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Salt-steered partitioning of proteins in polymeric two-phase systems based on *N*,*N*-dimethylformamide

Helle Truust, Göte Johansson*

Department of Biochemistry, University of Lund, P.O. Box 124, S-221 00 Lund, Sweden

Abstract

The polymeric two-phase (liquid–liquid) system, based on *N*,*N*-dimethylformamide (DMFA) and the two polymers Ficoll and poly(ethylene glycol), has been investigated. The partitioning of the water-insoluble protein fraction, zein (from corn), was affected by the addition of various salts that were soluble in DMFA. The effect of the salts has been correlated to their partition coefficients. The data have been fitted to a theory for partitioning of polyelectrolytes in aqueous two-phase systems. The two-phase system has been used for partial fractionation of zein by counter-current distribution.

Keywords: Aqueous two-phase systems; Partitioning; Proteins; Dimethylformamide; Zein

1. Introduction

Aqueous two-phase systems can be obtained by dissolving two kinds of polymers in water. These systems have found great use in protein purification by liquid–liquid extraction [1,2]. The partitioning of proteins between the two phases can be adjusted in a number of ways: By addition of various salts to the system, by changing the concentration or the average molecular mass of the polymers or by covalent linkage of charged, hydrophobic or affinity ligand groups to one of the phase-forming polymers. The effect of salts on partitioning is fairly well understood [3,4].

Two-phase systems may also be obtained by dissolving two polymers in organic solvents instead of water [5]. Such systems would be useful for fractionation of water-insoluble proteins, for example, gluten proteins from cereals, by using liquid–liquid extraction. Since both of the phases consist primarily of a single solvent, the solvation properties may not differ significantly between the two phases. However, to achieve good separation by repeated liquid–liquid extraction, it is important to create conditions such that the partition coefficients of the various proteins of a mixture differ as much as possible. In this work, we have studied the possibility of steering the partitioning of proteins in an organic two-phase system by the addition of salts that are soluble in the solvent. The system used here is composed of the solvent *N*,*N*-dimethylformamide (DMFA) and the two polymers, Ficoll and poly(ethylene glycol) (PEG). Crude zein extracted from

*Corresponding author.

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corn, which contains several proteins [6], has been used as a sample protein.

2. Experimental

2.1. Chemicals

Poly(ethylene glycol)s (PEGs) were supplied by Union Carbide (New York, NY, USA) (PEG 8000, M_r=8000) or by Serva (Heidelberg, Germany) (PEG 40 000, M_r=40 000 and PEG 400 000, M_r= 400 000). Ficoll 70 (M_r=70 000) and Ficoll 400 (M_r=400 000) were obtained from Pharmacia Biotech (Uppsala, Sweden). DMFA, analytical grade, was supplied by British Drug House (Poole, UK). Bicinchoninic acid (BCA) protein assay reagent was received from Pierce (Rockford, IL, USA). L-Tryptophan methyl ester–HCl, methyl guanidine–HCl and Zein from corn were obtained from Sigma (St. Louis, MO, USA). LiI, L-tyrosine methyl ester–HCl and L-phenylalanine methyl ester–HCl were purchased from Aldrich (Steinheim, Germany). LiCl was provided by Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

2.2. Assays

Protein concentration was determined photometrically [7], using a combination of the biuret reaction (protein reducing Cu^{2+} in an alkaline medium to produce Cu^{+}) with a selective detection reagent for Cu^{+}, BCA. The absorbance of purple reaction product was measured at 562 nm after 2 h of incubation at room temperature.

2.3. Two-phase systems and partitioning of proteins and salts

Organic two-phase systems, typically containing 9% (w/w) PEG 8000, 11% (w/w) Ficoll 70, 80% (w/w) DMFA and salt (normally at 2.5 m*M*), were prepared directly from the polymers, solvent and solid salt. The DMFA that was added was pure or contained dissolved zein at approximately 10 g l^{-1}. The partition of proteins was determined by first equilibrating the systems (8 g) at 23°C using careful mixing for 20 min. This was followed by centrifugation at 3000 *g* for 5 min. Only negligible amounts, <3% of added protein, were, in some cases, found at the interface. Samples (25 μl) were withdrawn from each phase and their protein content was determined. Partitioning of salts was determined by diluting samples of the phases with DMFA (typically by a factor of twenty) and estimating the salt concentration by measuring the electric conductivity with a Metrohm 644 conductometer (Herisau, Switzerland).

