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Influence of high concentration monovalent cations on the protein partitioning in polyethyleneglycol 1500-phosphate aqueous two-phase systems

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Abstract

The influence of chloride salts of Na^+, Rb^+ and Cs^+ at concentrations from 0.15 to 1.2 M was studied with bovine albumin, trypsin, ovoalbumin and lysozyme partitioning in an aqueous two-phase system formed by polyethyleneglycol 1500 and potassium phosphate at pH 7.4. Monovalent cations favoured the protein transfer to the polyethyleneglycol rich phase in the following order: $Rb^+ > Na^+ > Cs^+$. Structure making cations as Na^+ induced a poor loss of structured water, producing little diminution of the molar partial specific volume of polyethyleneglycol, while Rb^+ and Cs^+, structure breaking cations, induced a significant decrease in the specific volume of the polyethylene glycol. The increase of available solution free volume in the top phase favours the protein transfer to the polyethyleneglycol rich phase. Na^+ and Rb^+ induced a slight decrease in the alpha helix content of the proteins, while Cs^+ increased the secondary structure for all the proteins. All the cations induced a decrease in the hydrophobic surface of the proteins, this effect was more significant in the presence of Cs^+.

Keywords: Partitioning; Aqueous two-phase systems; Cations

1. Introduction

It is widely known that flexible chain polymers in aqueous solution are able to form an aqueous two-phase system where proteins have the capacity of partitioning. Polyethyleneglycol-phosphate is the system most frequently used in the biotechnology of protein isolation and purification due to its low cost and the possibility of recycling components. The method is based on the different affinity for proteins in one of the phases [1]. Several theories for phase separation behaviour of macromolecules have been provided [2]: the excluded volume theory, the lattice theory and the interaction polymer–protein theory which is based on the Flory–Huggins theory. However, the role of water that constitutes the bulk of the two-phases in these systems has been not considered as participating in the protein partitioning. The structure of water is altered in an aqueous solution, depending on the chemical nature and concentration of the solute added. Zaslavsky et al. [3] studied the aqueous two-phase system formation process from a non-ionic polymer and stressed the role of water structure as a factor controlling the two-phase formation. The phase separation in a two polymer mixture is a fine balance of water–polymer interaction, so any factor that modifies this interaction will induce a modification in the binodial diagram. Since a non-ionic flexible polymer chain such as polyethylenglycol (PEG) has a tendency to interact

Abbreviations: CD, circular dichroims; ANS, 1 anilino-8-naphthalen sulfonate magnesium salts; BSA, bovine serum albumin; OVA, ovoalbumin; LZ, lysozyme; TRY, trypsin; PEG1500, polyethyleneglycol of average molecular mass 1500.

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with water throughout the hydrogen bond and form structured water around the ethylene chain, any factor that modifies the water structure will induce a modification in the quality of this.

Some factors drive the protein partitioning process in aqueous two-phase systems such as: the interphase electrical potential [1], the molecular mass and hydrophobic surface area of the protein, the polymer molecular mass, etc. Except for the first factor, the other ones are all included in the protein flexible chain polymer interaction [4]. Therefore, the structured water should play an important role in driving the protein partitioning and so this should be reflected in the partition coefficient value of the protein.

It is known that salts modify the water structure [5] which interacts with hydrophilic polymers and proteins, thereby modifying the interaction between them. This effect has been used in many works to modify the partition coefficient value of a desired protein in favour of top or the bottom phases, increasing the recovery of the protein by the liquid–liquid extraction method. Previous publications have shown that the partition coefficient can be increased quite dramatically when higher concentrations of NaCl are added to aqueous two-phase systems. This effect has been attributed to a possible hydrophobic interaction.

Schmidt et al. [6] have used NaCl at 1–10% w/w concentration (about 0.2–2 M) to induce the amylase partition in favour of the polyethyleneglycol rich phase and thus to increase the amylase recovery. Oliveira et al [7] used NaCl concentration 0.1–1 M on the liquid–liquid extraction method for trypsin to increase the protein partition coefficient. Guan et al. [8] used NaCl 3% w/w to obtain a purification factor of 25 in the human interferon alpha 1 from recombinant *Escherichia coli*.

