Purification and Characterization of Crystallins by Aqueous Two-Phase Extraction

Ondrea Bermudez and Daniel Forciniti*

Chemical Engineering Department, University of Missouri-Rolla, Rolla, MO 65409, USA

Abstract Crystallins are a family of water-soluble proteins that constitute up to 90% of the water-soluble proteins in mammalian eye lenses. We present in this paper an alternative purification method for these proteins using polyethylene glycol/dextran aqueous two-phase extraction. Under the appropriate conditions, we were able to recover the γ-crystallin fraction essentially free of the remaining proteins. High concentrations of salt at a neutral pH maximize the recovery of γ-crystallins in the top phase and minimize the contamination by the other proteins present in the lenses. The proposed protocol decreases the separation time by about 50%. The complex partition behavior observed for these proteins reflects a delicate balance between protein/phase-forming species (various polymers and salts) and protein/protein interactions. This is evidenced, in part, by the role played by the largest proteins in this group as a "pseudo" phase-forming species.

Keywords: crystallins, purification, aqueous two-phase systems.

INTRODUCTION

Aqueous two-phase extraction has been used for the purification of a variety of proteins and for the fractionation of cell populations [1]. The high water content of these systems (which gives the technique its name) provides a gentle environment for biologically active proteins, cells, and cell organelles. Aqueous two-phase extraction is a technique that is ideally suitable for the removal of cell debris and simultaneous concentration of the target protein; furthermore, it can be used as a very selective procedure by adding affinity ligands to one of the phase-forming polymers [2,3]. For either industrial or recombinant proteins the method offers notable advantages. For industrial enzymes, the method's utility stems from the ease with which it can be adapted to continuous production and scaled up to meet industrial needs. For recombinant proteins, its utility is based on its attractiveness as a gentle first step or on its selectivity on a small scale by using affinity ligands. Aqueous two-phase systems can be also used for analytical purposes. For example, the protocols for the determination of isoelectric points and hydrophobicities are well-established [4-6].

The technique is based on the observation that when above-critical concentrations of an aqueous polymer solution or a polymer and a salt solution are mixed, the mixture splits into two phases separated by a sharp interface. One phase is rich in one polymer (or the salt) and the other is rich in the other one. Still, each phase is up to 90% water. Although there is a myriad of polymers and salts that can be used to generate aqueous two-phase systems, the most widely used ones are Poly(ethylene)glycol (PEG)/Dextran (Dx) and PEG/sodium phosphate.

Large molecules (like proteins) partition unequally between the phases. The separation power of a given aqueous two-phase system is given by the partition coefficient, K, of a protein. K is defined as the ratio of the protein concentration in the top and bottom phases This coefficient depends on the difference in chemical potential of the protein between top and bottom phases and therefore, it is a function of the chemical nature of the polymers, the protein, added electrolytes, and temperature. Consequently, the partition behavior of proteins can be manipulated by changing the concentration of the phase-forming species, their molecular weight, pH, type and concentration of added salts and by the addition of affinity ligands. By a judicious manipulation of these variables, separation of complex mixtures of proteins can be achieved. Because the partition behavior of a given protein depends on its solubility in both phases, the partition coefficient (or rather its dependence on various parameters) can be used to gain an insight into the solution behavior of proteins.

Manipulation of the pH is of primary importance in partitioning studies of proteins because partition coefficients are very sensitive to pH changes. In the analysis of partitioning data at different pHs we must consider any change in the physical properties of the proteins due to changes in the pH and how these changes may affect the protein partition coefficient. The molecular weight and concentration of the phase-forming species also affect the partition coefficient strongly. For example, in a PEG/Dx system, low PEG molecular weight favors the partitioning of proteins into the PEG-rich

* **Corresponding author**
Tel: +1-573-341-4427 Fax: +1-573-341-4377
e-mail: forcinit@umr.edu

phase. The effect of polymer concentration on the partition coefficient is also well known. If K is smaller than 1, an increase in either one of the phase-forming species concentration decreases K. Similarly, if K is larger concentration in either of the phase forming species increases K. The amount of the phase-forming species also affects the volume ratio between the phases; and therefore, the technique can be used for the simultaneous isolation and concentration of a target protein.

Small molecules have partition coefficients close to one. Still, the slightly uneven partition of electrolytes has a strong effect on the partitioning of large molecules. In general, the partition coefficient of proteins away from their isoelectric point depends on both the type and concentration of cation and anion. For example, for positively charged proteins the partition coefficient in PEG/Dx systems is higher in potassium chloride than in potassium phosphate; the reverse is true for negatively charged proteins.

