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INFLUENCE OF SALTS ON THE COEXISTENCE CURVE AND PROTEIN PARTITIONING IN NONIONIC AQUEOUS TWO-PHASE MICELLAR SYSTEMS

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Abstract - Aqueous two-phase micellar systems (ATPMS) can be exploited in separation science for the extraction/purification of desired biomolecules. Prior to phase separation the surfactant solution reaches a cloud point temperature, which is influenced by the presence of electrolytes. In this work, we provide an investigation on the cloud point behavior of the nonionic surfactant $C_{10}E_4$ in the presence of NaCl, Li_2SO_4 and KI. We also investigated the salts' influence on a model protein partitioning. NaCl and Li_2SO_4 promoted a depression of the cloud point. The order of salts and the concentration that decreased the cloud point was: Li_2SO_4 0.5 M > NaCl 0.5 M ≈ Li_2SO_4 0.2 M. On the other hand, 0.5 M KI dislocated the curve to higher cloud point values. For our model protein, glucose-6-phosphate dehydrogenase (G6PD), partitioning experiments with 0.5 M NaCl or 0.2 M Li_2SO_4 at 13.85 °C showed similar results, with K_{G6PD} ~ 0.46. The lowest partition coefficient was obtained in the presence of 0.5 M KI (K_{G6PD} = 0.12), with major recovery of the enzyme in the micelle-dilute phase (%Recovery = 90%). Our results show that choosing the correct salt to add to ATPMS may be useful to attain the desired partitioning conditions at more extreme temperatures. Furthermore, this system can be effective to separate a target biomolecule from fermented broth contaminants.
Keywords: Salt effect; Protein purification; $C_{10}E_4$; G6PD; Cloud point; Aqueous two-phase micellar systems.

INTRODUCTION

Advances in biotechnology have opened up numerous possibilities for the large-scale production of many biomolecules for industrial applications. Liquid-liquid extraction is a promising technique that can possibly be used instead of, or as a complementary process to more typical chromatographic operations in order to reduce the costs of downstream processing of different molecules (Kilikian *et al*., 2000; Mazzola *et al*., 2006; Hebbar *et al*., 2012; Lopes *et al*., 2014).

One type of liquid-liquid extraction is the aqueous two-phase micellar system (ATPMS), which has proved to be useful for extraction and purification of biomolecules such as proteins, viruses, DNA and antibiotics from unclarified microorganism cultures (Bordier, 1981; Albertsson, 1986; Kamei *et al*., 2002; Rangel-Yagui *et al*., 2003; Mazzola *et al*., 2008; Lopes *et al*., 2011; 2013). Compared with other traditional

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purification techniques, ATPMS presents several advantages, such as high water content of both phases (80%, w/w) and biocompatibility, low interfacial tension (minimizing degradation of biomolecules), high capacity and yield, besides the possibility of surfactant recycling. In addition, due to its simplicity, low cost and ease of scale-up, this process has been widely investigated for industrial applications (Mazzola *et al*., 2008).

In these systems, an aqueous surfactant solution, under the appropriate solution conditions, spontaneously separates into two predominantly aqueous, yet immiscible, liquid phases, one of which has a greater concentration of micelles than the other (Liu *et al*., 1998; Rangel-Yagui *et al*., 2004). The difference between the physicochemical environments in the micelle-rich and in the micelle-poor phases forms the basis for an effective separation and makes ATPMS a convenient and potentially useful method for the separation, purification, and concentration of biomaterials (Liu *et al*., 1996; Dutra-Molino *et al*., 2014).

The addition of inorganic salts may result in changes of both intra- and intermicellar interactions due to electrostatic screening. Therefore, it often leads to changes in the phase behavior of aqueous mixtures of oppositely charged surfactants, thereby improving biomolecule partitioning (Santos *et al*., 2011; Xu, 2012; Santos-Ebinuma *et al*., 2013). In general, the effect of inorganic salts (such as NaCl, Na_2SO_4, Li_2SO_4) on the properties of nonionic surfactant solutions is considered to be small, since the direct electrostatic interactions between ionic species and nonionic surfactants are relatively weak. However, a large amount of inorganic salt may induce changes in the solution properties through their influence on the properties of water.

For a micellar solution, the temperature at which the isotropic solution breaks up into two phases is known as the cloud point (Warr *et al*., 1990). In fact, it is well known that salts added at rather high concentrations depress or elevate the cloud point of nonionic surfactant solutions, depending on the salting-out or salting-in nature of the salt species (Weckström, Zulauf, 1985; Koshy *et al*., 1996; Schott, 1997). In the same way that salts may interfere with the cloud point of nonionic surfactants, these electrolytes can influence biomolecule partitioning in nonionic ATPMS. Although salts distribute almost evenly between the phases, there are small, but significant variations in the partition coefficient of ions due to the existence of an electrostatic potential difference between the phases, which can directly influence biomolecule partitioning (Sarubbo *et al*., 2000). Accordingly, a significant electrostatic potential difference can exist depending on the salt used to govern electroneutrality.

