Table 1
Effect of ionic strength, pH, and chaotrophic agent on the chromatographic profile of blue dextran, bovine serum albumin (BSA), and phenol red (Φ red) on Superose 6 columns

Test buffer	Expected effect	Elution volume (V_e)		
		Blue dextran	BSA	Φ Red
1. Tris 25 m*M*, pH 7.4	Low ionic strength buffer; reduced hydrophobic interaction between analyte and matrix.	6.7	14.7	29.1
2. Tris 25 m*M*, pH 7.4 Urea 100 m*M*	Low ionic strength with urea; decreased H-bond interaction (if any) of analyte with matrix.	6.8	14.7	28.4
3. Tris 25 m*M*, pH 8.3	Low ionic strength, high pH; to assess pH effects (cf. buffer 1).	6.7	13.6	22.8
4. Tris 25 m*M*, pH 8.3 NaCl 500 m*M*	High ionic strength, high pH; to assess effect of ionic effects on analyte–matrix interactions.	7.0	16.6	29.4
5. Tris 25 m*M*, pH 7.4 NaCl 500 m*M*	High ionic strength, lower pH than buffer 4; to assess effect of pH at higher ionic strength	6.8	16.5	37.8
TBE (Tris-borate 89 m*M*, EDTA 2 m*M*, pH 8.3)		6.6	13.8	22.4
PBS (NaCl 150 m*M*, Na Phosphate 10 m*M*, EDTA 1 m*M*, pH 7.4)		6.8	16.6	38.0

different lipid composition, or both. There are two apparent artefacts observed in PBS: excessive early elution of apoE and apoA-I (in the "LDL" fractions), and excessive late elution (in the "post-HDL" fractions). Although the late-eluting apoA-I and apoE peaks can probably be attributed to "tailing" due to non-ideal hydrophobic or ion-exchange interactions with the column matrix, the early elution artefact is more problematic. It may reflect ion-exclusion between the negatively-charged lipoproteins and the residual COOH and SO_4 groups on the matrix, or self association of apoA-I and apoE-containing lipoproteins in PBS, due to weak interactions which are eliminated by ultracentrifugation and by TBE buffer.

In summary, we have shown that non-ideal analyte–matrix interactions contribute significantly to the fractionation pattern of plasma or McA-RH7777 lipoproteins during size exclusion on Superose 6 columns in PBS running buffer. For the fractionation of plasma, the interactions actually enhance the chromatographic separation, improve the integrity of lipoprotein fractions, and thereby increase the correlation between size exclusion and ultracentrifugation fractions. For McA-RH7777 medium, the interactions introduce analytical discrepancies between size exclusion and ultracentrifugation, which can be avoided by using TBE running buffer. It is hoped that these observations will serve to increase the awareness of the limitations of the

size-exclusion technique as a surrogate for ultracentrifugation when analytes other than plasma are involved.

Acknowledgements

The authors thank Drs. Taira Ohnishi, Roger Davis, and Karl Weisgraber for providing antisera used in this study. Drs. Roger McLeod and Shinji Yokoyama made valuable comments and suggestions. This work was made possible by grants from the Medical Research Council of Canada, the Canadian Heart and Stroke Foundation, and the Alberta Heritage Foundation for Medical Research.

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Journal of Chromatography A, 718 (1995) 67–79

JOURNAL OF CHROMATOGRAPHY A

Effects of salts and the surface hydrophobicity of proteins on partitioning in aqueous two-phase systems containing thermoseparating ethylene oxide–propylene oxide copolymers

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First received 17 February 1995; revised manuscript received 6 June 1995; accepted 9 June 1995

Abstract

The partitioning of five well-characterised model proteins, bovine serum albumin (BSA), lysozyme, β-lactoglobulin A, myoglobin and cytochrome c, in aqueous two-phase systems has been studied. As top phase polymers PEG (polyethylene glycol, 100% EO) and the thermoseparating ethylene oxide (EO)–propylene oxide (PO) random copolymers, Ucon 50-HB-5100 (50% EO, 50% PO) and $EO_{30}PO_{70}$ (30% EO, 70% PO), respectively, were used. The top phase polymers are increasing in hydrophobicity with increasing content of PO. Reppal PES 200 (hydroxypropyl starch) was used as the bottom phase polymer. Phase diagrams for Reppal PES 200–PEG and Reppal PES 200–$EO_{30}PO_{70}$ two-phase systems were determined. The partitioning of four salts with different hydrophobicity, and also the effect of the salts on protein partitioning in these systems, was studied. It was found that the partitioning of the salts followed the Hofmeister series. The partitioning of proteins with low surface hydrophobicity, myoglobin and cytochrome c, was little affected by hydrophobic polymers and salts. However, the partitioning of a protein with higher surface hydrophobicity, lysozyme, was strongly affected when polymer hydrophobicity was increased and a hydrophobic counterion was used. A protein with a relatively hydrophobic surface can be partitioned to a phase containing a thermoseparating EO–PO copolymer by using a hydrophobic counterion. The partitioning of lysozyme and cytochrome c in the polymer–water system formed after temperature-induced phase separation was also examined. Both proteins partitioned exclusively to the water phase. A separation of the protein and polymer was obtained by temperature-induced phase separation on the isolated phase containing the EO–PO copolymer. The partitioning data also indicated that the hydroxypropyl starch polymer had a weak negative charge.

1. Introduction

An aqueous two-phase system consists of a mixture of two structurally different polymers which separate into two phases above a critical concentration. A protein or any other substance included in the system partitions between the two phases. The partitioning of a protein depends on its properties such as net charge, size and hydrophobicity. It can also be affected by including various salts in the system, changing the pH, changing polymers or polymer molecular mass [1]. Partitioning in an aqueous two-phase system is a mild method for the purification of a protein from a cell homogenate because of the

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0021-9673/95/$09.50
SSDI 0021-9673(95)00657-0

high content of water (75–90%) in the two phases.