2.4. Apparent pH

Apparent pH values were obtained by using a Radiometer pH meter, PHM 61 (Copenhagen, Denmark), equipped with a glass electrode, Radiometer PHC2406.

2.5. Volume ratio

The ratio between the volumes of the upper and lower phase was determined by equilibrating the systems (8 g) in calibrated graduated 10 ml centrifugation tubes, then recording the phase volumes.

2.6. Transition points

The concentrations of the two polymers Ficoll and PEG at which the systems became homogeneous were determined by turbidometric titration. Known weights of systems were titrated, while being shaken with DMFA, until the two phases just disappeared. The concentrations of polymers were calculated from the final total weight. Systems based on water as the solvent were likewise analysed, but these were titrated with water.

2.7. Counter-current distribution

The settling time of the phases was reduced by centrifugation. A centrifugal counter-current distribution (C-CCD) apparatus [8] containing 60 chambers and with bottom phase cavities with a volume of 0.81 ml was used. Mixed systems containing 0.75 ml of bottom phase and 1.35 ml of top phase were added to each chamber. Systems with sample were applied in chambers 0–1. The system containing the sample was prepared by dissolving polymers and salt in a solution of 1% zein in DMFA. Twenty-five

distribution steps were carried out at 23°C. Each cycle included mixing of the phases at unity gravity for 2 min and centrifugation for 10 min at 100 *g*. Finally, the two-phase systems were transformed into single-phase solutions by the addition of 2 ml of DMFA to each cavity, and the obtained fractions were collected and analyzed for protein content. The diagram obtained by plotting absorbance values as a function of tube number, the CCD diagram, shows the fractionation of the protein components.

3. Results and discussion

3.1. DMFA-based two-phase systems

Two-phase systems are formed by dissolving pairs of polymers not only in water but also in organic solvents [5,9]. This makes it possible to use two-phase systems containing a single organic solvent present in both phases for the separation of hydrophobic substances by liquid–liquid extraction. It is important when this is done to ensure that the solvent should be such that it does not react with the material to be partitioned.

To create a two-phase system based on an organic solvent, both of the polymers should have high solubility in the solvent. Both Ficoll and PEG are soluble in DMFA [5] and together they cause phase separation when mixed with the solvent in sufficiently high concentrations.

Fig. 1 shows the borderlines between the one- and two-phase regions (the binodal curve) for systems where the polymers were dissolved either in water or in DMFA. The phase transition occurs at somewhat lower concentrations of polymers for the system based on DMFA than for the corresponding aqueous two-phase system. The similarity in phase-forming properties indicates that the phase separation is mainly due to the structures of the polymers and not primarily to the solvent they are dissolved in.

3.2. Some properties of the DMFA-based system

The partitioning of soluble substances between the two phases of aqueous two-phase systems is known to depend on several factors. These factors include the concentration of the polymers and their molecular masses, the salt content and the pH. Increasing polymer concentrations produce greater differences between the compositions of the two phases [10]. When the molecular mass of one of the phase-forming polymers is reduced, proteins show an increased partitioning towards the phase that is rich in this polymer.

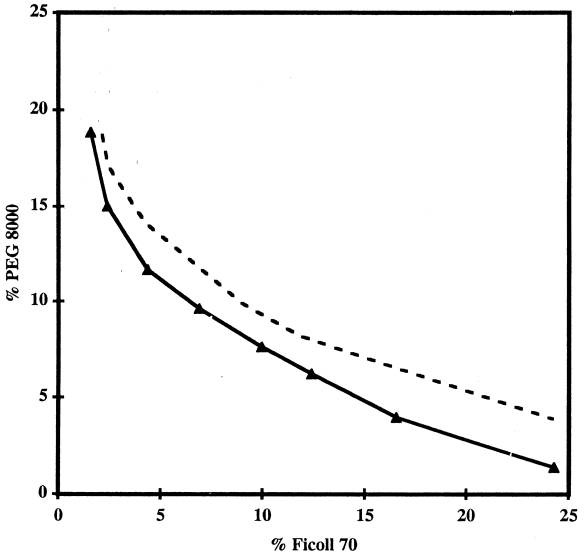

Fig. 1. Transition curve (between one and two phases) for a system composed of Ficoll 70, PEG 8000 and DMFA, ▲. Temperature, 23°C. The broken line indicates the corresponding curve with water as the solvent.