This study focuses on the protein partition behaviour in PEG1500 potassium phosphate aqueous two-phase systems in the presence of various monovalent cations of chloride salts that affect the water structure. The role of the salt concentration on some physical protein parameters such as percentage of alpha helix and hydrophobic surface has also been studied to elucidate the molecular mechanism which controlls the protein partitioning PEG1500 has been selected because is one of the most popular polyethylenglycol derivatives used.

2. Materials and methods

2.1. Chemicals

Lysozyme (LZ), trypsin (TRY), ovoalbumin (OVA), bovine serum albumin (BSA), polyethylene glycol of average molecular weight 1500 (PEG1500) and 1-anilino-8 naphthalene sulfonate magnesium salts (ANS) were purchased from Sigma Chem Co. (St. Louis, USA) and used without further purification. All the other reagents were of analytical quality.

2.2. Preparation of the aqueous two-phase system

Stock solution of the phase component PEG1500 40% w/w, and 24.8% w/w potassium phosphate solution were mixed in order to obtain 3 g of total system composition of 12.83% w/w phosphate and 12.4% w/w PEG1500, pH 7.40 according to the binodal partition diagram reported by Zaslavsky [5]. Then, low-speed centrifugation was used to increase phase separation through a gentle mixing of the system components. Then 1 ml of each phase (top and bottom) was mixed to reconstitute several two-phase systems in which the protein partition was assayed.

2.3. Determination of the partition coefficient (K)

The partition coefficient of the proteins between both phases was analyzed by dissolving increased amounts of protein solution 1000 μM (5–15 μl) in the two-phase preformed system containing 3 ml of each equilibrated phase, the change of the total volume of each phase being negligible. After mixing by inversion for 1 min and leaving it to settle for at least 30 min, the system was centrifuged at low-speed for adequate phase separation. Samples were withdrawn from separated phases and the protein content in each phase was determined by measuring the absorption at 280 nm after dilution. The partition coefficient was defined as:

$$K = \frac{[P]_{\text{top}}}{[P]_{\text{bottom}}} \quad (1)$$

where: $[P]_{\text{top}}$ and $[P]_{\text{botton}}$ are equilibrium concentrations of the partitioned protein in the PEG and phosphate enriched phases, respectively. In the assayed protein concentration range, a plot of $[P]_{\text{top}}$ versus $[P]_{\text{bottom}}$ showed a linear behavior, K being its slope. Absorbance measurements were made on a Spekol 1200 spectrophotometer.

2.4. Measurements of the protein surface hydrophobicity (So)

Proteins at 1000 μM concentration were prepared in 40 mM phosphate buffer pH 7.4. Aliquots of the protein were added to a sample containing 3 ml of the corresponding equilibrated phase (top or bottom) and 2.5 ml of ANS stock solution 8 mM. The final ANS concentration was 20 μM while the protein concentration varied from 0 to 3 mM. Relative fluorescence intensity was measured at a Jasco FP 770 spectrofluorometer, the excitation and emission wavelengths were 360 and 470 nm, respectively. Under the above mentioned conditions with excess probe (ANS), the initial slope (So) of the fluorescence intensity vs. protein concentration plot has been shown to be correlated to the relative surface hydrophobicity of the protein [9].

2.5. CD measurements

Circular dichroism spectra (CD) were performed in a Jasco 815 dichrograph, using a thermostatized cuvette of 10 or 1 mm of pathlength (according to the spectrum region). A repetitive scanning of five cycles was used for the determination of the alpha helix content for the four proteins assayed in a PEG1500 7% w/w medium in the presence of increasing concentration of Na^+, Rb^+ and Cs^+. Control of these cations was carried out in the absence of proteins. The percentage of alpha helix was calculated according to [10].