Partitioning in aqueous two-phase systems can be combined with temperature-induced phase separation if one of the polymers is thermoseparating [2–4]. After separation in the primary phase system, the phase containing the thermoseparating polymer is isolated in a separate container and the temperature is raised above the cloud point (CP) of the polymer. This leads to a new phase separation where the new top phase consists mainly of water and the new bottom phase consists mainly of the polymer [2–4]. Proteins partition almost exclusively to the water phase in this step and a separation of the polymer and protein is obtained. The thermoseparating polymer can be recycled in the process. It is desirable in a purification process to partition the target protein to the top phase in the primary phase system and then separate the protein from the polymer with temperature-induced phase separation at a temperature as low as possible to avoid denaturation of the target protein. The CP of the thermoseparating polymer can be lowered by either adding a suitable salt, e.g. sodium sulphate, to the system [2] or by making the polymer more hydrophobic [4].

Aqueous two-phase systems containing the polymers PEG (polyethylene glycol) and dextran have been extensively studied. In the present studies we have used the low-cost polymer hydroxypropyl starch, Reppal PES 200 [5] as bottom phase polymer instead of dextran. Hydroxypropyl starch polymers have earlier been studied as a substitute for dextran and were found to have similar phase-forming properties [6]. We have used three different top phase polymers having nearly similar molecular masses, PEG (100% EO, EO_{100}) and the random copolymers of ethylene oxide (EO) and propylene oxide (PO), Ucon 50-HB-5100 (50% EO, 50% PO; $EO_{50}PO_{50}$) and $EO_{30}PO_{70}$ (30% EO, 70% PO). EO–PO random copolymers are thermoseparating [2–4,7], and the polymer hydrophobicity increases with increasing content of PO.

The partitioning of five model proteins, bovine serum albumin (BSA), lysozyme, β-lactoglobulin A, myoglobin and cytochrome c, was studied. The proteins are well characterised with respect to structure and properties. The partitioning of the proteins was studied at different pH values, pH 7.1 and pH 5.0. The partitioning of four salts with different hydrophobicity, sodium phosphate ($Na^+HPO_4^{2-}/H_2PO_4^-$) (NaP), NaCl, $NaClO_4$ and triethylammonium phosphate (Et_3NH^+ $H_2PO_4^-$) (Et_3NP), was studied. The effect of the salts on protein partition in phase systems with EO–PO random copolymers was investigated. Ions have different affinity for the two phases and can therefore create a potential difference between the phases which will affect protein partitioning [1,8].

For the application of temperature-induced phase separation in protein purification it is necessary to partition the target protein to the top phase containing the thermoseparating polymer in the primary phase system. The purpose of this study was to investigate how the hydrophobicity of the thermoseparating polymer, and added salts with varying hydrophobicities, affected the partitioning of the model proteins, and how the effects could be correlated to the surface hydrophobicity of the proteins. We have also investigated the partitioning of lysozyme and cytochrome c in the polymer–water system formed by the temperature-induced phase separation.

2. Materials and methods

2.1. Proteins

The following proteins were obtained from Sigma (St. Louis, MO, USA): serum albumin, bovine fatty acid free (nr. A-6003); β-lactoglobulin A, bovine milk (nr. L-7880); myoglobin, horse heart (nr. M-1882); cytochrome c, horse heart (nr. C-2506). Lysozyme: hen egg white (nr. 107255) was obtained from Boehringer (Mannheim, Germany).

2.2. Polymers and chemicals

Bottom phase polymer was Reppal PES 200 (hydroxypropyl starch), molecular mass 200 000, from Reppe AB (Växjö, Sweden). The respective top phase polymers were PEG 4000 (EO_{100})

from Merck (Münich, Germany), Ucon 50-HB-5100 ($EO_{50}PO_{50}$) from Union Carbide (New York, NY, USA) and $EO_{30}PO_{70}$ from Shearwater Polymers (Huntsville, AL, USA). PEG and Ucon had a molecular mass of 4000, $EO_{30}PO_{70}$ had a molecular mass of 3200. All chemicals were of analytical grade. Triethylammonium phosphate was obtained by mixing triethylamine with phosphoric acid to the desired pH.

2.3. Phase diagrams

The specific rotation $[a]_D^{25}$ of Reppal PES 200 was determined by polarimetry (as the slope of a standard curve) to 192° ml g^{-1} dm^{-1}. Systems of 10 g with different concentrations (percent w/w) of Reppal, PEG (or $EO_{30}PO_{70}$) and water were prepared. The systems were centrifuged for 10 min at 5700 rpm. The top- and bottom phases were isolated and diluted six times. First the concentration of Reppal was determined in both phases by polarimetry. The concentration of PEG and $EO_{30}PO_{70}$, respectively, was determined by measuring the refractive index and by subtracting the Reppal contribution to the refractive index readings. A few points in the phase diagram, around the critical point, were determined by titration of the two-phase system with water until the formation of a one-phase system [1].

2.4. Two-phase systems for the partitioning of proteins

The phase diagrams of the two-phase systems using different polymers differ and, hence, to be able to compare the partition coefficients comparable two-phase systems were chosen. $\Delta\text{Reppal} = C_{\text{Reppal bottom phase}} - C_{\text{Reppal top phase}}$ was chosen to be the same (14%) in each system. Systems of 5 g with the following concentrations were mixed: 13.8% Reppal–6.5% PEG, 11% Reppal–5% Ucon, 10.6% Reppal–6.8% $EO_{30}PO_{70}$. All polymer concentrations in this paper are given as percent w/w. The systems also contained 10 m*M* buffer (sodium phosphate buffer at pH 7.1, sodium acetate buffer at pH 5.0), 0.5 mg/ml protein, 100 m*M* salt (NaCl, $NaClO_4$ or Et_3NP). When no salt was included the buffer concentration was 100 m*M*.