The organic two-phase systems based on DMFA were tested with polymers of various molecular masses. The effect of one or both polymers having a higher molecular mass was a prolonged settling time due to increased viscosity of the phases (see Table 1). The combination of Ficoll 70 and PEG 8000 was chosen for further study because of the rapid settling of the phases, which is particularly important for multistep extraction processes such as CCD.

3.3. Effect of salt on protein partitioning

The effect of salts on the partitioning behaviour of charged macromolecules in aqueous two-phase systems has been studied previously [3]. This steering effect of salts has been related to the chemical affinities of ions for the two phases, which may cause an orientation of the positive and negative ions

Table 1
Effect of polymer concentration on time for the phase separation of some organic two-phase systems at 23°C

Composition of system (w/w)	Settling time
8.5% Ficoll 400 8.5% PEG 8000 83% DMFA	70 min
4.5% Ficoll 70 4.5% PEG 400 000 91% DMFA	No visible phase separation within 2 h
10% Ficoll 70 5% PEG 40 000 85% DMFA	35 min
11% Ficoll 70 9% PEG 8000 80% DMFA	20 min

Height of systems, 6 cm.
The settling time was taken as the time when the mixed zone was not more than 5% of the total volume of the system.

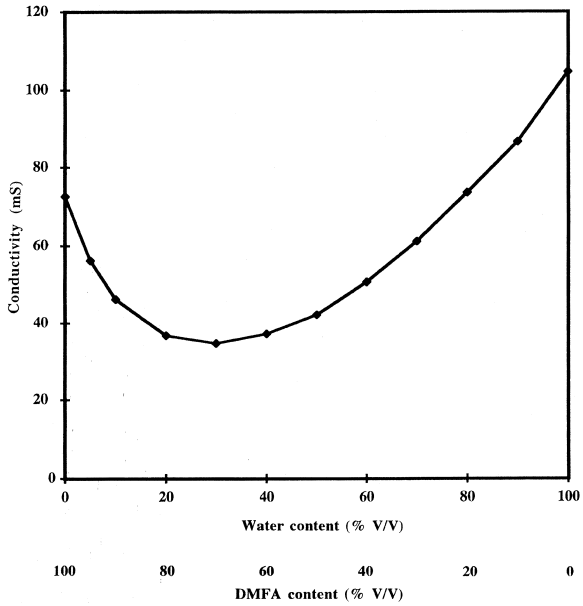

Fig. 2. Effect of the relative volume of water and DMFA on the electric conductivity of a 5 m*M* LiCl solution using a liquid mixture as the solvent. Temperature, 23°C.

at the interface between the two phases, resulting in an electrical potential difference across this interface [11]. Dissociation of the salt into free (solvated) ions is a necessary condition for this effect, which results in the influence of various salts on the partition coefficient of the charged material, proteins for example. The ability of an organic solvent to dissociate a salt is usually assumed to be quite low, which should strongly reduce the influence of salts on the partitioning of proteins in a system based on organic solvent. Fig. 2 shows the conductivity in different mixtures consisting of DMFA and water containing a fixed lithium chloride concentration. A solution of LiCl in 100% DMFA has a conductivity that is roughly 70% of that of an aqueous solution under the same conditions. This shows that DMFA has considerable dissociating strength. The conductivity minimum (32% of that in water) occurs at approximately 70 volumes of DMFA and 30 volumes of water, corresponding to a molar ratio of roughly two to one between water and DMFA, respectively. The low conductivity could be the result of an interaction between two water molecules and one DMFA molecule, generating a solvent that has less ability to dissociate ion pairs. Alternatively, the ions may be solvated by larger molecule aggregates, resulting in increased frictional force.

The use of salts for adjusting the partitioning of proteins in an organic two-phase system is limited by the low solubility of many salts in organic solvents. A number of organic and inorganic salts (mainly lithium salts) were included in the DMFA-based systems at various concentrations. In all cases, a strong positive relationship between salt concentration and phase volume ratio could be seen (Table 2). This may indicate that one of the ions of the salt bind to the top phase polymer (PEG), and the repelling of charges present cause the expansion of the top phase.