In this paper we explore the partition behavior of a group of proteins called crystallins. Crystallins constitute approximately 90% of all water-soluble proteins of mammalian lenses. Their unique solution properties give the lenses the necessary optical properties for vision. Physical or chemical changes in these proteins are responsible for the development of cataracts -by far the most common of the serious vision problems. Crystallins are a heterogeneous family of proteins, which is subdivided into three subfamilies: α-crystallin (molecular mass ~ 800,000) is an oligomeric globular protein, β-crystallin (molecular mass from 50,000 to 300,000) and γ-crystallins (molecular mass ~25,000). Both α and β crystallins are present in the eye lenses as aggregates of various molecular sizes. γ-crystallins are monomeric and they are subdivided into seven or eight subgroups of almost identical molecular weight and subtle differences in their isoelectric point. The main subgroups are γ I, II, III and IV crystallins. The differences in size and isoelectric point of this group of proteins (Table 1) make them an ideal target for their separation by aqueous two-phase systems.

There is a cataract-related phenomenon known as cold cataracts in which cooling of the lenses below a critical temperature induces a liquid/liquid phase transition in the cytoplasm of the lenses (similar to the one observed in aqueous two-phase systems) [7]. In cold cataracts, a phase rich in protein and a phase rich in water coexist as a fine emulsion, which makes the lenses opaque. Because it is believed that understanding cold cataracts may lead to a better understanding of pathologic cataracts and because γ-crystallins undergo a similar phase transition, intensive experimental and theoretical research has been done on these particular proteins [8-10]. This amount of experimental work is possible if large amounts of the protein are available. Crystallins are usually purified for further studies from mammalian lenses (most commonly bovine) by (1) tissue grinding, (2) centrifugation to eliminate cell debris, (3) gel permeation chromatography to separate the α/β fraction from the γ fraction, and (4) ion exchange chromatography to fractionate γ-crystallins into its individual components. The entire process takes approximately five days and most of the time is used in the gel permeation step.

Table 1. Isoelectric points and molecular weights of crystallins.

Protein	Molecular weight	Isoelectric point
α	1,070,000	5.1
β	165,000,	7.0
γI	22,000	7.0
γII	19,100	7.8
γIII	20,100	7.45-7.8
γIV	20,000	7.85
γIIIa	20,900	7.45
γIIIb	19,900	7.8
γIVa	20,800	7.9
γIVb	N/A	8.6

Our primary goal is to explore the use of aqueous two-phase systems to separate γ-crystallins from their larger cousins in a single (and fast) step. Replacement of the gel permeation step by aqueous two-phase extraction would considerably reduce the separation time. This is critical because under prolonged exposure to air during long separation procedures these proteins oxidize irreversibly forming large aggregates. A secondary objective is to use their partition behavior to gain new knowledge about the solution behavior of these proteins, which we have already studied by small angle neutron scattering and by dynamic light scattering [11-12].

MATERIALS AND METHODS

Materials

The lenses (of various ages) were bought from Animal Technologies, Inc. (Tyler, TX, USA) or obtained from a local farm. The polymers (PEG: 1,300, PEG 3,350, PEG 8,000, PEG 10,000 and Dx 265,000 and Dx 75,000) were analytical grade batches from Sigma (St. Louis, MO, USA). The salts and buffers were all analytical grade.

Methods

Preparation of cell extract. Bovine lenses were homogenized in four volumes of 100mM sodium phosphate buffer with 3 mM sodium azide and 100 mM mercapto ethanol using a tissue grinder at room temperature. The homogenate was centrifuged at 21,000 × g for one hour at 5°C to eliminate cell debris.

Preparation of aqueous two-phase systems. Here we present a synopsis of the steps followed to prepare the systems. The interested reader is referred to Ref. 13 for further details. Stock solutions of the various polymers were prepared in nanopure water (30% w/w). All the partition experiments were performed at room tem-

perature. To a centrifuge tube we added: stock solution of PEG, stock solution of dextran, the buffer containing a salt (either NaCl, LiBr, or LiCl), from 1 g to 0.25 g of the cell homogenate (either previously centrifuged or containing the cell debris) and enough buffer to complete either 5 or 9 g. The systems were mixed for about 10 minutes and centrifuged for 20 minutes at 2,000 × g. Duplicates and in some cases triplicates were prepared. Approximately one-gram samples of the top and bottom phases were carefully removed with a pipette and diluted as needed. A slight positive pressure was used to sample the bottom phase to avoid contamination by the top phase. Absorbance at 280 nm against blanks of the phase systems properly diluted was used to determine protein concentration. This is preferred to a colorimetric assay because it is more accurate in spite of the fact that different proteins have different molar absortivities (colorimetric assays do not measure absolute protein concentrations but rather relative amounts with respect to a standard curve, whose position depends on the standard protein used for calibration). The partition coefficient was estimated as $K = A^{280}_{top} / A^{280}_{bottom}$.