2.6. Measurements of the partial molar specific volume (v_2) of PEG1500

v_2 is the volume variation of solution when the mass of PEG1500 is increasing $(\partial V/\partial m_2)_{P,\,T,\,nj}$ at constant pressure, temperature and other components. It was determined by density measurements using the following equation [11]:

$$v_2 = \frac{(1 - \partial\delta/\partial c_2)}{\rho^{o}} \tag{2}$$

where ρ and ρ^{o} are the density of the solution and the solvent respectively and c_2 the concentration of PEG solution measured in grams of PEG per ml of solution. Density measurements were carried out at 20 °C using an Anton Paar 35 DMA 35N density meter.

3. Results and discussion

3.1. Cation effect on the protein partitioning in PEG1500-potassium phosphate aqueous two-phase systems

Table 1 resume same physical properties of the proteins assayed. The protein partitioning was assayed at pH 7.4. Under this pH condition, BSA and OVA have negative electrical charges, while LZ and TRY have positive net electrical charges. Fig. 1 shows the dependence of the partition coefficient (K) for the assayed proteins in PEG1500-phosphate buffer aqueous two-phase systems at increasing concentration of salts. It can be seen that all the cations assayed induce a significant increase in the K. The observed general sequence for the proteins was $K_{Rb}{}^{+} > K_{Na}{}^{+} > K_{Cs}{}^{+}$, although, the order was $K_{Rb}{}^{+} > K_{Cs}{}^{+} > K_{Na}{}^{+}$ for the BSA.

The uneven ion partitioning creates an electrical potential difference ($\Delta\psi$) between the phases. $\Delta\psi$ has been reported to be constant for ion concentrations larger than 0.1–0.2 M [12]. This effect has been found at a low ion concentration (10–100 mM). Therefore, the influence of $\Delta\psi$ on the protein partition is observed only at low ion concentration. A tendency to protein partition would not be caused by a $\Delta\psi$ variation in this case because, when the ion concentration increases, the K value for all proteins studied increases in a similar way. This suggests that it is regardless of the cation type. Shibukawa et al. [13] have measured the partitioning coefficient of different salts in PEG phosphate systems. They found that the most hydrophobic ions tend to go to the rich PEG phase. Two well defined effects can be observed from Fig. 1. Firstly, Rb^+ is the cation which strongly induced the protein transfer to the PEG rich phase. Secondly, the K versus salt concentration curves reached, in general, a plateau at high salt concentrations. This last finding suggests the presence of a process associated to a saturation effect.

Table 1

Protein	Molecular mass	Isoelectric point
BSA	69.000	4.80
OVO	45.000	4.90
TRY	23.000	10.70
LZ	14.600	11.00

From the thermodynamic point of view, the salt effect on the protein partitioning can be analyzed applying the linked function relationship developed by Wyman and Gill [14]. It has been shown that for any reaction which depends on the solvent, as it is the case of protein partition, the equilibrium constant (K) will vary with the solvent composition as follows:

$$\frac{\partial \ln K}{\partial \ln a_3} = \Delta\upsilon_{\text{pref}} \tag{3}$$

where K is the partitioning constant, a_3 is the cosolute activity present in the solution; i.e., the monovalent chloride activity and υ_{pref} is the cosolute interaction parameter for the two states of the reaction under discussion. Eq. (3) can be adapted for an aqueous two-phase system, therefore $\Delta\upsilon_{\text{pref}}$ can be expressed as:

$$\Delta\upsilon_{\text{pref}} = \left(\frac{\mathrm{d}m_3}{\mathrm{d}m_2}\right)_{\text{Top}} - \left(\frac{\mathrm{d}m_3}{\mathrm{d}m_2}\right)_{\text{Bottom}} \tag{4}$$

where $\mathrm{d}m_2$ and $\mathrm{d}m_3$ are the protein and cosolute concentration variations at the top and bottom phases respectively and $\Delta\upsilon_{\text{pref}}$ the variation of the preferential interaction parameter of the protein in the top and bottom phases. The left term of the Eq. (4) is positive. In this way, the $\Delta\upsilon_{\text{pref}}$ terms are also positive and are arranged as follows: $\Delta\upsilon_{\text{pref Rb+}} > \Delta\upsilon_{\text{pref Na+}} > \Delta\upsilon_{\text{pref Cs+}}$, which suggests a major protein–cosolute interaction in the rich PEG phase.