A commercial preparation of zein from corn was used as the test protein for partitioning in the DMFA-based two-phase systems. The protein had been extracted by ethanol from corn flour. It consists, according to Landry and Guyon [6,12], of several monomeric and dimeric proteins with monomeric masses of 22–24 kDa and with isoelectric points in the range 7.2–8.5, determined by isoelectric focusing in a concentrated urea solution. The partition coefficient of zein proteins is affected by the addition of various inorganic and organic salts. The protein partition coefficient, K=concentration of protein in upper phase/concentration in lower phase, in the

Table 2
Influence of various salts and their concentrations on the phase volume ratio (top phase/bottom phase) of a system with a composition of 11% (w/w) Ficoll 70, 9% (w/w) PEG 8000 and 80% (w/w) DMFA, at 23°C

Salt	Volume ratio at salt concentrations of:			
	0 m*M*	1 m*M*	2.5 m*M*	5 m*M*
L-Phenylalanine methyl ester–HCl	1.6	1.6	1.7	1.9
L-Tyrosine methyl ester–HCl	1.6	1.7	1.8	2.8
N-Carbobenzoxy-L-tryptophan	1.6	1.8	2.0	2.5
L-Tryptophan methyl ester–HCl	1.6	1.9	2.1	3.8
LiCl	1.6	2.0	2.3	3.3

presence of different salts is shown in Table 3. It was observed that the three methyl esters all decrease the partition coefficient, that is, they promote the affinity of the proteins for the bottom phase. Moreover it can be seen that the presence of L-tyrosine methyl ester–HCl produced the largest change in partitioning behaviour, followed by L-phenylalanine methyl ester–HCl, LiI and L-tryptophan methyl ester–HCl. In contrast, LiCl and methylguanidinium chloride gave rise to higher *K* values, which were close to unity. These results show that it is possible to steer the partitioning of proteins by adding various salts to this DMFA-based two-phase system, more or less in the same way as in aqueous two-phase systems. The salts were chosen because of their solubility in DMFA.

3.4. Partitioning of salts

Table 3 shows the partition coefficients of the salts that were included in the system. The salt concentrations in the phases were measured (after dilution with DMFA) via their electric conductivity. The values show that all of the salts have a greater affinity for the bottom phase (rich in Ficoll) than for the PEG-containing upper phase, and that the partition coefficients have more extreme values (0.3–0.9) than salts in aqueous two-phase systems (0.7–1.1) [3]. Comparing the *K* values of the salts with their effect on partitioning of zein (Table 3), the methyl esters of amino esters cause a more pronounced partitioning of protein into the bottom phase. In such cases, the protein partitions more into the lower phase as the *K* value of the salt increases. Likewise, LiCl gave a higher *K* value of zein than LiCl, while the latter has the highest partition coefficient. Methylguanidinium chloride, on the other hand, has the highest *K* value (0.9) and also causes the highest *K* value of protein (1.2).

Table 3
Mean partition coefficient, *K*, of zein proteins (5 g l^{-1}) with various salts included (5 m*M*) in systems containing 11% (w/w) Ficoll 70, 9% (w/w) PEG 8000 and 80% (w/w) DMFA at 23°C, as well as partition coefficients, K_{salt}, for the salts in a protein-free system

Salt	*K*	K_{salt}
–	1.03	–
L-Tyrosine methyl ester–HCl	0.40	0.59
L-Tryptophan methyl ester–HCl	0.70	0.31
L-Phenylalanine methyl ester–HCl	0.50	0.43
Methyl guanidine–HCl	1.21	0.90
LiCl	1.06	0.46
LiI	0.60	0.82

3.5. Influence of protein charge on partitioning

In aqueous two-phase systems, the influence of pH on partitioning has been studied to increase our understanding of system behaviour. The pH value is only defined for aqueous solutions, and it normally is measured with a glass electrode that is calibrated with aqueous buffer solutions. Nevertheless, it was found that glass electrodes could be used to quantify the degree of acidity in a DMFA solution by measuring the electromotive force (the voltage) over the glass electrode. The voltage increases when HCl is added to the DMFA, and drops when tetrabutylammonium hydroxide is added. Fig. 3 shows the observed voltage values when solutions of 10 m*M* tetrabutylammonium hydroxide in DMFA and 10 m*M* HCl in DMFA were mixed in various proportions. From the curve, it can be seen that