Samples of the top and bottom phases were isoelectric focused to determine the degree of separation achieved. This was done on polyacrylamide gels (under native or under denaturing conditions) covering a pH range from 3 to 10 or from 5 to 8. The isoelectric focusing gels were prepared by mixing 24.25 g acrylamide, 0.75 g Bis-(*N,N'*-methylene-bis-acrylamide), 25 g glycerol, 50 μL 0.1% (w/v) riboflavin 5'-phosphate, 15 μL 10% (w/v) ammonium persulfate, and 3 μL *N,N,N',N'*-tetramethylethylenediamine in 200 mL of water. Polymerization of the gels was achieved by UV illumination for one hour. The gels were focused on a BioRad apparatus for approximately 30 min after the voltage reached 1,500 V. All gels were stained with Commassie Blue with some being restained with silver nitrate.

A few samples of top and bottom phases as well as the supernatant of the cell homogenate were run through a 60 × 1.5 cm column packed with Sephadex 75 and eluted with phosphate buffer at pH 7. This was done to quantify the separation achieved by aqueous two-phase extraction.

RESULTS AND DISCUSSION

One of the typical characteristics of mammalian lenses is a decrease in the amount of the smallest proteins (γ-crystallins) upon aging. One accepted explanation is that γ-crystallins oxidize upon aging forming large aggregates that become insoluble. These changes in the lenses' composition may have an effect on the partition behavior of the cell homogenate. Therefore, it was important to determine which age group would potentially yield a better separation of these proteins using aqueous two-phase systems (although calf lenses are routinely used in the traditional separation method). With that in mind, we partitioned cell homogenates of bovine lenses of different ages at pHs 4.2, 7, and 9.7

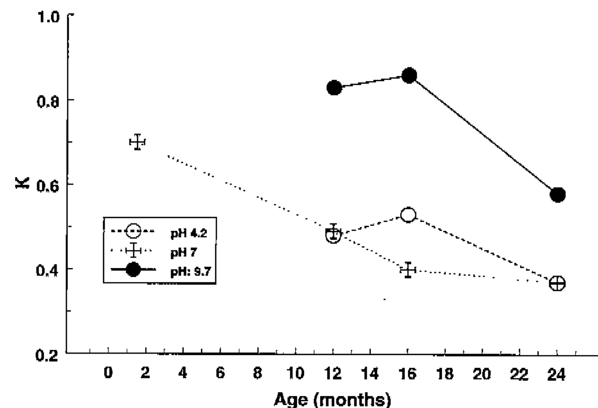

Fig. 1. Partition coefficient versus age of the animal at three pHs.

using a PEG 3,350/Dx 267,000 aqueous two-phase system. The polymer concentrations were 7.64% PEG and 10.5% dextran; these polymer concentrations correspond to a tie line length of 25% w/w. The length of the tie line is defined as the square root of $\Delta PEG^2 + \Delta Dx^2$, where Δ PEG= $[PEG]^{top} - [PEG]^{bottom}$ and $\Delta Dx = [Dx]^{top} - [Dx]^{bottom}$. The data is shown in Fig. 1. It is clear from the figure that the partition coefficient at pH 7 decreases as the age of the animal increases. The partition coefficients for 24 month old lenses is smaller than those of younger animals at pHs 4.2 and 9.7, but the differences between 12 and 16 months old lenses are not significant. A decrease in the partition coefficient is associated with an increase in the molecular weight of the sample -- it is well known that the partition coefficient decreases nearly exponentially with molecular weight. Therefore, it is safe to assume that the average molecular weight of the homogenate increases as the age of the specimen increases. The increase in the molecular weight of the preparation upon aging can be due to two related phenomena. First, the amount of γ-crystallins is lower in older specimens; therefore, the average molecular weight of the preparation will increase with age and the decrease in the partition coefficient just reflects this fact. Second, in older animals some of the γ-crystallins may be naturally oxidized (the early stages of cataracts are characterized by oxidation of crystallins); and therefore, present as large aggregates. Here again, the average molecular weight increases and therefore the partition coefficient decreases. These results are a vivid example of the power of aqueous two-phase systems as an analytical tool. Because the partition coefficient is sensitive to the composition of the lenses, it is conceivable that the technique could be eventually used to rapidly quantify either the γ-crystallin content of the preparation or to determine their degree of oxidation. Another obvious trend shown by the data in this figure is that there are not major differences in the partition coefficient between pHs 4.2 and 7 but the partition coefficient substantially increases as the pH becomes more basic. This is discussed

in detail in the following paragraphs.