The partition of a substance is described by the partition coefficient K, which is defined as $K = C_T/C_B$, where C_T is the concentration of the substance in the top phase and C_B is its concentration in the bottom phase. All partition coefficients are average values from at least two experiments. Experiments with different salts at pH equal to p*I* for the protein were done with one of the top phases (Ucon). The systems were equilibrated at room temperature and were left standing for one hour. The top- and bottom phases were isolated and diluted 7–15 times. The phases were analysed for their protein content. BSA and myoglobin were analysed according to Bradford [10] with Coomassie Brilliant Blue G at 595 nm and with the respective protein as standard. Lysozyme and β-lactoglobulin A were analysed with the BCA method [11] with the respective protein as standard. Cytochrome c was analysed by measuring the absorbance at 408 nm.

2.5. Temperature-induced phase separation

The cloud points for Ucon and $EO_{30}PO_{70}$ are approximately 50°C [7] and 35°C [12], respectively. To obtain a macroscopic separation of the polymer- and water phases in a reasonable time the temperature must be raised 5–10°C higher than the cloud point. Temperature-induced phase separation was studied in systems with $EO_{30}PO_{70}$. After separation and isolation of the top- and bottom phases from the primary two-phase system at room temperature, the top phase was heated in a water bath to 44°C and was left standing for one hour. The new top- and bottom phases were isolated and diluted. The protein content was determined in the same way as in the primary two-phase system.

2.6. Two-phase systems for the partitioning of salts

Systems with the polymer concentrations (percent w/w) of 20% Reppal–9% PEG, 15.3% Reppal–8.1% Ucon and 16% Reppal–9.1% $EO_{30}PO_{70}$, 100 m*M* of the salts (NaP, NaCl,

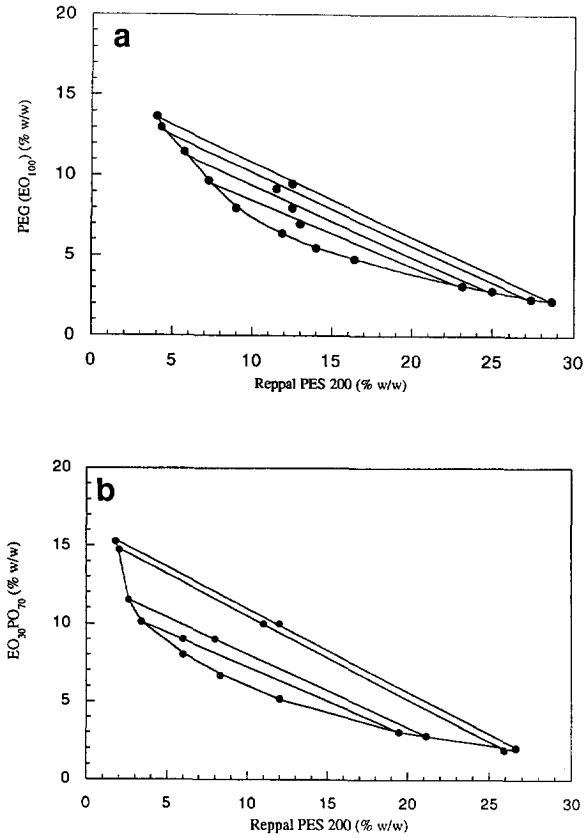

Fig. 1. (a) Phase diagram of Reppal PES 200–PEG 4000 (EO_{100})–water system at 20°C. (b) Phase diagram of Reppal PES 200–$EO_{30}PO_{70}$–water system at 20°C.

$NaClO_4$ or Et_3NP) and water to 5 g were prepared. The systems were equilibrated at room temperature and were left standing for one hour. The top- and bottom phases were then isolated and diluted 80 times. The partitioning of the salts was analysed by measuring the conductivity of the top and bottom phase, respectively.

3. Results and discussion

3.1. Phase diagrams

The phase diagram and the composition of the phases for the Reppal PES 200–PEG 4000–water system at 20°C are shown in Fig. 1a and Table 1. Table 2 shows the compositions of the phases for the Reppal PES 200–Ucon 50-HB-5100–water system at 20°C and the phase diagram can be found in Ref. [13]. The phase diagram and the composition of the phases for the Reppal PES 200–$EO_{30}PO_{70}$–water system at 20°C are shown in Fig. 1b and Table 3.

3.2. The partitioning of salts

The polymer concentrations that were used for the partitioning of the proteins showed very small difference in partition coefficients between different salts. Higher concentrations of the polymers are known to result in more extreme partition coefficients for a substance [1]. Hence we chose to partition the salts at higher polymer concentrations than that used for the proteins. In the three different systems Δ(top phase polymer) was kept constant (15%), where Δ(top phase polymer) is equal to the concentration of the dominating polymer in the top phase minus the

Table 1
Compositions of the phases in the Reppal PES 200–PEG 4000 (EO_{100})–water system at 20°C

Total system			Top phase			Bottom phase		
Reppal (% w/w)	PEG (% w/w)	H_2O (% w/w)	Reppal (% w/w)	PEG (% w/w)	H_2O (% w/w)	Reppal (% w/w)	PEG (% w/w)	H_2O (% w/w)
13.0	7.0	80.0	7.3	9.7	83.0	23.1	3.2	73.7
12.5	8.0	79.5	5.7	11.5	82.8	25.0	2.8	72.2
11.5	9.2	79.3	4.3	13.0	82.7	27.4	2.4	70.2
12.5	9.5	78.0	4.0	13.7	82.3	28.7	2.3	69.0

Table 2
Compositions of the phases in the Reppal PES 200–Ucon 50-HB-5100–water system at 20°C. The phase diagram is shown in Ref. [13]