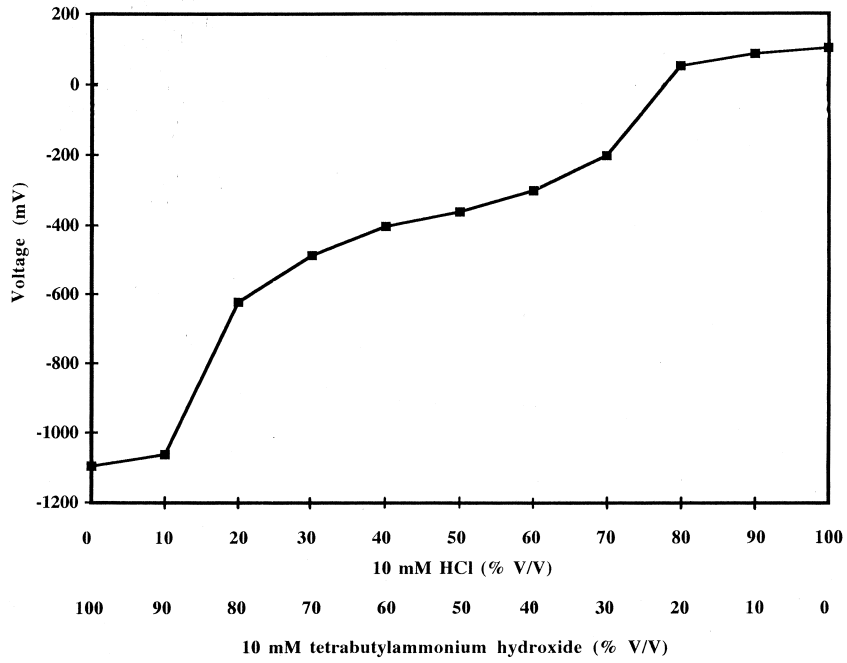

Fig. 3. Relationship between the voltage measured with a pH glass electrode and the relative proportions of 10 m*M* tetrabutylammonium hydroxide in DMFA and 10 m*M* HCl in DMFA. Temperature, 23°C.

tetrabutylammonium hydroxide behaves as a weak base in DMFA. The partitioning of zein was studied in DMFA systems that were adjusted with either HCl, tetrabutylammonium hydroxide, or not at all. These systems had glass electrode voltages of 0, −560 and −200 mV, respectively. The degree of acidity was found to affect the partition coefficient of protein. By adjusting the measured voltage to 0 mV, the proteins were assumed to be mainly positively charged, while a decrease to −560 mV would give mostly negatively charged proteins. This assumption is based on the isoelectric points (7.2–8.5) found for zein proteins [12]. In Fig. 4, the influence of the acidity of the system and the correlated protein charge on the partition coefficient of zein are shown. The common effect is that more positively charged proteins (in an acidic system) have a lower partition coefficient (increased affinity for the bottom phase) while the partition coefficients are higher for more negatively charged proteins (base-containing systems).

3.6. Ionic effects on partitioning and theory

The results above, with partitioning of zein at different acidities, indicate that the top phase side of the interface is positively charged relative to the bottom phase side, i.e. the cation of the salt has a relatively higher affinity to the upper phase compared with the anion. The relative affinity of ions for the two phases can be expressed by hypothetical partition coefficients for the cation (K_+) and the anion (K_-) [3,11]. These are the partition coefficients that the ions would have if they could partition independently of each other. When LiI is included in the system, the glass electrode voltage of the system has only a minor influence on the K value of the proteins (Fig. 4). This indicates that Li^+ and I^- have nearly the same relative affinity for the two phases, which can be expressed as $K_+ = K_-$. Lithium iodide can therefore be said to be a salt that generates a stable zero potential over the interface. Furthermore, the partition coefficient of LiI, $K_{LiI} = K_+(Li^+) =$