The remaining experiments were all performed with eye lenses from six-week-old calves because no major differences in the distribution of proteins from different ages were observed and because newborn calf lenses contain the largest amount of γ-crystallins. All experiments were performed with centrifuged cell homogenates unless noted differently. Before exploring different conditions, we performed a few exploratory experiments to determine whether it is feasible to separate γ-crystallins from the other proteins. Cell homogenates were partitioned in PEG/Dx systems (three different PEGs were used, 1,300, 3,000, and 8,000 and one dextran MW: 265,000; the total concentration of polymers was: 6 and 10% w/w respectively) at pH 9 without the addition of salts. The resulting partition coefficients were quite low (K=0.58, 0.35 and 0.11 for the PEG 1,300, 3,000, and 8,000 containing systems, respectively). Because the partition coefficient, as defined by us, represents the distribution of total protein between top and bottom phases, its value does not provide any direct evidence about the degree of separation achieved. Therefore, samples of the top and bottom phases were run through a gel permeation chromatography column to quantify the separation achieved. The composition of the top and bottom phases can be estimated by isoelectric focusing of the different fractions. Fig. 2 shows a gel permeation chromatogram of the cell homogenate as well as chromatograms corresponding to the top and bottom phases. The fraction eluting at approximately 50 mL corresponds to a mixture of α and β-crystallins whereas the fraction eluting at approximately 150 mL corresponds to γ-crystallins. It is obvious that the separation between γ and α/β-crystallins is almost complete. Repeated extractions with the top phase can be done to improve the yield. The remaining experiments were aimed (1) to improve the recovery of γ-crystallins in the top phase by adding various salts, changing the molecular weight of the polymers and by varying the pH and (2) to highlight the differences in the partition behavior between the different species.

The partition coefficient of proteins in aqueous two-phase systems is quite sensitive to the pH of the phases because the solubility of proteins is a strong function of pH. This sensitivity makes the selection of the pH one important design parameter in any aqueous two-phase systems separation. Although there is not a comprehensive model that allows the prediction of the pH dependence of the partition coefficients of a target protein in a complex mixture, some empirical correlations can be used. For example, the partition coefficient has been correlated with the charge of the protein (and therefore the pH of the solution) by [14]:

$$\ln K = \ln K_0 + \Gamma z \quad (1)$$

where K_0 is the partition coefficient at zero charge, z is the protein's charge and Γ is a coefficient that depends on the type of protein, the type of phase system, and the type and concentration of salt used. One can expect

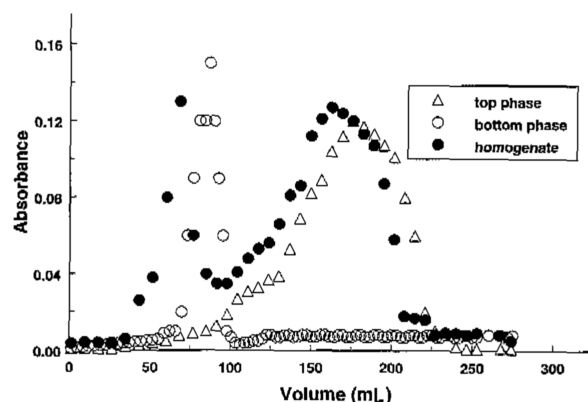

Fig. 2. Gel permeation chromatography of (a) cell homogenate; (b) top phase; and (c) bottom phase of PEG 1,300/Dx 265,000 at pH 9.