3.2. PEG effect on the protein structure

Fig. 2 shows the alpha helix content for three of the four proteins assayed in a of PEG1500 7% w/w medium in the presence of increased concentration of Na^+, Rb^+ and Cs^+. Due to the low content alpha helix in TRY, the cation effect cannot be measured on the CD of this protein. The PEG1500 increased the alpha helix content for OVA and LZ but decreased it for BSA. Cs^+ was, in general, the only cation that induced a light increase in the alpha helix content of the proteins; the most hydrophobic proteins, OVA and BSA, show a

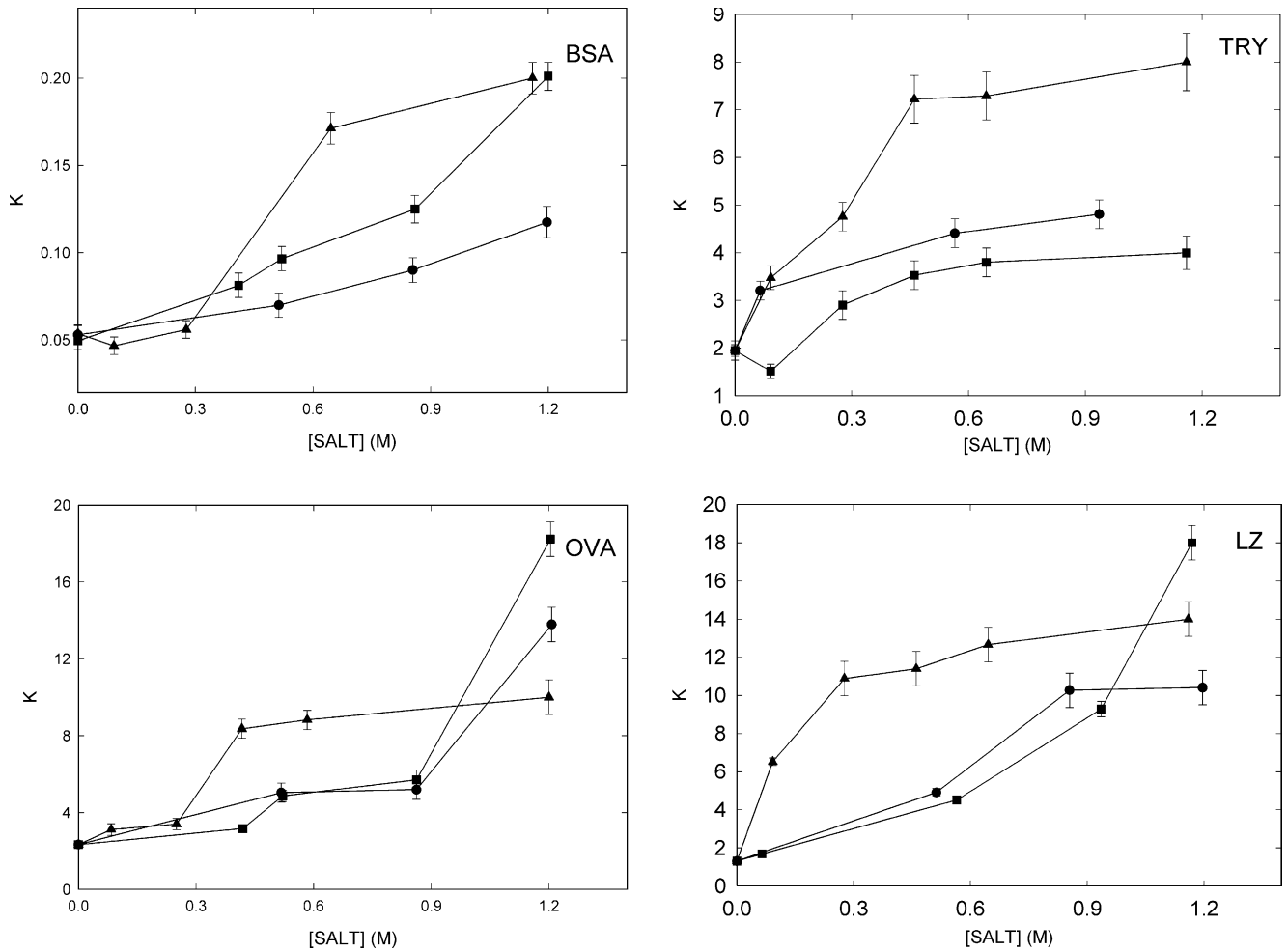

Fig. 1. Partitioning coefficient in PEG1500-potassium phosphate for the proteins in the presence of chloride of monovalents cation. Temperature 20 °C. pH 7.0. (●) Na^+, (▲) Rb^+ and (■) Cs^+. The error bars indicate the deviation standard of three independent measurements.

major variation in their alpha helix content, while the effect on the LZ was very poor. The general tendency observed for Rb^+ and Na^+ was a slight decrease in the alpha helix content. This finding is a proof that the presence of these cations slightly affects the protein structure.

3.3. Cation effect on the protein hydrophobic surface in the presence of PEG15000