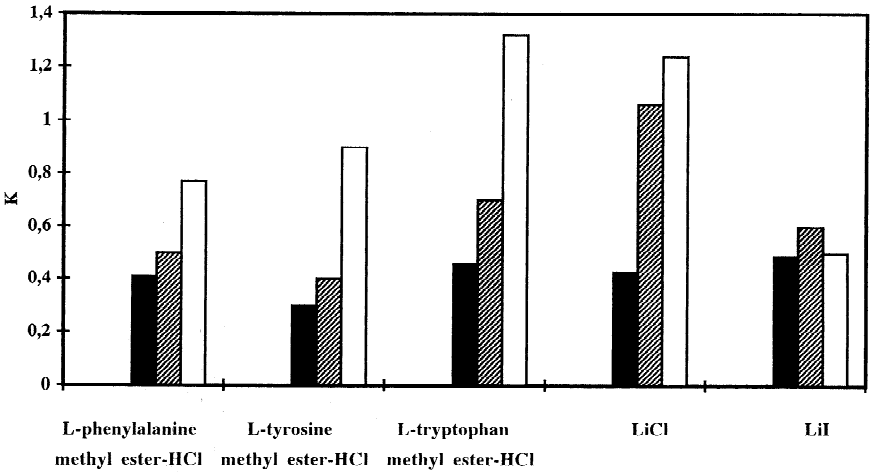

Fig. 4. Partition coefficient, *K*, of zein proteins, titrated to various degrees of acidity by the addition of HCl or tetrabutylammonium hydroxide, in the presence of various salts. System composition: 11% (w/w) Ficoll 70, 9% (w/w) PEG 8000, 79% (w/w) DMFA, 1% Zein, and 5 m*M* salt at 23°C. Symbols: filled column, proteins with a strongly positive net charge (at 0 mV); diagonal lined column, proteins with an intermediate net charge (at −200 mV); and unfilled column, proteins with a strongly negative net charge (at −560 mV).

$K_-(I^-)$. From this relationship, the hypothetical partition coefficients can be calculated from the K_{salt} values for all ions of 1–1-electrolytes, where one ion is either Li^+ or I^- by using Eq. (1).

$$K_{salt} = \sqrt{K_+ \cdot K_-} \quad (1)$$

The hypothetical partition coefficient for various ions calculated in this way are given in Table 4 and their relative effects on the partitioning of the proteins can be seen in Fig. 5. The bigger the difference between log K_+ and log K_- (or the more extreme the ratio

Table 4
Logarithms of hypothetical partition coefficients (K_+ and K_-) for various ions in a system with a composition of 11% (w/w) Ficoll 70, 9% (w/w) PEG 8000 and 80% (w/w) DMFA at 23°C

Cation	log K_+	Anion	log K_-
Methyl guanidinium$^+$	+0.50	I^-	−0.09
L-Tyrosine methyl ester.H$^+$	−0.44	Cl^-	−0.59
Li^+	−0.09		
L-Phenylalanine methyl ester.H$^+$	−0.14		
L-Tryptophan methyl ester.H$^+$	−0.43		

Calculated from K_{salt} using Eq. (1), assuming K_+ for lithium to be equal to K_- for iodide.

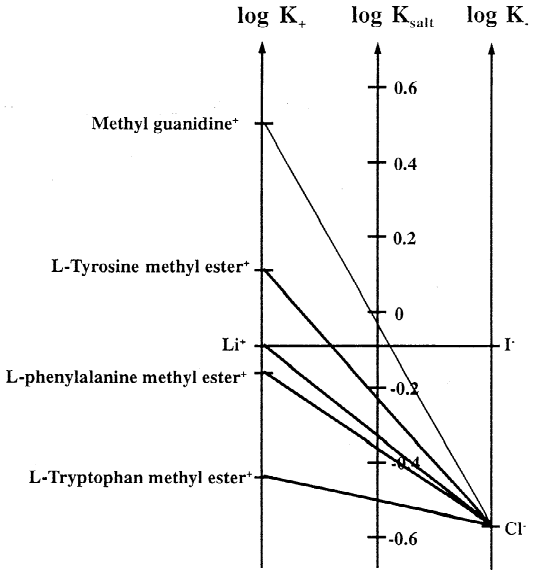

Fig. 5. Hypothetical partition coefficients of cations, K_+, and anions, K_-, of salts in a system containing 11% (w/w) Ficoll 70, 9% (w/w) PEG 8000 and 80% (w/w) DMFA. Temperature, 23°C. The K_+, K_- and K_{salt} values are related by log K_{salt} = (log K_+ + log K_-)/2, obtained from Eq. (1). The effect of the salt on the partitioning of a protein with a given net charge is proportional to the difference log K_- − log K_+.

K_-/K_+), the larger the effect of the corresponding salt on the partitioning of the proteins. The use of a salt for which $K_+ > K_-$ results in a larger percentage of the positively charged proteins in the bottom phase, while a salt with $K_+ < K_-$ drives the positively charged protein molecules to the top phase. The partition coefficient of protein, K_p, can then be expressed (as is the case for aqueous two-phase systems [3]) by Eq. (2),

$$K_p = K_0\left(\frac{K_-}{K_+}\right)^Z \quad (2)$$

or the logarithmic form (Eq. (3)),

$$\log\left(\frac{K_p}{K_0}\right) = Z \cdot \log\left(\frac{K_-}{K_+}\right) \quad (3)$$

where Z is the net charge of the protein and K_0 is the partition coefficient of the protein in the presence of a salt for which $K_+ = K_-$. From Eq. (3), it can be seen that $\log(K_p/K_0)$ is proportional to the protein's net charge.