Total system			Top phase			Bottom phase		
Reppal (% w/w)	Ucon (% w/w)	H_2O (% w/w)	Reppal (% w/w)	Ucon (% w/w)	H_2O (% w/w)	Reppal (% w/w)	Ucon (% w/w)	H_2O (% w/w)
6.9	7.5	85.6	3.9	8.8	87.3	18.8	1.5	79.7
7.5	8.0	84.5	3.3	10.1	86.6	19.7	1.7	78.6
10.0	8.0	82.0	2.8	11.5	85.7	24.4	0.6	75.0
9.2	10.0	80.8	2.1	13.9	84.0	25.8	0.8	73.4
11.6	12.5	75.9	1.2	18.5	80.3	31.9	0.5	67.6

Table 3
Compositions of the phases in the Reppal PES 200–$EO_{30}PO_{70}$–water system at 20°C

Total system			Top phase			Bottom phase		
Reppal (% w/w)	$EO_{30}PO_{70}$ (% w/w)	H_2O (% w/w)	Reppal (% w/w)	$EO_{30}PO_{70}$ (% w/w)	H_2O (% w/w)	Reppal (% w/w)	$EO_{30}PO_{70}$ (% w/w)	H_2O (% w/w)
6.0	9.0	85.0	3.4	10.1	86.5	19.5	3.0	77.5
8.0	9.0	83.0	2.6	11.5	85.9	21.2	2.8	76.0
11.0	10.0	79.0	2.0	14.8	83.2	25.9	1.9	72.2
12.0	10.0	78.0	1.8	15.3	82.9	26.6	2.0	71.4

concentration of this polymer in the bottom phase.

The well-known Hofmeister or lyotropic series describes the salting-out effects of ions on proteins, as well as the effect of ions on the surface tension of water [14]. The Hofmeister series can be used as a measure of the relative hydrophobicity of an ion. The hydrophobicity of the anions studied in this work is in the following order $ClO_4^- > Cl^- > CH_2COO^- > HPO_4^{2-}/H_2PO_4^-$, while the hydrophobicity of the cations is $Et_3NH^+ > Na^+$. Et_3NH^+ is an ion with hydrophobic groups and Na^+ is hydrophillic due to its high surface charge density. Fig. 2 shows that the partitioning of the salt to the relatively more hydrophobic top phase was enhanced with increase in hydrophobicity of the anion or cation, in accordance with Hofmeister series. The difference in hydrophobicity of the top phase polymer affected the partitioning of the salts very little.

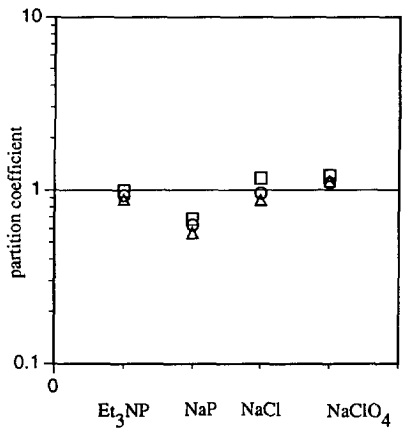

Fig. 2. The partitioning of the salts, at 100 m*M* concentration, triethylammonium phosphate (Et_3NP), sodium phosphate (NaP), sodium chloride (NaCl) and sodium perchlorate ($NaClO_4$) in the two-phase systems. The phase systems were 20% (w/w) Reppal–9% (w/w) EO_{100} (□), 15.3% (w/w) Reppal–8.1% (w/w) $EO_{50}PO_{50}$ (○) and 16% (w/w) Reppal–9.1% (w/w) $EO_{30}PO_{70}$ (△).

3.3. Protein properties

The hydrophobic interaction chromatography (HIC) of the proteins BSA, lysozyme, β-lactoglobulin A and myoglobin on butyl epoxy-Sepharose [18] has been studied earlier. Hydrophobic partitioning using fatty acids bound to PEG has been performed with BSA, β-lactoglobulin A and cytochrome c [19]. Studies in aqueous two-phase systems with hydrophobic groups, benzoyl and valeryl, bound to dextran have been made [20]. The influence of the average surface hydrophobicities on partitioning of the proteins BSA, lysozyme and β-lactoglobulin A in PEG–salt systems has been studied [21]. The partitioning in PEG–salt systems was better correlated with data from ammonium sulphate precipitation than with HIC data. Other studies in PEG–salt systems have shown that the content of the aromatic amino acid tryptophan (Trp) exposed on the surface is of importance in the partitioning of proteins [22]. Trp has also been found to partition very strongly to the hydrophobic Ucon phase in Ucon–water two-phase systems compared with other amino acids [23]. Literature data for the five model proteins on retention in HIC, ammonium sulphate precipitation, hydrophobic partitioning, the total content of Trp and Trp exposed on the surface are shown in Table 4, together with other properties of importance for partitioning: molecular mass, pI and the net charge at pH 7.1.

