systems were mixed at 3–4°C. (PB = 5 m*M* sodium phosphate buffer, pH 7.1; AB = 5 m*M* sodium acetate buffer, pH 4.8 or 5.1; NaCl = addition of 0.1 *M* NaCl to systems containing PB).

leaving them at room temperature for 15–20 min, and their absorbances were measured at 562 nm against a reagent blank, which contained sample from a phase system without any protein added. The concentrations were determined from calibration curves for the respective proteins.

The partitioning of a substance is described by the partition coefficient K, which is defined as $K = C_T/C_B$ where C_T and C_B are the equilibrium concentration of the partitioned substance in the upper and lower phases, respectively [4]. The protein yields were determined by calculating the total protein amount added to the system and the amounts in the different phases. The total and the phase volumes were determined by the use of volume-calibrated test tubes. The enzyme activity of lysozyme was determined by the rate of lysis of *Micrococcus lysodeikticus* cells [25]. All results are average values after partition of protein in two equal systems with two repeated measurements of protein content and lysozyme activity in each of them.

2.5. Phase boundary and composition determination

Phase boundaries were obtained by the cloud-point method [4], in a cold room at 3–4°C. Samples were prepared in the two-phase region. Water, or solvent, with the appropriate salt concentration was added until the one phase region was reached. A sample was regarded as monophasic when it was completely clear.

The compositions of the phases in the mixtures were determined by the following procedure. The systems (5 g) were mixed and left to separate overnight at 3–4°C. The compositions are shown in Table 2. The phases were collected separately using a syringe and diluted as appropriate for the concentration determination. The Dx and DxS concentrations were determined by polarimetry (optical rotation) against calibration curves. The polarimetric measurements were performed at room temperature using a digital polarimeter (Model AA-10) from Optical Activity (London, UK). The concentration of surfactants in the different phases was determined by refractive index measurements, by the use of calibration curves determined for Dx, DxS and $C_{12}E_5$ with/without SDS or DoTAC and were performed in a differential refractometer from Carl Zeiss (Germany) connected to a Julabo C water circulator (at 20°C) from Juchheim Labortechnik (Seelbach, Germany). The ratio of ionic–non-ionic surfactant was assumed to be equal in the two coexisting phases. The buffers were assumed to be distributed evenly

between the two phases. In the concentration range used, the contributions to the refractive index were additive. After subtracting the polymer and buffer contributions to the refractive index, the surfactant concentration was determined.

3. Results

3.1. Partitioning of proteins in a polymer–surfactant aqueous two-phase system

The study includes partitioning of five water-soluble model proteins, BSA, β-lactoglobulin, myoglobin, cytochrome *c* and lysozyme. The proteins in the experiments have different net charges, from −18, for BSA, to +7, for lysozyme, at pH 7.1 (Table 1). This pH is the p*I* of myoglobin. The proteins were partitioned in an aqueous two-phase system composed of Dx and $C_{12}E_5$.

The partition coefficients and yields for the different proteins are shown in Table 3, and in Fig. 1, the partition coefficient is shown as a function of the net charge of the different proteins. A decrease in *K* value is observed when the proteins are arranged in order of their increasing net charge at pH 7.1, i.e. the proteins partitioned in increasing degree to the Dx phase. The *K* values are from 0.7 (BSA and β-lactoglobulin) to 0.3 (cytochrome *c* and lysozyme), with the uncharged myoglobin at 0.49.

3.2. Partitioning of proteins in weakly charged polymer–surfactant mixtures

The same model proteins were partitioned in Dx–surfactant mixtures, where negative charges were introduced either by the use of the polyelectrolyte DxS with a DS of 0.025, or by the use of mixed micelles formed by $C_{12}E_5$ plus SDS of either 0.5 or 1.5% (w/w of non-ionic surfactant). Also, mixtures of DxS and $C_{12}E_5$ + 1.5% SDS were examined. In this mixture at 1.5% SDS, maximum segregation occurred and it was proposed to contain approximately equal concentrations of counter-ions in the two-phases [22].