3.7. Counter-current distribution

The ability to steer partitioning in organic systems by including a salt is a big advantage when the two-phase system is used for multiple extractions, as is the case with CCD [9]. The resolution of proteins in the CCD is related to the average G value, that is, the amount (mass) of partitioned material in the top phase divided by the amount in the bottom phase. Fig. 6 shows the results of CCD of zein using a DMFA-based two-phase system. The diagrams illustrate the effects of LiCl and LiI, respectively. The distribution curves are much too broad to be generated by a homogeneous protein and comparison with theoretical curves suggests that the zein used consists of (at least) three main components, localised under the broad peak in the middle of the diagram. By testing various G values and relative amounts, theoretical distribution curves were calculated [13] using Eq. (4):

$$T_i = \frac{n!}{i!(n-i)!} \cdot \frac{G^i}{(1+G)^n} \quad (4)$$

where T_i is the fraction of the substance recovered in tube i and n is the number of transfers of upper phases relative to lower phases. The above values were varied to get a good fit between the sum of the peaks of the three assumed components and the experimental curve. The G values obtained for the three assumed components under the main peak were 0.32, 0.85 and 1.63, in the case of LiCl, and 0.56, 1.34 and 3.63, in the case of LiI. In the case of LiCl, two more theoretical peaks are included, one to the far left and one to the far right. The G values of the three hypothetical proteins are lower for a system with LiI than with LiCl. This correlates with the fact that the partition coefficient for zein with LiCl (Table 3), at intermediate net charge conditions, is 1.8 times higher than that with LiI. Since the phase volume ratio (top to bottom phase) was 1.8, the K values of the three assumed components would be 0.18, 0.47 and 0.91, in the case of LiCl, and 0.31, 0.74 and 2.0, in the case of LiI. The latter K values must be close to K_0 for the proteins since K_+ and K_- seem to be approximately equal for LiI. Thus, according to Eq. (3), the ratios between the log (K_p/K_0) values, 0.22:0.20:0.35, for the three peaks, also gives the ratios between the (negative) net charges of the hypothetical proteins. The CCD experiments shows that a fractionation of zein is possible but that a greater number of transfers are necessary. Furthermore, the net charge of the protein fractions may be estimated. Work to compare these charges with the electrophoretic mobility of zein proteins in DMFA is in progress.

4. Conclusions

Polymeric two-phase systems based on DMFA can be used for partitioning and separation of water-insoluble proteins. In addition, their partitioning can be adjusted by including DMFA-soluble salts in the systems. The salt steering effect is similar to that in aqueous two-phase systems and can be related to the relative affinity of the salt ions for each of the two phases. By using the DMFA-based system for a counter-current distribution process, non-water-soluble proteins can be fractionated.