3.4. The partitioning of proteins

The logarithm of the partition coefficient ($\log K$) of a protein can be divided into contributions from different factors influencing the partitioning [1]:

$$\log K = \log K^0 + \log K_{el} + \log K_{hphob} + \log K_{size} + \cdots$$

where $\log K_{el}$ is the contribution from electrostatics, $\log K_{hphob}$ is the contribution from the surface hydrophobicity of the protein, $\log K_{size}$ depends on the size (molecular mass) of the protein and $\log K^0$ includes other factors. The $\log K_{el}$ is influenced by the partitioning of ions which creates an electrical potential difference between the phases. The partition of a protein with a positive or negative net charge is affected by this potential difference [8]. Log K_{el} can be shown to be linearly dependent on the protein net charge [1]. At pH equal to the pI of the protein there are no salt effects because the net

Table 4
Properties of the five model proteins

	Bovine serum albumin	Lysozyme	β-Lactoglobulin A	Myoglobin	Cytochrome c
Molecular mass	69 000 [28]	13 900 [27]	35 000 [28]	17 500 [28]	13 000 [27]
pI	5.0 [28]	11.0 [29]	5.1 [28]	7.1 [28]	9.4 [28]
Net charge at pH 7.1	−18 [25]	+7 [30]	−5 [24]	0 [28]	+6 [31]
Retention in HIC	10.33[a]	7.49[a]	4.41[a]	2.99[a]	– –
Hydrophobic partition	1.8[b]	– –	1.25[b]	– –	−0.1[b]
Precipitation	0.310[c]	0.416[c]	0.287[c]	– –	– –
Total content of Trp	0.3% [25]	4.6% [32]	1.1% [24]	1.3% [33]	0.9% [34]
Content of Trp exposed on the surface	0% [25]	4.0% [32]	0.6% [24]	– –	0% [34]

[a] Retention coefficient in hydrophobic interaction chromatography on butyl epoxy-Sepharose, determined in Ref. [18].
[b] Maximal Δ log K determined by hydrophobic partition using fatty acids bound to PEG, determined in Ref. [19]. Δ log K is the difference in log K of protein in a PEG–dextran system with and without fatty acid esterified groups. A high value indicates hydrophobic interaction with the polymer-bound ligand.
[c] The inverse of the concentration of ammonium sulphate needed for the precipitation of the protein, determined in Ref. [21]. A high value indicates a high protein surface hydrophobicity.

charge of the protein is zero, and the protein–polymer interaction will then mainly determine the partition [9]. In systems containing hydrophobic polymers the interaction will depend on the protein surface hydrophobicity. At pH values different from the p*I* a combination of protein–polymer interactions and charge effects will affect protein partitioning.

The model proteins were partitioned in phase systems with top phase polymers of increasing hydrophobicity. Comparable phase systems were used (see Material and Methods). The salts were included at a concentration of 100 m*M*; they were dominating over the buffer because the concentration of the salt was ten times higher than the concentration of the buffer [1]. The salts give rise to slightly different ionic strengths in the two-phase systems. It has been shown [8] in PEG–dextran systems that the partitioning of a protein at salt concentrations higher than 50 m*M* is independent of the ionic strength.

Bovine serum albumin

BSA has a negative net charge at pH 7.1 (Fig. 3a) and the tendency for the partition coefficients for BSA with different anions in the system were $K_{NaP} > K_{NaCl} > K_{NaClO_4}$. BSA was partitioned more to the top phase by the partitioning of the hydrophillic $HPO_4^{2-}/H_2PO_4^-$ to the bottom phase and the opposite effect was obtained with Cl^- and ClO_4^-. The hydrophobic triethylammonium ion has a strong tendency to partition to the top phase and the partition coefficients of BSA should, therefore, be higher when triethylammonium is the counterion compared to sodium, which is also seen in Fig. 3a. The top phase polymer change between $EO_{50}PO_{50}$ and $EO_{30}PO_{70}$ did not affect the partitioning as much as when $EO_{50}PO_{50}$ or $EO_{30}PO_{70}$ was exchanged with EO_{100}. The more hydrophillic top phase resulted in higher partition coefficients, especially when sodium phosphate was included in the system.

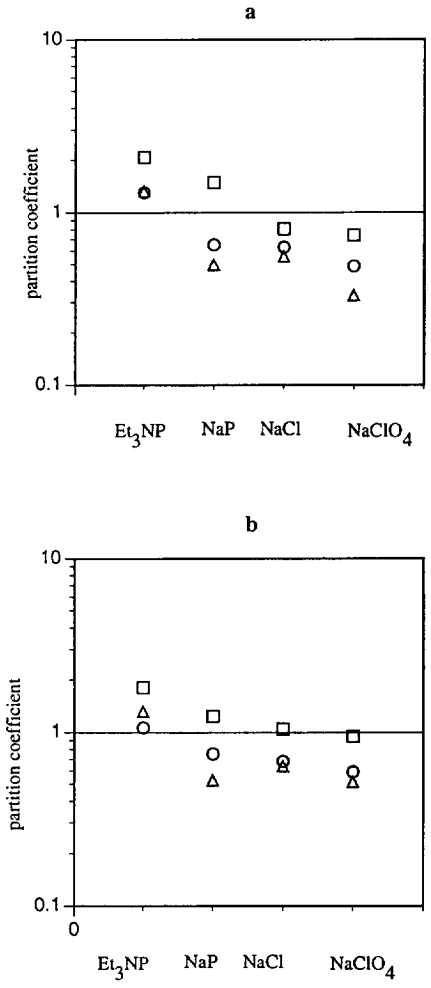

Fig. 3. (a) Partitioning of BSA at pH 7.1 (negatively charged) in two-phase systems. All systems were in 10 m*M* or 100 m*M* sodium phosphate buffer (100 m*M* when no salt other than NaP was included). NaCl, $NaClO_4$ and Et_3NP were at 100 m*M* concentration. The phase systems were 13.8% (w/w) Reppal–6.5% (w/w) EO_{100} (□), 11% (w/w) Reppal–5.0% (w/w) $EO_{50}PO_{50}$ (○) and 10.6% (w/w) Reppal–6.8% (w/w) $EO_{30}PO_{70}$ (△). Standard deviation 0.06. (b) Partitioning of β-lactoglobulin at pH 7.1 (negatively charged) in two-phase systems. All systems were in 10 m*M* or 100 m*M* sodium phosphate buffer (100 m*M* when no salt other than NaP was included). NaCl, $NaClO_4$ and Et_3NP were at 100 m*M* concentration. The phase systems were 13.8% (w/w) Reppal–6.5% (w/w) EO_{100} (□), 11% (w/w) Reppal–5.0% (w/w) $EO_{50}PO_{50}$ (○) and 10.6% (w/w) Reppal–6.8% (w/w) $EO_{30}PO_{70}$ (△). Standard deviation 0.11.