The partition coefficients and yields for the different systems are shown in Table 3, and in Fig. 1 the partition coefficient is shown as a function of the net charge of the different proteins. Proteins with a negative net charge (BSA and β-lactoglobulin) were partitioned to the surfactant phase when a negatively charged polymer was used. The negative proteins were partitioned to the Dx phase, when negative charges were incorporated into the micelles. The partitioning was more extreme when more negative charges were incorporated into the mixed micelles, i.e. when the SDS concentration was increased from 0.5 to 1.5% (w/w of non-ionic surfactant). The partitioning was also more one-sided for the most negatively charged protein (BSA), compared to the less charged β-lactoglobulin. Myoglobin is un-

Table 3
Partition coefficients of five water-soluble model proteins, BSA, β-lactoglobulin, myoglobin, cytochrome *c* and lysozyme, in polymer–surfactant aqueous two-phase systems of Dx and the non-ionic surfactant, $C_{12}E_5$, and in weakly charged polymer–surfactant mixtures, where charges were included either by sodium dextran sulphate and/or by mixed micelles of $C_{12}E_5$ plus SDS

Protein type	Net charge	*K*				
		Dx–$C_{12}E_5$	DxS–$C_{12}E_5$	DxS–($C_{12}E_5$ +1.5% SDS)	Dx–($C_{12}E_5$ +0.5% SDS)	Dx–($C_{12}E_5$ +1.5% SDS)
BSA	−18	0.69	7.27	0.85	0.45	0.11
β-Lactoglobulin	−5	0.74	1.48	0.65	0.63	0.31[a]
Myoglobin	0	0.49	0.44	0.50	0.55	0.64
Cytochrome *c*	+6	0.33[a]	Precipitated	Precipitated	0.74	1.92
Lysozyme	+7	0.31	Precipitated	Precipitated	0.70	2.74

Protein yields were 90–100%; total and phase compositions are shown in Table 2; all systems contained 5 m*M* sodium phosphate buffer, at pH 7.1; temperature 3–4°C; $K > 1$ is equivalent to a partitioning to the surfactant-rich phase.
[a] Protein yield is 80%.