Fig. 3A and B shows the effect of Na^+ and Cs^+ cations on the surface hydrophobicity of four assayed proteins. The most hydrophilic proteins such as LZ and TRY, showed neither an appreciable variation in their hydrophobicity in presence of Na^+ nor a slight increase in the presence of Cs^+. The most hydrophobic protein such as BSA, showed a great variation in its hydrophobicity. The OVA is a glycoprotein,which shows a decrease in the hydrophobicity at low cation concentration. However, at high Na^+ concentration, So increases. This effect was more significant in the presence of Cs^+.

This finding suggests that the hydrophobic surface of hydrophobic protein (BSA) assayed is highly influenced by the presence of salts, while hydrophilic proteins are not influenced by them. Water structure making cation such as Na^+, induced a significant decrease in So with respect to the breaking cations like Cs^+ or Rb^+.

3.4. Cation effect on the partial molar specific volume (υ_2) of the PEG1500

PEG1500 solution density versus PEG1500 concentration curves in presence and absence of increasing salt concentrations were analyzed. A linear relationship between the solution density and its PEG concentration was observed for all salt concentrations (data not shown). The υ_2 value was calculated from the slope (M^*/M_{c2}) and the density limit value at zero PEG concentration of these plots and by applying Eq. (2). Fig. 4 shows the cation concentration dependence versus υ_2. A net decrease in the υ_2 at increasing cation concentration can be seen, the sequence of this effect was $Cs^+ > Na^+ > Rb^+$, which shows that the PEG specific volume is highly influenced by the salt concentration.