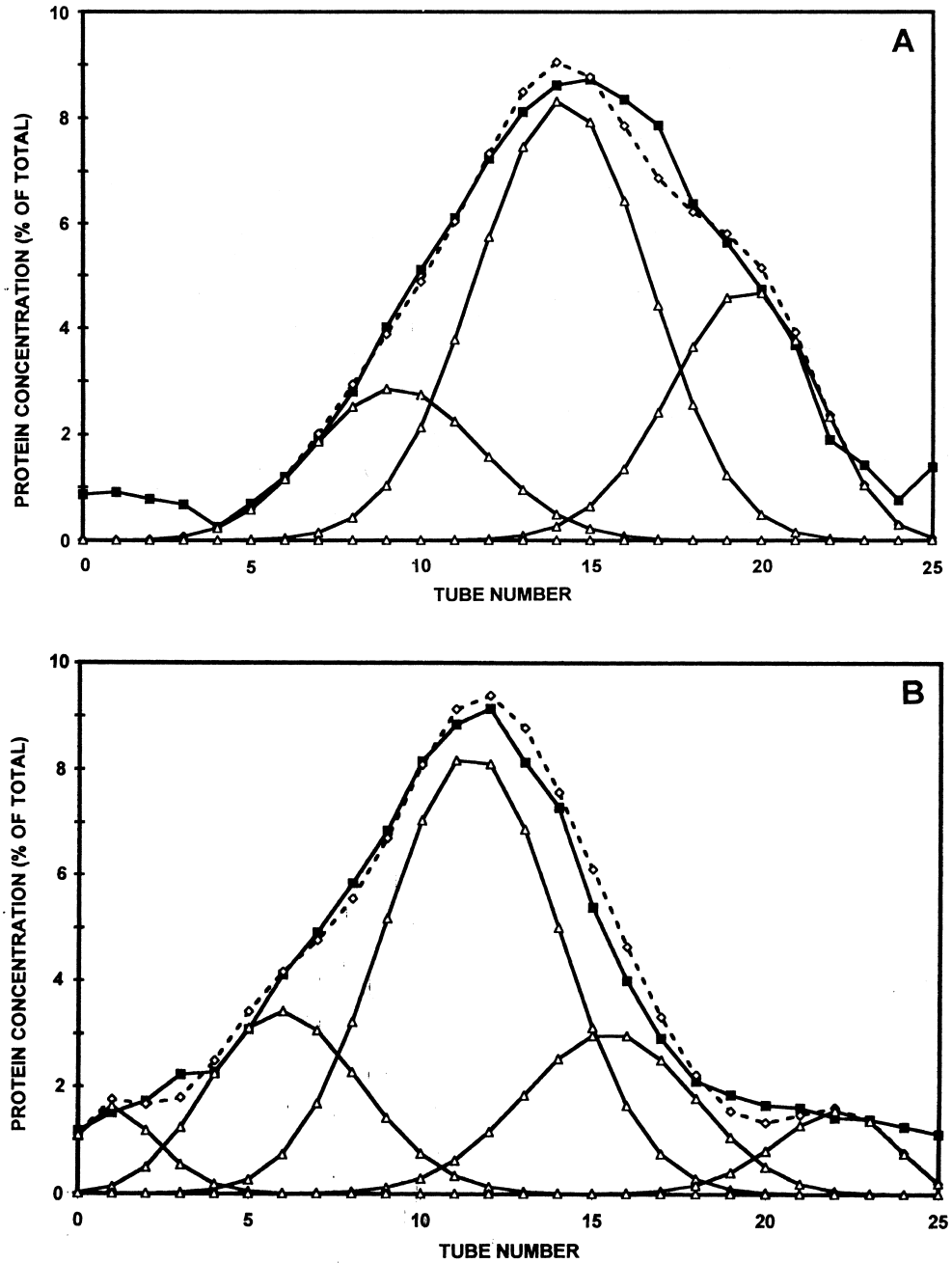

Fig. 6. Counter-current distribution of zein in a DMFA-based two-phase system (for experimental details, see Section 2) using 25 transfers (A) in the presence of 2.5 m*M* LiCl and (B) in the presence of 2.5 m*M* LiI. (■), Experimental values; (△), theoretical curves and (◇), the sum of the theoretical curves. Temperature, 23°C.

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References

[1] P.-Å. Albertsson, Partition of Cell Particles and Macromolecules, Wiley, New York, 3rd ed., 1986.
[2] H. Walter, G. Johansson (Editors), Methods in Enzymology, Vol. 228, 1994.
[3] G. Johansson, Acta Chem. Scand., Ser. B28 (1974) 873–882.
[4] G. Johansson, Methods Enzymol. 228 (1994) 28–42.
[5] G. Johansson, M. Joelsson, J. Chromatogr. 464 (1989) 49–59.
[6] J. Landry, P. Guyon, Biochimie 66 (1984) 451–460.
[7] 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, D.C. Klenk, Anal. Biochem. 150 (1985) 76–85.
[8] H.-E. Åkerlund, P.-Å. Albertsson, Methods Enzymol. 228 (1994) 87–99.
[9] A. Dobry, F. Boyer-Kawenoki, J. Polymer Sci. 2 (1947) 90–100.
[10] G. Johansson, G. Kopperschläger, P.-Å. Albertsson, Eur. J. Biochem. 131 (1983) 589–594.
[11] P.-Å. Albertsson, Partition of Cell Particles and Macromolecules, Wiley, New York, 3rd ed., 1986, pp. 65–67.
[12] J. Landry, P. Guyon, Biochimie 66 (1984) 461–469.
[13] C.J.O.R. Morris, P. Morris, Separation Methods in Biochemistry, Pitman, London, 1964, pp. 559–619.