β-Lactoglobulin

The partitioning behaviour of the negatively charged β-lactoglobulin at pH 7.1 (Fig. 3b) was similar to the partitioning behaviour of BSA at the same pH (Fig. 3a). Similar effects of salt and polymer hydrophobicity were observed.

The partitioning behaviours of BSA and β-lactoglobulin were similar although the two proteins differ in molecular mass and net charge. However, β-lactoglobulin has a strong tendency to form dimers in the pH interval studied here [24], and β-lactoglobulin as dimer and BSA have relatively similar molecular masses and net charge at pH 7.1. Both proteins exhibited lower affinity for the top phase when the hydrophobicity of the top phase polymer increased and seem therefore to have relatively hydrophillic surfaces. Another indication of their relatively hydrophillic surfaces is that all partition coefficients at pH 5.0 (Fig. 4) were equal to or less than 1 for both proteins. Earlier results from HIC [18] and partitioning in aqueous two-phase systems with hydrophobic groups coupled to one of the polymers [19,20] indicated higher surface hydrophobicity for these proteins. Hydrophobic sites on the surface of the protein may give a high retention coefficient in HIC and may also interact with hydrophobic groups coupled to a polymer. BSA has a number of hydrophobic sites that can bind long-chain fatty acids [25], which can explain the HIC and hydrophobic partitioning results [18–20].

Lysozyme

The partitioning of the positively charged lysozyme at pH 7.1 (Fig. 5) was strongly affected by the hydrophobicity of the anions. Lysozyme could be transferred from the lower to the upper phase by the use of the relatively hydrophobic ClO_4^- instead of the hydrophillic phosphate ion. The partition coefficients were lowered when Et_3NH^+ was added to the system instead of Na^+. The partition coefficients were relatively independent of the difference in hydrophobicity of the top phase polymer, except when $NaClO_4$ was included in the system. With $NaClO_4$ the partition coefficients were highest for the most hydrophobic top phase polymer, $EO_{30}PO_{70}$. Partitioning at the p*I* for lysozyme could not be performed because of its low solubility at this pH.

Cytochrome c

The partitioning of the positively charged cytochrome c at pH 7.1 (Fig. 6) was also affected

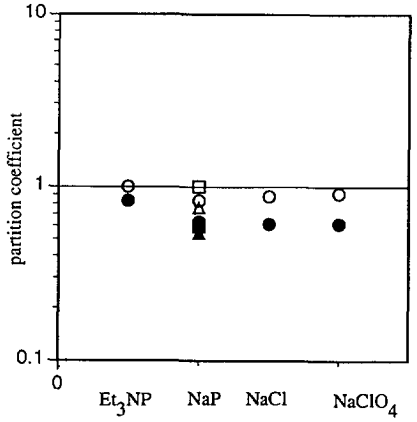

Fig. 4. Partitioning of BSA (filled symbols) and β-lactoglobulin (open symbols) at pH 5.0 (at the p*I*). All systems were in 10 m*M* or 100 m*M* sodium acetate buffer (100 m*M* when no salt other than NaAc was included). NaCl, $NaClO_4$ and Et_3NP were at 100 m*M* concentration. The phase systems were 13.8% (w/w) Reppal–6.5% (w/w) EO_{100} (■, □), 11% (w/w) Reppal–5.0% (w/w) $EO_{50}PO_{50}$ (●, ○) and 10.6% (w/w) Reppal–6.8% (w/w) $EO_{30}PO_{70}$ (▲, △). Standard deviation 0.08.

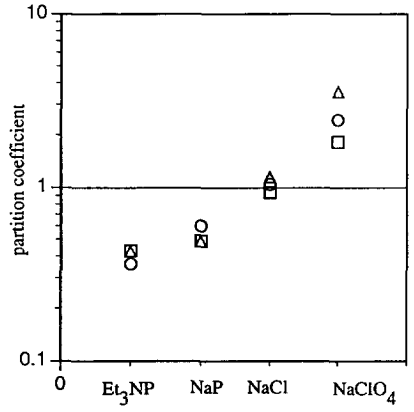

Fig. 5. Partitioning of lysozyme at pH 7.1 (positively charged). All systems were in 10 m*M* or 100 m*M* sodium phosphate buffer (100 m*M* when no salt other than NaP was included). NaCl, $NaClO_4$ and Et_3NP were at 100 m*M* concentration. The phase systems were 13.8% (w/w) Reppal–6.5% (w/w) EO_{100} (□), 11% (w/w) Reppal–5.0% (w/w) $EO_{50}PO_{50}$ (○) and 10.6% (w/w) Reppal–6.8% (w/w) $EO_{30}PO_{70}$ (△). Standard deviation 0.14.