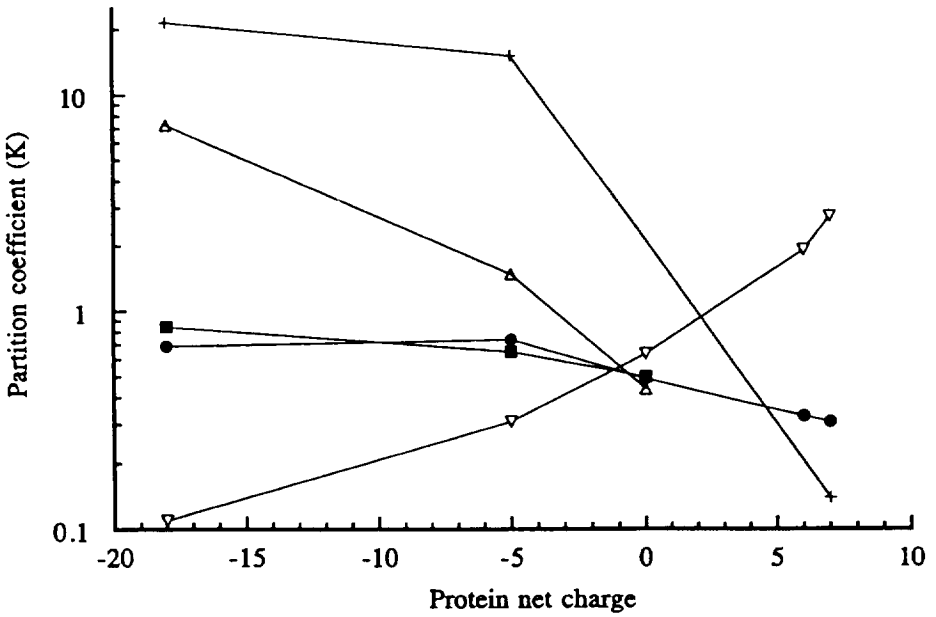

Fig. 1. Partition coefficients for five hydrophilic proteins, bovine serum albumin (net charge −18), β-lactoglobulin (−5), myoglobin (0), cytochrome *c* (+6) and lysozyme (+7), shown as function of protein net charge in weakly charged polymer–surfactant aqueous two-phase systems. The systems are: dextran–$C_{12}E_5$ (●), dextran sulphate–$C_{12}E_5$ (△), dextran sulphate–($C_{12}E_5$ + 1.5% SDS) (■), dextran–($C_{12}E_5$ + 1.5% SDS) (▽), and dextran–($C_{12}E_5$ + 1.37% DoTAC) (+). Results are from Table 3 and Table 4. Total and phase compositions are given in Table 2. All systems contained 5 m*M* sodium phosphate buffer, pH 7.1; temperature, 3–4°C; $K > 1$ is equivalent to a partitioning to the surfactant-rich phase.

charged at this pH and had an almost constant K value in the different systems. The positively charged proteins (cytochrome *c* and lysozyme) partitioned in the opposite manner, compared with the negatively charged proteins. Cytochrome *c* and lysozyme were partitioned to the surfactant phase when a negative surfactant was incorporated into the micelles. However, the positively charged proteins were precipitated in systems which contained DxS. The precipitation was caused by the polyelectrolyte and not by the mixed micelles containing the strong protein denaturing agent, SDS. This was concluded after experiments where cytochrome *c* and lysozyme, respectively, were mixed in two different solutions, one containing DxS and the other mixed micelles of $C_{12}E_5$ plus SDS. The positively charged proteins were precipitated in the DxS solution but not in the mixed micelle solution. In these experiments the concentrations of protein, salt, polymer and surfactants were the same as in the corresponding two-phase systems.

3.3. Partitioning of proteins in weakly positively charged systems

As a comparison to the weakly negatively charged polymer–surfactant system (Section 3.2), three model proteins (BSA, β-lactoglobulin and lysozyme) were partitioned in polymer–mixed micelle systems, where positive charges were incorporated into the micelles by the addition of DoTAC. The use of DoTAC slightly increased the miscibility of the systems, compared to SDS-containing mixed micelle systems, Dx–($C_{12}E_5$ + SDS). Both DoTAC and SDS remain in the mixed micelles and do not bind to oppositely charged proteins, since no protein precipitation was observed and lysozyme retained its activity (see Section 3.5).

Our results with positive surfactant added to the micelles (Table 4) show that the proteins partitioned in the opposite manner than systems with negative charges in the mixed micelle phase (Fig. 1). The two negatively charged proteins, BSA and β-lactoglobu-

Table 4
Partition coefficients of three hydrophilic model proteins, BSA, β-lactoglobulin and lysozyme in dextran–positively charged mixed micelle aqueous two-phase systems, where positive charges have been introduced by addition of DoTAC to $C_{12}E_5$ micelles

Protein type	Net-charge	*K*		
		Dx–$C_{12}E_5$	Dx–($C_{12}E_5$ + 0.45% DoTAC)	Dx–($C_{12}E_5$ + 1.37% DoTAC)
BSA	−18	0.69	1.94[a]	21.6[a]
β-Lactoglobulin	−5	0.74	1.49	15.2
Lysozyme	+7	0.31	0.28	0.14

Protein yields were 90–100%; system compositions are shown in Table 2; all systems contained 5 m*M* sodium phosphate buffer, pH 7.1.; temperature 3–4°C; $K > 1$ is equivalent to a partitioning to the surfactant-rich phase.
[a] Protein yield of 80%.

lin, were partitioned to the positively charged surfactant phase. The partitioning to the surfactant phase was more one-sided for BSA than for β-lactoglobulin, i.e. the *K* value increased with increasing net negative charge of the proteins. The positively charged protein, lysozyme, was enriched in the Dx phase. Partitioning was more extreme in the system that contained a higher amount of DoTAC [1.37% (w/w) of non-ionic surfactant] compared to a system with less DoTAC [0.45% (w/w) of non-ionic surfactant] (Table 4).