This work focused on evaluating the influence of salts, in particular NaCl, Li_2SO_4, and KI), on the coexistence curve of the nonionic surfactant $C_{10}E_4$. We also investigated the partitioning of a model protein, the enzyme glucose-6-phosphate dehydrogenase (G6PD), in ATPMS composed of $C_{10}E_4$ with and without salt addition. G6PD is obtaind by bioprocess and presents great interest as an analytical reagent, being used in various quantitative assays, including the measurement of creatin-kinase activity, hexose concentrations, and as a biomarker in enzyme immunoassays (Bassi *et al*., 1999; Lojudice *et al*., 2001). Therefore, ATPMS coud be an alternative for this biomolecule extraction/purification.

MATERIALS AND METHODS

Materials

The glucose-6-phosphate dehydrogenase enzyme from *Leuconostoc mesenteroides*, glucose-6-phosphate, β-nicotinamide adenine dinucleotide phosphate (β-$NADP^+$) and octylphenol ethoxylate (Triton™ X-114 in this work abbreviated to TX-114) were all purchased from Sigma (St. Louis, MO). The nonionic surfactant *n*-decyl tetraethylene oxide ($C_{10}E_4$) was purchased from NIKKO Chemicals (Japan). All the solutions were prepared in McIlvaine's buffer, pH 7.2, consisting of 16.4 mM disodium phosphate and 1.82 mM citric acid in water purified through a Millipore Milli-Q ion-exchange system (Bedford, MA). The glassware used was washed in a 50:50 ethanol:1 M sodium hydroxide bath, followed by a 1 M nitric acid bath, rinsed copiously with Milli-Q water, and finally dried in an oven. Other chemicals were of reagent grade and used as received, unless otherwise stated.

Determination of G6PD Concentration

The determination of G6PD concentration in aqueous surfactant solutions was based on a well-established enzymatic assay (Bergmeyer, 1983). The activity of G6PD was measured by determining the rate of NADPH formation, which absorbs at 340 nm, using a Beckman DU-640 (Fullerton, CA) spectrophotometer. To prevent phase separation during the assay, a temperature of 15 °C was employed. One G6PD unit was defined as the amount of enzyme that catalyzes the reduction of 1 μmol of $NADP^+$ per minute under the assay conditions.

Mapping the Coexistence Curve of the $C_{10}E_4$/ Buffer System in the Presence or Absence of Salts

Coexistence curves of the $C_{10}E_4$/Buffer system were mapped out in pure McIlvaine's buffer solutions, and in the presence of salts, as specified: NaCl 0.5 M, Li_2SO_4 0.2 and 0.5 M, KI 0.5 M. The curves were obtained by the cloud-point method (Albertsson, 1986; Blankschtein *et al*., 1986). Briefly, buffered $C_{10}E_4$ solutions of known concentrations (in the presence or absence of salts) were prepared and placed in a transparent thermo-regulated device with temperature control within 0.2 °C. A magnetic stirrer was used to ensure temperature and concentration homogeneity. The temperature was first lowered such that the solution exhibited a single, clear phase. Then, the temperature was raised slowly, and the temperature at which the solution first became cloudy, indicating the onset of phase separation, was taken as the cloud point (T_{CLOUD}). The procedure was repeated three times for each data point to ensure reproducibility and the coexistence curves were obtained by plotting the T_{CLOUD} values as a function of surfactant concentration.

G6PD Partitioning in Aqueous Two-Phase Micellar Systems

Buffered solutions, each with a total mass of 3 g, were prepared in 10 mL graduated test tubes by the addition of the desired amounts of surfactant ($C_{10}E_4$), G6PD and salt solution as required. Since the enzymatic assay for determination of G6PD concentrations is very sensitive, there was no need to use large amounts of the enzyme and, therefore, the overall G6PD concentration in each partitioning experiment was 0.007 %w/w (0.7 mM). The systems were mixed and equilibrated at 4 °C in order to form a clear and homogeneous single phase. Subsequently, the systems were placed in a thermo-regulated device, previously set at the desired temperature, and maintained for 3 hours to attain partitioning equilibrium. After that, the two coexisting micellar phases formed were withdrawn separately and the concentration of G6PD in each phase was determined. Each partitioning experiment was carried out in triplicate to verify reproducibility.