this equation to be valid for single proteins in a relatively narrow pH range. Still, it can be used in preliminary experimental design. The isoelectric point of these proteins ranges from 5.1 (α-crystallins) to 8.6 (γIVb-crystallins). Therefore, we conducted a series of experiments at pHs 5, 7 and 9 to cover a wide range of charge combinations. At both pH 9 and 5 all the proteins are either negatively or positively charged. At pH 7, on the contrary, α-crystallins are negatively charged whereas β-crystallins are almost at their isoelectric point and γ-crystallins are positively charged. The polymer concentrations used were: PEG 3,350: 7% w/w, Dx 265,000: 7% w/w; PEG 8,000: 5.9% w/w, Dx 265,000: 5.8% w/w; PEG 10,000: 3.8% w/w, Dx 265,000: 5.20% w/w. These concentrations yield tie line lengths of 17, 15, and 12% w/w respectively. The buffer used in all cases was 0.050 M sodium phosphate. The partition coefficients versus pH are shown in Fig. 3. The value of Γ for sodium phosphate is negative (Γ= -23); so, we might expect a minimum in the partition coefficient at pH 5 because all proteins are positively charged at this pH and this combination of charges will result in the largest subtraction from $\ln K_0$ (Eq. (1)). At pH 7 γ-crystallins remain positively charged, β-crystallins are isoelectric and α-crystallin is negatively charged; therefore, the partition coefficient increases from pH 5 to 7. At pH 9 all the proteins are negatively charged and the partition coefficient reaches a maximum. Although the reasoning based on Eq. (1) indicates that the increase in the partition coefficient could be due mainly to an increase in the amount of α-crystallin in the top phase, the series of gels shown in Fig. 4 depicts a different picture. These gels confirm that the highest partition coefficient is achieved at pH 9, but they also indicate that a better separation is achieved at pH 7. The sequence of gels shows that at pH 7 the contamination of the top phase by α-crystallin has nearly disappeared --as evidenced by the absence of marks in the acidic region of the gel. It is impossible to rationalize these findings by looking at established trends of protein partitioning with pH and

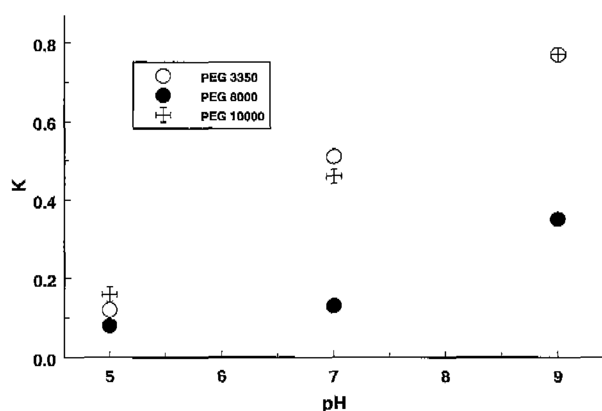

Fig. 3. Partition coefficient versus pH for systems containing PEG 3,350, 8,000 and 10,000 and Dx 265,000.

polymer molecular weight, which predict a maximum in the partition coefficient of γ-crystallins at pH 9. The arguments must be based on the effect that protein-protein interactions have on partitioning (one aspect that has been overlooked in the past). At pH 7 α-crystallins are negatively charged; so, they should partition into the top phase. This would decrease the purity of the γ-crystallin fraction in that phase. However, the top phase has little room for the bulkier α-crystallin. Therefore, α-crystallin partitions into the bottom phase displacing the smallest γ-crystallin into the top phase. We may think of α-crystallins as another phase forming species that is being concentrated in the bottom phase.

Dramatic changes in the partition coefficient can be achieved by adding salts at moderate to high concentrations. Their effect can be estimated by correlations between the parameter Γ in Eq. (1) [14,15] and the type of cation and anion added to the system. Salt effects can be rationalized as follows. Small ions distribute unequally (but only slightly) between the two phases. This uneven distribution of electrolytes creates a ionic atmosphere in one phase that differs from the ionic atmosphere in the other one. The electrostatic contribution to the free energy of the system can be estimated by calculating the necessary work of insertion of a point charge as it is brought from infinity into the phase. Because the ionic atmospheres in both phases are different, there will be a difference in the work of insertion; and therefore an electrostatic potential difference will be created across the interface (similar to the Donnan potential observed in cells). Accepted values for Γ for NaCl, and LiBr (at moderate salt concentrations, $<$ 1 M) are: $\Gamma_{NaCl} = 6$ and $\Gamma_{LiBr} = -1$. So, we expect NaCl to increase K for positively charged proteins and LiBr to decrease K for positively charged proteins. The reverse effect is expected for negatively charged proteins. The partition coefficients of the preparation upon addition of the two salts at two pHs and for three different phase systems are presented in Table 2. They cover three PEG molecular weights (1,300, 8,000, and 3,350) and two pHs, 7 and 9. The total polymer concentration is 6% w/w PEG and 10% w/w dextran; and the molecular weight of dextran was fixed at 265,000. The partition behavior reflects the differences in charge of these proteins. At pH 7, the addition of NaCl increases the partition coefficient and the effect of the salt is more noticeable at the lowest PEG molecular weight. At this pH α-crystallins are negatively charged whereas γ and β-crystallins are positively charged or near their isoelectric point. The fact that K increases upon addition of this salt ($\Gamma > 0$) reflects an increase in the concentration of γ-crystallins in the top phase. At pH 9, the partition coefficients increase even further; again, the effect of the added salt is more pronounced at low molecular weight PEG. All proteins are negatively charged at this pH, and Eq. (1) breaks down. We speculate that strong repulsion between the various proteins is responsible for this anomalous behavior. A high concentration of charged molecules in one phase is unfavorable and, therefore, they distribute between the phases. The addi-