3.4. Partitioning of proteins in systems containing 0.1 M salt

The partitioning of BSA in the presence of 0.1 *M* NaCl was studied in the same phase system as in Section 3.1 and Section 3.2. To get comparable results, the phase compositions had to be modified in some cases, due to the decreased miscibility of the phase components caused by the addition of salt (see Table 2). The strongest salt dependence was found in systems with uncharged polymer–charged micelles and charged polymer–non-ionic surfactant.

In Table 5, the partition coefficients and yields are shown with and without the addition of salt. BSA was partitioned in almost the same way in all systems containing 0.1 *M* NaCl, with *K* values between 0.33 and 0.48 in contrast to the strong effect of charged phase components observed in the systems without salt addition, with *K* values between 0.11 and 7.27.

3.5. Lysozyme activity and protein yields in weakly charged polymer–surfactant aqueous two-phase systems

The enzyme activity of lysozyme in the different systems was measured. The results are shown as yields of enzyme activity (Table 6). Lysozyme retained its activity in studied systems, except systems which contained DxS where lysozyme was precipitated (see Section 3.2). This indicates that the surfactants do not have any denaturing effects on the model proteins. The protein yields were measured

Table 5
Partition coefficients of bovine serum albumin in weakly charged polymer–surfactant aqueous two-phase systems, without or with 0.1 *M* NaCl

System	*K* (no salt addition)	*K* (with 0.1 *M* NaCl)
Dx–$C_{12}E_5$	0.69	0.44
DxS–$C_{12}E_5$	7.27	0.43
DxS–($C_{12}E_5$ + 1.5% SDS)	0.85	0.48
Dx–($C_{12}E_5$ + 0.5% SDS)	0.45	0.44
Dx–($C_{12}E_5$ + 1.5% SDS)	0.11	0.33

Protein yields were 90–100%; system compositions are shown in Table 2. All systems contained 5 m*M* sodium phosphate buffer, pH 7.1, without or with 0.1 *M* NaCl; temperature 3–4°C; $K > 1$ is equivalent to a partitioning to the surfactant-rich phase.

Table 6
Lysozyme activity after partitioning in weakly charged polymer–surfactant aqueous two-phase systems

System	Lysozyme activity yield (%)
Dx–$C_{12}E_5$	98
DxS–$C_{12}E_5$	Precipitated
DxS–($C_{12}E_5$+1,5% SDS)	Precipitated
Dx–($C_{12}E_5$+0.5% SDS)	113
Dx–($C_{12}E_5$+1.5% SDS)	98
Dx–($C_{12}E_5$+0.45% DoTAC)	nd
Dx–($C_{12}E_5$+1.37% DoTAC)	103

The yield is the sum of activity in the top and bottom phases; system compositions are shown in Table 2; all systems contained 5 m*M* sodium phosphate buffer, pH 7.1; temperature 3–4°C; nd=not determined.

also in the partitioning experiments described in Tables 3–5 and in Table 7. The yields were high, 90–100%, except in the experiments with DxS and oppositely charged proteins, which resulted in precipitates.

3.6. Partition of proteins at the isoelectric point

BSA, β-lactoglobulin and myoglobin were partitioned at their isoelectric points, 4.8, 5.1 and 7.1, respectively. The polymer–surfactant aqueous-two phase systems were the same as in Section 3.1 and Section 3.2. Sodium acetate buffer (5 m*M*) was used instead of sodium phosphate buffer for the partitioning of BSA and β-lactoglobulin. The systems tended slightly towards increased miscibility of the phase components by this buffer change. The effect was particularly pronounced for the system with the highest amount of SDS, Dx–($C_{12}E_5$ + 1.5% SDS).