The partitioning behavior of G6PD in the ATPMS was quantified in terms of the partition coefficient (K_{G6PD}), defined as follows:

$$K_{G6PD} = \frac{C_{G6PD,c}}{C_{G6PD,d}} \tag{1}$$

where C_c is the G6PD concentration in the surfactant concentrated top phase (micelle-rich phase), and C_d is the GFPuv concentration in the surfactant dilute bottom phase (micelle-poor phase). The $C_{10}E_4$ interference in the method was taken into consideration.

The volume ratio between the phases of the system was calculated as:

$$R = \frac{V_c}{V_d} \tag{2}$$

where V_c and V_d are the volumes of the micelle-rich and micelle-poor phases, respectively.

The activity balance ($\%AB_{G6PD}$) was also calculated, as follows:

$$\%AB_{G6PD} = \frac{C_{G6PD,c}V_c + C_{G6PD,d}V_d}{C_{G6PD,i}V_{G6PD,i}} x100\% \tag{3}$$

where $C_{G6PD,c}$, $C_{G6PD,d}$ and $C_{G6PD,i}$ correspond to the G6PD activity in the micelle-rich (top) phase, in the micelle-poor (bottom) phase, and in the G6PD solution initially added to the system, respectively. V_c, V_d and V_i correspond to the volumes of the micelle-rich phase, micelle-poor phase, and the G6PD solution initially added to the system, respectively.

Based on the partitioning theory proposed by Blankschtein *et al*. (1986), the excluded-volume effect - *EV* was calculated with the help of the following equation:

$$EV \equiv \frac{(\phi_c - \phi_d)}{100} \tag{4}$$

where ϕ_c and ϕ_d are the surfactant volume fractions in the concentrated (micelle-rich) and dilute (micelle-poor) phases, respectively (Nikas *et al*., 1992; Lue and Blankschtein 1996). The $(\phi_t - \phi_b)$ value can be obtained from the coexistence curve, since both phases have densities of approximately 1 g/mL, and therefore, weight fractions can be approximated as volume fractions. According to this theory, excluded-volume interactions are the main interactions governing the partitioning of hydrophilic proteins in nonionic ATPMS.

RESULTS AND DISCUSSION

Coexistence Curves of the $C_{10}E_4$/Buffer Systems

The coexistence curve represents the boundary separating the one-phase region from the two-phase region of an appropriate phase diagram. For polyethylene oxide derived nonionic surfactants, such as

$C_{10}E_4$, the attractive forces between the micelles increases with temperature (Blankschtein *et al*., 1986; Chevalier and Zemb, 1990). Moreover, the phase behavior of aqueous micellar solutions might change in the presence of electrolytes and impurities (Balzer and Lüders, 2000). So, the influence of different salts, namely NaCl, Li_2S0_4 and KI, on the phase diagram of the $C_{10}E_4$/buffer system was investigated (Figure 1, Table 1).

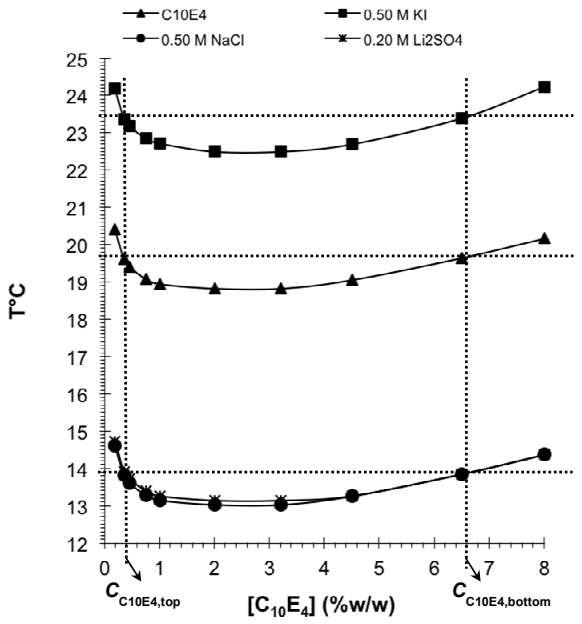

Figure 1: Experimentally determined coexistence curves of the $C_{10}E_4$ micellar system, showing the corresponding tie-lines employed in the partitioning experiments. The operational tie-lines are indicated as horizontal dashed lines. The experimental error was not superior to ± 0.2 °C.

Table 1: Experimentally determined coexistence curves of the $C_{10}E_4$ micellar system for different temperatures, $C_{10}E_4$ concentrations, salts and salt concentrations. A $C_{10}E_4$ micellar solution having any $C_{10}E_4$ concentration along the tie-line will phase separate into top and bottom phases having concentrations $C_{C10E4,top}$, $C_{C10E4,bottom}$, respectively. The excluded-volume effect (*EV*) was also calculated.