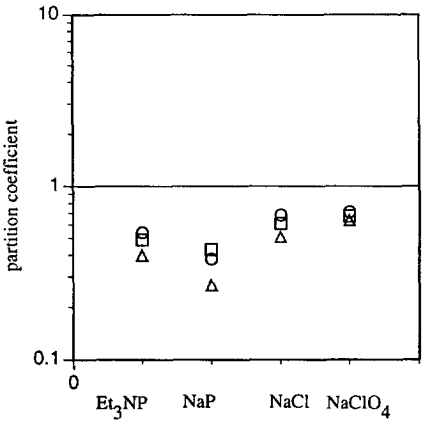

Fig. 6. Partitioning of cytochrome C at pH 7.1 (positively charged). All systems were in 10 m*M* or 100 m*M* sodium phosphate buffer (100 m*M* when no salt other than NaP was included). NaCl, $NaClO_4$ and Et_3NP were at 100 m*M* concentration. The phase systems were 13.8% (w/w) Reppal–6.5% (w/w) EO_{100} (□), 11% (w/w) Reppal–5.0% (w/w) $EO_{50}PO_{50}$ (○) and 10.6% (w/w) Reppal–6.8% (w/w) $EO_{30}PO_{70}$ (△). Standard deviation 0.04.

by the hydrophobicity of the anions but not to the same extent as lysozyme. The hydrophobicity of the cation had a small effect on the partitioning of cytochrome c, but in the opposite direction relative to lysozyme. The expected effect from the partitioning of the Et_3NH^+ ion was to lower the *K*-value, as in the case of lysozyme, but instead an increase was observed compared with the *K*-value for Na^+. A possible explanation can be that the hydrophobic Et_3NH^+ induces a conformational change in cytochrome c leading to exposure of hydrophobic residues. The partition coefficients varied very little with the hydrophobicity of the top phase polymer.

Cytochrome c and lysozyme have similar molecular mass and net charge (+6 and +7 at pH 7.1, Table 4) but they behaved differently in the two-phase systems, indicating a difference in surface hydrophobicity. Lysozyme has a relatively more hydrophobic surface than cytochrome c, which was also observed in studies with hydrophobically modified dextrans [20]. When ClO_4^- is added, the combined effect of a hydrophobic counterion and hydrophobic protein–polymer interaction directs lysozyme to the top phase. The effect is increased with increased polymer hydrophobicity (EO_{100} to $EO_{30}PO_{70}$). The hydrophobicity shown by lysozyme is in accordance with precipitation studies and the retention observed in HIC (Table 4). Cytochrome c, on the other hand, could not be partitioned to the top phase by ClO_4^- and in general the salt effects were small. This indicates that the protein has a low surface hydrophobicity, which is also in agreement with hydrophobic partitioning data [19,20] (Table 4).

Myoglobin

The partitioning of myoglobin at pH 5.0, positively charged, (Fig. 7b) was very little affected by salts and the difference in hydrophobicity of the top phase polymer. The protein was precipitated when $NaClO_4$ was included. Myoglobin could not be partitioned to the top phase with any of the top phase polymers or salts, which indicates a relatively hydrophillic surface. This is also in accordance with HIC [18] (Table 4) and partitioning with hydrophobically modified dextrans [20].

3.5. Salt effects on protein partitioning

At the p*I* of the proteins, when the protein had nearly zero net charge, the salts had little effect on their partitioning (Figs. 4 and 7a). At a pH different from the p*I* of the protein the partitioning followed Hofmeister series for almost all proteins and all top phase polymers. The partition coefficient for a positively charged protein increased with increasing anion hydrophobicity and for a negatively charged protein the partition coefficient decreased with increasing anion hydrophobicity. The only exception in this study was BSA and β-lactoglobulin with $EO_{30}PO_{70}$ as top phase polymer, where the partition coefficients were not lowered by the more hydrophobic Cl^- compared with phosphate.

Salts have a strong effect on the partitioning of a protein with a positive or negative net charge, which shows the importance of the term $\log K_{el}$. The $\log K_{el}$ is linearly dependent on the net charge of the protein, hence BSA (−18 at pH

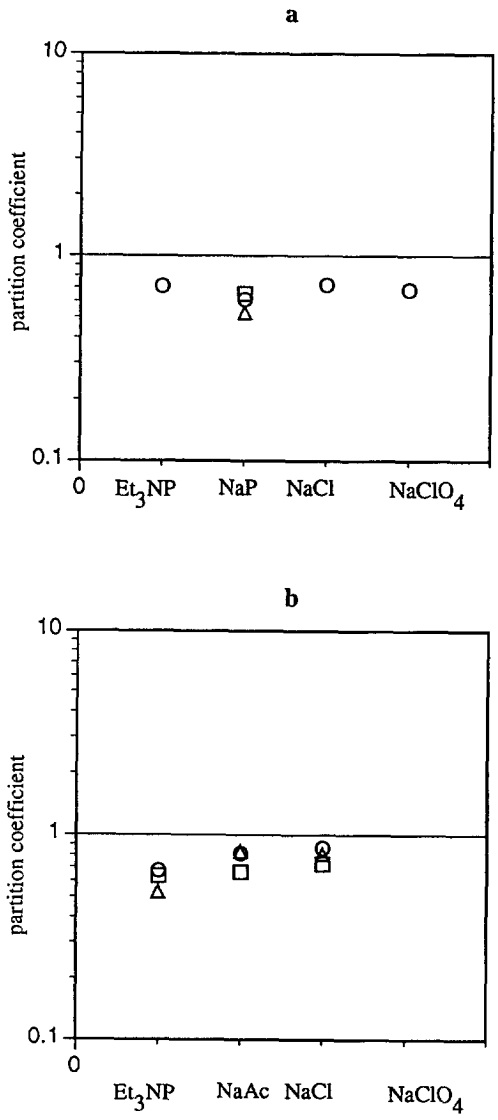

Fig. 7. (a) Partitioning of myoglobin at pH 7.1 (at the p*I*). All systems were in 10 m*M* or 100 m*M* sodium phosphate buffer (100 m*M* when no salt other than NaP was included). NaCl, $NaClO_4$ and Et_3NP were at 100 m*M* concentration. The phase systems were 13.8% (w/w) Reppal–6.5% (w/w) EO_{100} (□), 11% (w/w) Reppal–5.0% (w/w) $EO_{50}PO_{50}$ (○) and 10.6% (w/w) Reppal–6.8% (w/w) $EO_{30}PO_{70}$ (△). Standard deviation 0.02. (b) Partitioning of myoglobin at pH 5.0 (positively charged). All systems were in 10 m*M* or 100 m*M* sodium acetate buffer (100 m*M* when no salt other than NaAc was included). NaCl and Et_3NP were at 100 m*M* concentration. The phase systems were 13.8% (w/w) Reppal–6.5% (w/w) EO_{100} (□), 11% (w/w) Reppal–5.0% (w/w) $EO_{50}PO_{50}$ (○) and 10.6% (w/w) Reppal–6.8% (w/w) $EO_{30}PO_{70}$ (△). Standard deviation 0.04.