Partition coefficients and yields are shown in Table 7. In the studied systems [with the exception of Dx–($C_{12}E_5$ + 1.5% SDS)], BSA, β-lactoglobulin and myoglobin were partitioned to the Dx phase with about the same *K* values (0.37–0.63). The *K* values for these proteins at the isoelectric point were similar to the *K* values for BSA (0.44–0.48) in systems containing 0.1 *M* NaCl (Table 5). In the Dx–($C_{12}E_5$ + 1.5% SDS) system, both BSA and β-lactoglobulin, at their p*I*, were slightly enriched in the surfactant phase, with *K* values of 1.2–1.3. It should be noted that BSA was precipitated in both DxS-containing systems at pH 4.8. At pH 5.1, BSA was precipitated in DxS–$C_{12}E_5$ but not in the DxS–($C_{12}E_5$ + 1.5% SDS) system.

4. Discussion

An increased effect on partitioning has been shown in polyelectrolyte-containing aqueous two-phase systems compared to conventional polymer–polymer aqueous two-phase systems [4,10,12]. Also, in weakly charged polymer–surfactant mixtures, the partition is more extreme than in uncharged polymer–surfactant systems (Table 3 and Fig. 1). The partition of the hydrophilic model proteins depends on the charge of the phase-forming components and on the net charge of the proteins. An increasing charge, both of the proteins and of the mixed micelles, made the partitioning more extreme (Table

Table 7
Partition coefficients of BSA, β-lactoglobulin and myoglobin at their isoelectrical points, pH 4.8, 5.1 and 7.1, respectively

System	*K*		
	BSA	β-Lactoglobulin	Myoglobin
Dx–$C_{12}E_5$	0.58	0.48	0.49
DxS–$C_{12}E_5$	Precipitated[b]	nd	0.44
DxS–($C_{12}E_5$+1,5% SDS)	0.37[a]	nd	0.50
Dx–($C_{12}E_5$+0.5% SDS)	0.63	0.50	0.55
Dx–($C_{12}E_5$+1.5% SDS)	1.25	1.29	0.64

Protein yields were 90–100%; system compositions are shown in Table 2; buffers: 5 m*M* sodium acetate, pH 4.8 and 5.1; 5 m*M* sodium phosphate buffer, pH 7.1; temperature 3–4°C; $K > 1$ is equivalent to a partitioning to the surfactant-rich phase; nd=not determined.
[a] 5 m*M* sodium acetate buffer, pH 5.1.
[b] Precipitated both at pH 4.8 and 5.1.

3 and Fig. 1). The partition coefficient for BSA could be shifted from 0.1 to 20 by changing the charge of the mixed micelle, by exchanging SDS with DoTAC (Table 3 and Table 4, Fig. 1). BSA could also be transferred from one phase to the other by the use of a polyelectrolyte instead of similarly charged mixed micelles, i.e. the use of the DxS–$C_{12}E_5$ instead of the Dx–($C_{12}E_5$ + SDS) system (Fig. 1). Consequently, a protein can be extracted into either the polymer phase or the surfactant phase in the polymer–surfactant aqueous two-phase system by the use of a suitable polyelectrolyte or charged surfactant in the mixed micelles. The protein net charge is dependent on the pH. It should be noted that both the pH and the charge of the system can be selected independently, when non-titrating phase components are used. This makes it possible to obtain extreme partitioning by the use of an appropriate pH and a suitable polymer–surfactant aqueous two-phase system. In practice, the simplest way will be to regulate the charge in the surfactant phase, by the addition of non-titrating charged surfactants (e.g., SDS or DoTAC) and retain the polymer in an uncharged form.

The partitioning of water-soluble proteins in weakly charged polymer–surfactant aqueous two-phase systems shows the same dependency of net charge of the biomolecule as in polyelectrolyte systems [10,12]. In aqueous two-phase systems containing polyelectrolytes, the partition of charged biomolecules can be explained by an electrical interfacial potential theory [4]. The similarities in protein partition patterns between the latter systems and weakly charged polymer–surfactant aqueous two-phase systems makes it likely that partition in this system also can be understood by the creation of an electrical potential difference over the interface. This is further strengthened by the fact that BSA partitioned relatively similarly in the different weakly charged polymer–surfactant systems after the addition of 0.1 *M* NaCl (Table 5), which screens the electrical interfacial potential. Also, the partition experiments for myoglobin, β-lactoglobulin and BSA, at their isoelectric points (Table 7), are in agreement with this, i.e. approximately the same *K* values for these proteins were obtained as in the partitioning of BSA with the addition of 0.1 *M* NaCl.