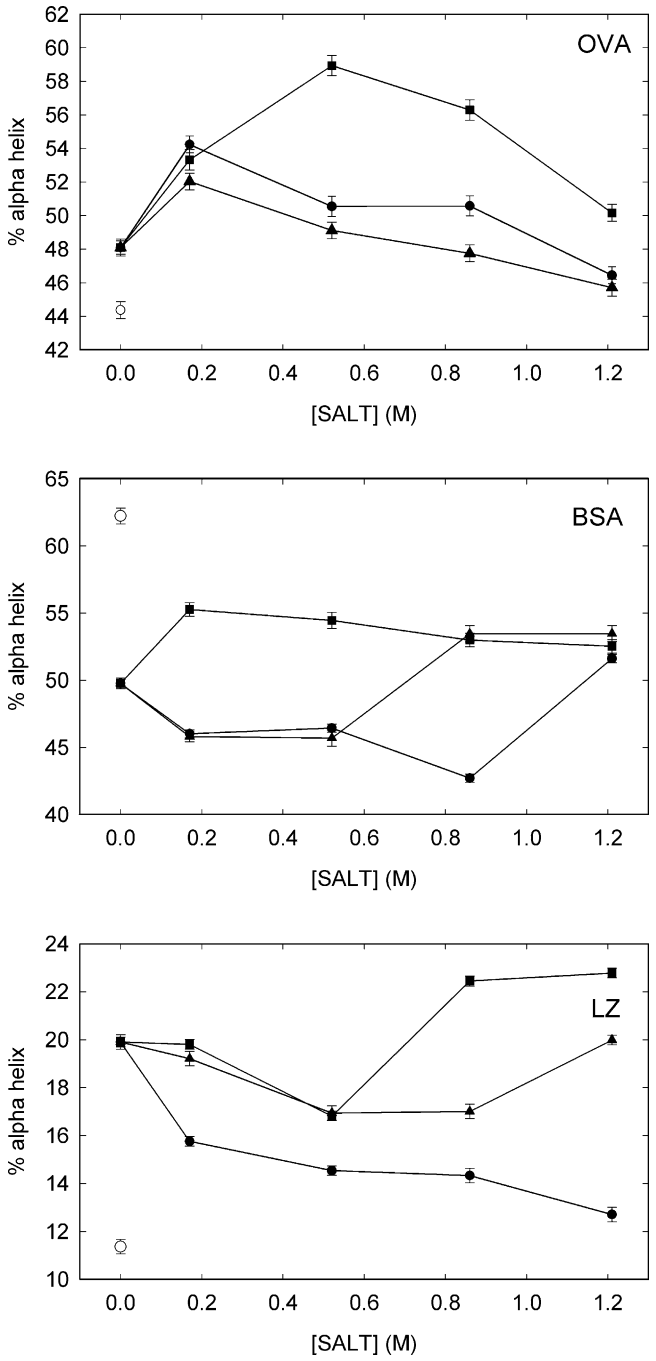

Fig. 2. Dependence of the alpha helix content of the protein at increasing concentration of the monovalent salts (●) Na^+, (▲) Rb^+ and (■) Cs^+. The empty symbol corresponds to the value of protein in buffer phosphate alone. All the results are the mean of five independent measurements.

PEG has been demonstrated to be a hydrophilic molecule in where each oxi ethylene group interacts with 16 water molecules: two, by hydrogen binding formation with the oxygen ether and 14 with ethylene, forming an ordered water structure around it [15]. This ordered water is very sensitive to the temperature changes and to the presence of the solute which modifies the water structure. Salts have been classified as structure breaking and structure making to describe