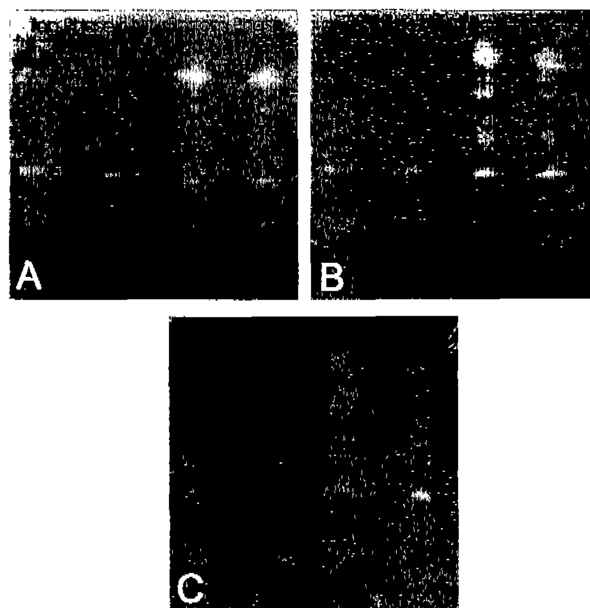

Fig. 4. Isoelectric focusing gels of top and bottom phases of a PEG 10,000/Dx 265,000 system at three pHs. The pH range is from 3 (top) to 10 (bottom). (a) top and bottom phases at pH 5; (b) top and bottom phases at pH 7; (c) top and bottom phases at pH 9.

Table 2. Partition coefficients at two pHs with and without the addition of NaCl or LiBr for Dx 265,000 and PEG 1,300, 3,350 and 8,000

	PEG 1,300			PEG 3,350			PEG 8,000		
	No Salt	0.1 M NaCl	0.1 M LiBr	No Salt	0.1 M NaCl	0.1 M LiBr	No Salt	0.1 M NaCl	0.1 M LiBr
K (pH 7)	0.15	1.00	1.30	0.15	0.32	0.15	0.36	0.39	0.12
K (pH 9)	0.58	1.70	1.40	0.35	0.21	1.10	0.11	0.14	0.07

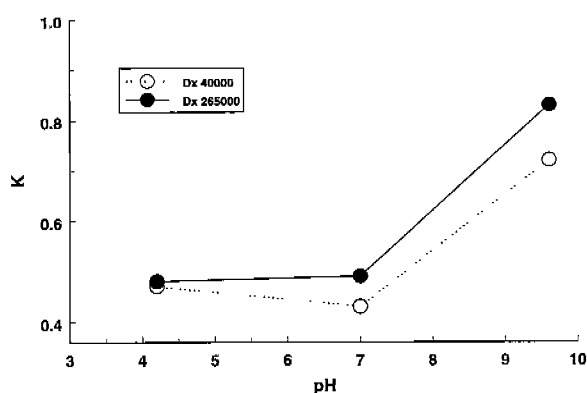

Fig. 5. Partition coefficient versus pH at two Dx molecular weights.

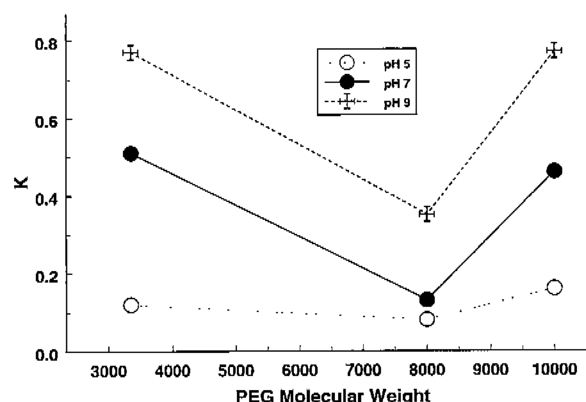

Fig. 6. Partition coefficient versus PEG molecular weight.