In the uncharged system, Dx–$C_{12}E_5$, the proteins partitioned slightly more to the Dx phase as the net charge of the proteins increased (Fig. 1). In these experiments we have used a sodium phosphate buffer. Phosphate ions (HPO_4^{2-}–$H_2PO_4^-$) have been shown to partition into the Dx phase in conventional aqueous two-phase systems of PEG–Dx. The uneven salt distribution has been suggested to create a small electrical potential difference between the phases [4,26]. A similar distribution of phosphate ions in Dx–$C_{12}E_5$ systems can explain this increased protein partition to the Dx phase, with increasing net charge. Note that the systems resemble each other, as the head group of $C_{12}E_5$ is the same as the monomer of PEG.

In the partitioning experiments of myoglobin, at the p*I*, in the system containing the highest SDS concentration, Dx–($C_{12}E_5$ + 1.5% SDS), myoglobin partitioned mainly into the Dx phase (K=0.64, Table 7). However, both BSA and β-lactoglobulin were enriched in the surfactant phase in this system, both to the same extent (K=1.2–1.3). This unexpected partitioning of BSA and β-lactoglobulin to the surfactant phase may be related to the higher concentration of phase components in this system. Due to increased miscibility of the system when acetate buffer was used instead of phosphate buffer (the latter buffer was used in the partitioning of myoglobin at its p*I*), a more concentrated phase system was used (Table 2), but the concentration difference between the phases was kept in the same range as in the other systems.

Hydrophilic proteins do not seem to bind to non-ionic surfactant micelles [18]. Ionic surfactants interact much more strongly with proteins compared with non-ionic surfactants. Anionic surfactants, like SDS, have to be considered as strong protein denaturation agents. SDS is almost totally incorporated into non-ionic micelles, i.e. the concentration of free SDS molecules is insignificant [27,28]. This also seems to be true for mixed micelles of $C_{12}E_5$ with SDS or DoTAC in mixtures with oppositely charged proteins, since no protein precipitation was observed. It has been found that an addition of a non-ionic surfactant reduces the denaturing effect of an ionic surfactant, like SDS [29]. In our investigations, lysozyme retained its enzymatic activity when in

contact with mixed micelles of $C_{12}E_5$ with SDS or DoTAC (Table 6). This indicates that proteins are not denatured either in systems with non-ionic micelles or in the charged mixed micelle systems containing strong protein denaturing agents, like SDS or DoTAC. We obtained high protein yields, 90–100%, which shows that we do not have any protein precipitation in polymer–charged mixed micelle systems. However, we did get precipitation of proteins in systems which contained the oppositely charged polyelectrolyte, DxS. Polyelectrolytes usually interact more strongly with biological materials when compared with non-ionic polymers, due to strong electrostatic attraction between oppositely charged proteins and polyelectrolytes.

5. Conclusions

Polymer–surfactant mixtures can be used for separation of macromolecules. By using charged mixed micelles in the systems, effective separations based on protein charge were obtained. The proteins in this study were all hydrophilic and were not incorporated into the micelles. The partitioning in polymer–surfactant systems is thus analogous to partitioning in polymer–polymer aqueous two-phase systems. In the systems with charged mixed micelles, the sign of the charge and its magnitude can be changed independently of pH, by addition of different amounts of negatively or positively charged surfactants. The partitioning of proteins can, in this way, be changed from one phase to the other. Consequently, a hydrophilic protein can be partitioned into either phase by selecting an appropriate pH and a suitable polymer–charged mixed micelle system.