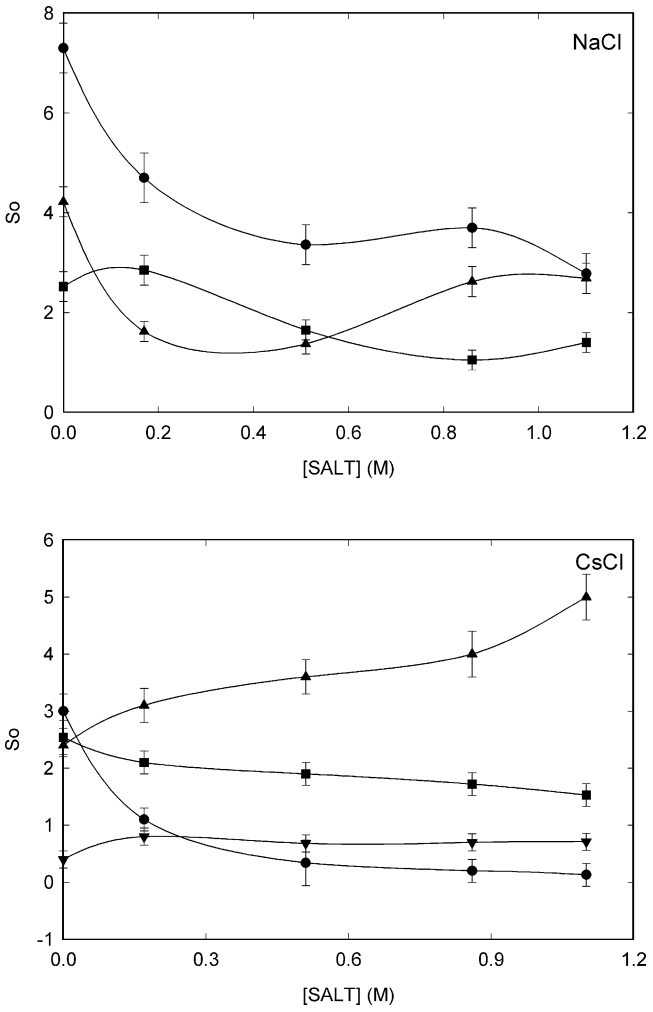

Fig. 3. Effect of Na^+ and Cs^+ in chloride form on the surface hydrophobicity of TRP (■), OVO (▲), LZ (▼) and (●) BSA. in the presence of PEG1500 in buffer potassium phosphate 50 mM pH 7.0. Temperature 20 °C. All the results are the mean of three independent measurements.

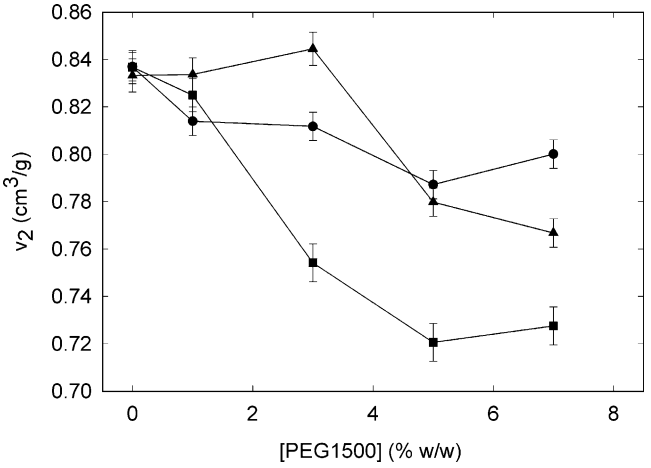

Fig. 4. Dependence of the partial molar specific volume of PEG1500 (v_2) with the salt concentration (●) Na^+, (▲) Rb^+ and (■) Cs^+. Medium potassium phosphate pH 7.0. Temperature 20 °C. All the results are the mean of five independent measurements.

their effects on the water structure. The addition of salts to the aqueous PEG solution leads to an arrangement of ordered water molecules around the polymer molecule. This induces a decrease in the excluded volume of the PEG molecule due to the loss of ordered water by the formation of a water layer around the cation. It also induces a more compact structure with a minor volume of PEG molecule. As a result of this process, an increase in the volume available for the protein in the PEG phase takes place. This mechanism agrees with the increase in the protein transfer to the PEG rich phase by an increase in the salt concentration.

An important decrease in the second coefficient value was reported by Haynes et al. [16] for different proteins reaching a low value in a medium of salt concentrations between 0 to 0.3 M, at pH 7.0. Between 0.4 to 1.0 M, the virial coefficient does not change its value, which suggests that the protein-solution interaction remains constant and is very poor. This finding confirms that a considerable decrease in the PEG excluded volumen is produced during the protein transfer process from the phosphate phase to the PEG rich one, which favors the protein transfer to the PEG rich phase.

The results showed in this work allow us to demonstrate that only the polymer excluded volume is the factor that drives protein partition in aqueous two-phase systems at high salt concentration.

Thus Cs^+, as structure breaking cation induces an increase in the alpha helix content of the protein which suggests a stabilization effect on the protein. This finding is in agreement with the observed increase in the protein thermodynamic stability induced by structure breaking salts [17].

On the other hand, the observed decrease of alpha helix induced by Na^+ and Rb^+ is a proof that an increase in the ordered water in the solution bulk induces a loss of ordered water around the protein structure. The modification of the water structure on the protein hydrophobic zone also leads to a modification in the hydrophobic surface of the protein exposed to the solvent.

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