7.1) should be most affected among all the proteins studied here. However, this was not the case in our study; lysozyme is affected even stronger than BSA, so other factors such as hydrophobic interaction with the polymer are important in these systems. When the effects are in the same direction in the two-phase system the total effect is strong, lysozyme is strongly partitioned to the top phase with the hydrophobic top phase polymer $EO_{30}PO_{70}$ and the hydrophobic counterion ClO_4^- as compared with the relatively hydrophillic cytochrome c, which remains in the bottom phase in a similar system.

Our results indicate that lysozyme has the highest total surface hydrophobicity of the five proteins studied. BSA and β-lactoglobulin show similar and relatively low total surface hydrophobicity, which is also the case for myoglobin and cytochrome c. Partitioning of proteins in these kinds of systems seems to reflect the total surface hydrophobicity in the same way as precipitation studies do. Interestingly, the content of Trp exposed on the surface of the proteins (Table 4) corresponds well with the results obtained for partitioning in EO–PO copolymer systems. Lysozyme has the highest content of Trp (4.65%) and the residues are to a high degree (4.0%) exposed on the surface of the protein. On the contrary, cytochrome c has only 0.9% Trp, none of which is exposed on the surface of the protein. β-Lactoglobulin has one (0.6% of total) Trp exposed on the surface, and BSA has no exposed Trp's. This can explain the relatively low total surface hydrophobicity for cytochrome c, β-lactoglobulin and BSA shown in partitioning in EO–PO copolymer systems.

3.6. Temperature-induced phase separation

$EO_{30}PO_{70}$ was further subjected to temperature-induced phase separation, as it has the lowest CP of the three top phase polymers studied. Lysozyme was the model protein with the highest affinity for the thermoseparating polymers (see Fig. 5), and this protein was chosen in order to examine protein partitioning in temperature-induced phase separation. The purpose was to see if a protein with affinity for

the thermoseparating polymer would partition to the water phase in the polymer–water phase system formed after temperature-induced phase separation. $NaClO_4$ was added as this salt could direct the partitioning of lysozyme to the $EO_{30}PO_{70}$-containing phase in the primary phase system. For comparison the partitioning of cytochrome c in a similar system was studied because of the low affinity of cytochrome c for the thermoseparating polymers. The primary phase system was composed of 6.8% $EO_{30}PO_{70}$ and 10.6% Reppal. After phase separation, the top phase was isolated in a separate container and placed in a water bath at 44°C. A new top (water) and bottom (EO–PO copolymer) phase was formed. For both lysozyme and cytochrome c 100% of the protein content was obtained in the water phase after separation at 44°C. Thus a separation of the polymer and the protein was achieved with the hydrophillic cytochrome c as well as with the more hydrophobic lysozyme.

3.7. Partitioning with amino acids as buffers and the effect of salt concentration

Reppal, the bottom phase polymer, has been suggested to possess negative charges [15]. Experiments were carried out in order to study the effect of negative charges of Reppal on the partitioning of proteins. Partitioning experiments for BSA and lysozyme with the amino acids β-alanine (p*I* 6.9 [16]) and glycine (p*I* 6.1 [17]) as buffers without any other salt in the system were done (Table 5). The top phase polymer was Ucon and the bottom phase polymers were Reppal or dextran. The proteins were partitioned at the p*I* of the buffers. By use of zwitterionic buffers it is possible to have a buffered phase system with zero electrical potential difference between the phases. BSA is negatively charged and lysozyme is positively charged, both at pH 6.1 and 6.9. In the system with Reppal, BSA was partitioned to the top phase while lysozyme was partitioned strongly to the bottom phase. In the system containing dextran as bottom phase polymer the opposite partitioning behaviour for the two proteins was obtained, BSA was partitioned to the bottom phase while lysozyme to the top phase. For both proteins the *K*-values were close to unity in the dextran systems.

We also examined how the buffer concentration, 10 and 100 m*M* sodium phosphate or triethylammonium phosphate buffer at pH 7.1, influenced the partitioning of two of the proteins (BSA and cytochrome c) in PEG–Reppal systems (Table 6). The concentration of buffer had a significant effect on the partitioning of both BSA and cytochrome c. BSA, negatively charged at pH 7.1, was partitioned to the top phase while cytochrome c, positively charged, was partitioned to the bottom phase. The partition coefficients were more extreme for both proteins at 10 m*M* buffer concentrations than at 100 m*M* concentration.

The results shown in Table 5 and the observation that the partition coefficients were more extreme at 10 m*M* than at 100 m*M* of the salts (Table 6) support the conclusion that Reppal has a small negative charge. A concentration of 10 m*M* of the salt is not enough to screen the negative charges on Reppal. The negative charge

Table 5
Partition coefficients of BSA, negatively charged, and lysozyme, positively charged, at pH 6.1 and 6.9

	Reppal–Ucon system		Dextran–Ucon system	
	BSA	Lysozyme	BSA	Lysozyme
Glycine	3.13	0.11	0.76	1.13
β-Alanine	3.69	0.18	0.64	1.14

The phase systems were 11% (w/w) Reppal–5% (w/w) Ucon–100 m*M* glycine, pH 6.1, or 100 m*M* β-alanine, pH 6.9, and 5.1% (w/w) dextran–4.4% (w/w) Ucon–100 m*M* glycine or 100 m*M* β-alanine.