tion of LiBr to the systems at pH 7 increases the partition coefficient at low molecular weight PEG, it does not affect K for PEG 3,350 containing systems, and decreases K for PEG 8,000 containing systems. We can rationalize this observation as follows: α-crystallins are negatively charged at pH 7 and they are bulky; so, they can move into the PEG rich phase of a PEG 1,300/Dx system because the PEG size is quite small. As the molecular weight of the polymers increases, excluded volume effects dominate and the partition coefficient decreases. All proteins are negatively charged at pH 9. Therefore, LiBr should increase the partition coefficient. This is true for the PEG 1,300 and 3,350 containing systems. However, the partition coefficient slightly decreases for the PEG 8,000 containing system. As the PEG molecular weight increases, the proteins are completely excluded and the partition coefficient is very small. Once again, excluded volume effects dominate electrostatic effects when high molecular weight PEGs are used.

Another known feature of the partition behavior of proteins in aqueous two-phase systems is that an increase in the molecular weight of Dx increases the partition coefficient whereas an increase in the PEG molecular weight decreases it (an increase in the excluded volume as the polymer molecular weight increases is believed to be the phenomenon underlying this trend). The experiments were performed using two PEG/Dx systems (PEG 3,350 8.8% w/w/ Dx 40,000 10.55% w/w and PEG 3,350 7.64% w/w/Dx 265,000 10.5% w/w). The preparation behaves as expected, at least at alkaline pH, upon changes in the molecular weight of dextran (Fig. 5), which shows an increase in the partition coefficient at pH 9 as the molecular weight of dextran increases. There are marginal differences between the two dextrans at pH 4, but these differences seem to be amplified as the pH increases. This amplification of pH effects by low polymer molecular weight has been ob served before in the partitioning of several pure proteins in a series of PEG/Dx systems [16,17]. Fig. 6 shows the partition coefficient of these proteins versus PEG molecular weight. The systems used were: PEG 3,350 7.02% w/w/Dx 265,000 7% w/w; PEG 8,000 5.82% w/ w/Dx 265,000 5.83% w/w; and PEG 10,000 3.8% w/w/ Dx 265,000 5.2% w/w. We expect a monotonic decrease in K as the PEG molecular weight increases only when the overall polymer concentration remains constant. However, the partition coefficients seem to go through a minimum for a PEG molecular weight of 8000. These types of observations are relatively common because it is not always possible to isolate the effects of polymer molecular weight and polymer concentration (for a discussion see Ref. 17). As the molecular weight of PEG increases (Fig. 6), the polymer concentration in the phases becomes smaller (shorter tie line length). Therefore, molecular weight effects dominate partitioning as the size of PEG increases from PEG 3,500 to PEG 8,000, and the partition coefficient decreases. On the contrary, the increase in the partition coefficient caused by a decrease in the tie line length overcompensates the decrease in the partition coefficient caused by increasing the molecular weight of PEG from 8,000 to 10,000 (nearly 20%), and therefore, the partition coefficient increases.

Finally, we performed a few experiments using the cell homogenate without centrifugation. By doing this, we are eliminating two steps in the traditional separation method: centrifugation and gel permeation. Since γ-crystallins are recovered in the top phase, PEG (the main contaminant in the phase) can be eliminated by ultrafiltration [16] before ion exchange chromatography. This does not add an additional step to the separation procedure since either ultrafiltration or dialysis is needed between the two chromatography steps simply to change buffers. We performed a series of partition experiments with Dx 265,000 (10.46% w/w) and PEG 3,350 (7.64% w/w) at pH 7 with and without the addition of 0.05 M or 2 M NaCl or LiCl (Γ=-7) using our results with the supernatant as guidelines. The partition coefficients for 0.02 M salt were K= 0.63 and 0.49 for LiCl and NaCl, respectively. For the highest salt concentration, the partition coefficients were K=0.48 and 1.73 for LiCl and NaCl, respectively. Isoelectric focusing (Fig. 7) clearly shows that whereas the addition of LiCl

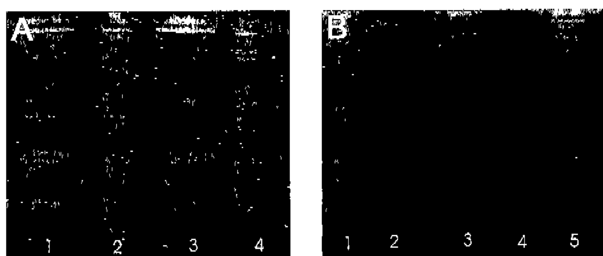

Fig. 7. Isoelectric focusing gels of top and bottom phases of systems containing either NaCl or LiCl. The pH range is from 4 (top) to 9 (bottom). (a) lines 1 and 3 are the top phases of a system without added salt and lines 2 and 4 are the corresponding bottom phases. (b) Line one is the cell homogenate, line 2 is the top of the system containing LiCl; line 3 is the bottom phase of the system containing LiCl; line 4 is the top phase of the system containing NaCl and line 5 is the bottom phase of the system containing NaCl.

increases the amount of α-crystallins in the top phase (and therefore, it decreases the purity of the γ-crystallin fraction recovered in the top phase) the addition of NaCl improves the recovery of the γ-crystallin fraction in the top phase. Overall, the partition behavior as well as the effect of salts observed do not differ from those obtained with the supernatant of the cell homogenate.