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References

[1] P.-Å. Albertsson, Nature, 177 (1956) 771–774.
[2] P.-Å. Albertsson, Biochim. Biophys. Acta, 27 (1958) 378–395.
[3] H. Walter, D.E. Brooks and D. Fisher (Editors), Partitioning in Aqueous-Two Phase Systems, Academic Press, London, 1985.
[4] P.-Å. Albertsson, Partition of Cell Particles and Macromolecules, Wiley, New York, 3rd ed., 1986.
[5] H. Walter and G. Johansson, Methods Enzymol., 228 (1994).
[6] M.R. Kula, Bioseparation, 1 (1990) 181–189.
[7] M. Lu, P.-Å. Albertsson, G. Johansson and F. Tjerneld, J. Chromatogr. A, 668 (1994) 215–228.
[8] H. Walter, G. Johansson and D.E. Brooks, Anal. Biochem., 197 (1991) 1–18.
[9] G. Johansson and V.S. Shanbhag, J. Chromatogr., 284 (1984) 63–72.
[10] G. Johansson, A. Hartman and P.-Å. Albertsson, Eur. J. Biochem., 33 (1973) 379–386.
[11] G. Johansson and M. Joelsson, J. Chromatogr., 393 (1987) 195–208.
[12] G. Johansson, Biochim. Biophys. Acta, 222 (1970) 381–389.
[13] P.-Å. Albertsson and B. Andersson, J. Chromatogr., 215 (1981) 131–141.
[14] C. Bordier, J. Biol. Chem., 256 (1981) 1604.
[15] J.G. Pryde, Trends Biochem. Sci., 11 (1986) 160.
[16] A. Sánchez-Ferrer, M. Pérez-Gilabert, E. Núnez, R. Bru and F. García-Carmona, J. Chromatogr. A, 668 (1994) 75–83.
[17] T. Minuth, J. Thömmes and M.-R. Kula, J. Biotechnol., 38 (1995) 151–164.
[18] Y.J. Nikas, C.-J. Liu, T. Srivastava, N.L. Abbott and D. Blankschtein, Macromolecules, 25 (1992) 4797–4806.
[19] C. Wormuth, Langmuir, 7 (1991) 1622.
[20] L. Piculell and B. Lindman, Adv. Colloid Interface Sci., 41 (1992) 149-178.
[21] S.M. Clegg, P.A. Williams, P. Warren and I.D. Robb, Langmuir, 10 (1994) 3390–3394.
[22] K. Bergfeldt and L. Piculell, submitted (1995).
[23] L. Piculell, S. Nilsson, L. Falck and F. Tjerneld, Polym. Commun., 32 (1991) 158–160.
[24] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson and D.C. Klenk, Anal. Biochem., 150 (1985) 76–89.
[25] L.A. Decker (Editor), Worthington Enzyme Manual, Worthington Biochemical Co., Freehold, NJ, USA, 1977.
[26] G. Johansson, Acta Chem. Scand., B 28 (1974) 873–882.
[27] M. Jonströmer and R. Strey, J. Phys. Chem., 96 (1992) 5993–6000.
[28] R. Schomäcker and R. Strey, J. Phys. Chem., 98 (1994) 3908–3912.
[29] K.P. Ananthapadmanabhan, in E.D. Goddard and K.P. Ananthapadmanabhan (Editors), Interaction of Surfactant with Polymers and Proteins, CRC Press: Boca Raton, FL, 1983, Ch. 8, p. 320.

[30] P.G. Righetti and T. Caravaggio, J. Chromatogr., 127 (1976) 1–28.
[31] G. Zubay, Biochemistry, Macmillan, New York, 2nd ed., 1988, p. 88.
[32] T. Peters, Adv. Prot. Chem., 37 (1985) 161–245.
[33] M. Wahlgren. Ph. D. Thesis, University of Lund, Lund, 1992.
[34] H.A. McKenzie, Milk Proteins: Chemistry and Molecular Biology, Academic Press, New York, 1971, p. 257.
[35] H. Theorell and Å. Åkesson, J. Am. Chem. Soc., 63 (1941) 1818–1820.
[36] C. Tanford and M.L. Wagner, J. Am. Chem. Soc., 76 (1954) 3331–3336.