In summary, aqueous two-phase extraction is a viable option for the purification of γ-crystallins from eye lenses. The method is quick and does not require special instrumentation. High concentrations of salt at a neutral pH maximize the recovery of the target protein in the top phase and minimize the contamination by the other proteins present in the lenses.

REFERENCES

[1] Albertsson, P. Å. (1986) *Partition of Cell Particles and Macromolecules*. 3rd edition, Wiley (Interscience), New York, USA.

[2] Hart, R. A. and J. E. Bailey (1991) Purification and aqueous two-phase partitioning properties of recombinant *Vitreoscilla* hemoglobin. *Enzyme Microb. Technol.,* 13:788-795.

[3] Plunkett, S. D. and F. H. Arnold (1990) Metal affinity extraction of human hemoglobin in an aqueous polyethylene glycol-sodium sulfate two-phase system. *Biotechnol. Tech.* 4:45-48.

[4] Johansson, G. (1994) Charge determination by Partitioning. *Methods Enzym.* 228: 234-240.

[5] Zaslavsky, B. Y. (1995) *Aqueous Two-Phase Partitioning*. Marcel Dekker, NY, USA

[6] Walter, H. and D. Forciniti (1994) Cross-partitioning: Determination of isoelectric point by partitioning. *Methods Enzymol.* 228: 223-233.

[7] Broide, M. L., C. R. Berland, J. Pande, O. O. Ogun, and G. B. Benedek (1991) Binary-liquid phase separation of lens protein solutions. *Proc. Natl. Acad. Sci. USA* 88: 5660-5664.

[8] Thomson, J. A., P. Schurtenmberger, G. M. Thurston, and G. B. Benedek. (1987) Binary liquid phase separation and critical phenomena in a protein/water solution. *Proc.Natl. Acad. Sci. USA.* 84: 7079-7083.

[9] Clark, J. I. and G. B. Benedek (1980) The effects of glycols, aldehydes, and acrylamide on phase separation and opacification in the calf lens. *Inves. Ophthalmol. Vis. Sci.* 19: 771-776.

[10] Tardieu, A., F. Vérétout, B. Krop, C. Slingsby. (1992) Protein interactions in the calf eye lens: Interactions between β-crystallins are repulsive whereas in γ-crystallins they are attractive *Eur. Biophys. J.* 21: 1-12.

[11] Edwards, M., P. Petitt, and D. Forciniti (1997) A small angle neutron scattering study of proteins at their isoelectric point. *Eur. J. Biochem.* 243: 415-421.

[12] P. Petitt and D. Forciniti (2000) Cold cataracts: A naturally occurring aqueous two-phase system. *J. Chromatogr. B* 743 431-441.

[13] Forciniti, D. (1999) Preparation of aqueous two-phase systems. pp. 23-33. In: R. Hatti-Kaul (ed.), *Aqueous Two-Phase Systems – Methods and Protocols.* Humana Press, New Jersey, USA.

[14] Johansson, G. (1984) Partitioning of Proteins. pp. 161-226. In: H. Walter, D.E. Brooks, and D. Fisher, (eds.). *Partitioning in Aqueous Two-Phase Systems*, Academic Press, NY, USA.

[15] Forciniti, D. (2001) Hemoproteins purification and characterization by using aqueous two-phase systems. pp. 185-208. In: A.G. Smith and M. Witty (eds.). *Heme, Chlorophyll and Bilins: Methods and Protocols.* Humana Press, New Jersey, USA.

[16] Forciniti, D., C. K. Hall, and M.-R. Kula (1991) Protein partitioning at the isoelectric point: Effect of polymer concentration and polymer molecular weight. *Biotechnol. Bioeng.* 38: 986-994.

[17] Forciniti, D., C. K. Hall, and M.-R. Kula (1992) Protein partitioning: Effect of pH and polymer molecular weight. *Chem. Eng. Sci.* 47:165-175.

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