SALT [Molar]	T (°C)	$C_{C10E4,top}$	$C_{C10E4,bottom}$	*EV*
Without salt	19.70			
0.5 M NaCl	13.85			
0.2 M Li_2SO_4	13.85	0.40	6.60	0.062
0.5 M Li_2SO_4	8.11			
0.5 M KI	23.40			

As can be seen in Figure 1, coexistence curves shifted considerably in the presence of electrolytes as compared to the $C_{10}E_4$ curve in buffer. This phenomenon is based on the complex competition between the inorganic salts and the surfactant for water molecules and the existence of specific interactions (Ulloa *et al*., 2012). The differences observed were dependent on the type and concentration of salt added to the system. Hofmeister (1888), in his original work, reported that certain salts either decrease or increase protein solubility in water. The effectiveness of salts is governed by the properties of the anions and is observed typically at concentrations of approximately one molar, but may be seen at concentrations as low as 0.01 M, in some cases (Marcus, 2009). For a given cation, the series is generally written as $CO_3^{2-} > SO_4^{2-} > S_2O_3^{2-} > H_2PO_4^- > OH^- > F^- > HCO_2^- > CH_3CO_2^- > Cl^- > Br^- > NO_3^- > I^- > ClO_4^- > SCN^-$. This means that carbonate is the most kosmotropic anion (water-structure-making), whereas thiocyanate is the most chaotropic one (water-structure-breaking). Regarding the cations, the following sequence was described by Marcus (2009): $(CH_3)_4N^+ > (CH_3)_2NH_2^+ > K^+ \sim Na^+ > Cs^+ > Li^+ > NH_4^+ > Mg^{2+} > Ca^{2+} > C(NH_2)_3^+$.

This sequence of ions can suffer variations due to interaction of ions with macromolecules present in the solution. In this sense, the ions of the Hofmeister series are classified on the basis of their influence on the water structure in two different ways (Collins and Washabaugh, 1985). Chaotropic ions (such as thiocyanate, perchlorate) are usually large and have weak electric fields and a loose hydration shell, which can be easily removed. On the contrary, kosmotropic ions are usually small, have high electric fields at short distances and bind water molecules strongly. Due to their strong tendency to hydrate, they compete for water around the PEO chains in the surfactant assemblies such as micelles.

Considering the Hofmeister series for the anions employed in this work, the following sequence represents the kosmotropic action $SO_4^{2-} > Cl^- > I^-$, whereas for the cation the following sequence has to be considered: $K^+ \sim Na^+ > Li^+$. As can be observed in Figure 1, NaCl and Li_2SO_4 promoted a depression in the T_{CLOUD}. Probably these results were achieved due to the salts strong tendency for hydration; the ions compete for water around the PEO chains of the surfactant molecules assembled as micelles, leading to dehydration of the PEO chains, which in turn results in micelle-micelle van der Waals interactions and depression of T_{CLOUD}. The order and concentration of salts that decrease the cloud point is: Li_2SO_4 0.5 M > NaCl 0.5 M ≈ Li_2SO_4 0.2 M. The addition of 0.5 M Li_2SO_4 drastically decreased T_{CLOUD} and, therefore, it was not reported in this work. Our results are in agreement with the ones obtained by

Molina-Bolivar *et al*., (2002), who evaluated the influence of different chloride salts, namely NaCl, LiCl and CsCl, on the cloud point of Triton X-100 in aqueous solution. According to these authors, the decrease in cloud point occurred in the following order $Li^+ > Na^+ > Cs^+$.

Regarding NaCl and Li_2SO_4, the decrease in the cloud point was around 6 °C compared with the system without salt. It is known that the cation with higher global charge is highly hydrated and can decrease the water molecules in the micelle hydration layer, leading to an increase in micelle-micelle interactions that results in lower cloud point temperatures.

On the other hand, KI presents chaotropic ions, which are known as water structure breakers, promoting salting-in behavior (Yanjie and Paul, 2006). So, as expected, 0.5 M KI dislocated the curve to higher T_{CLOUD} values. The water-structure-breaking ions have weak electric fields and a loose hydration shell, which can be easily removed. Therefore, chaotropic ions disrupt the "icebergs" of water molecules in bulk water and increase the concentration of free water molecules that can form hydrogen bonds with the PEO chains of nonionic surfactants (Schott, 1997; Leontidis, 2002).

Micellar aggregation is also changed by the presence of salts, because the critical micellar concentration (CMC) and the aggregation number (average number of monomers in each micelle) are dependent on ion solvation (Anacker and Ghose, 1963; Tonova and Lazarova, 2005). Moreover, the binding of ions affects micellar organization and the electrostatic interactions among surfactant polar head groups. Consequently, these effects influence biomolecule partitioning in micellar solutions (Brochztain *et al*., 1990; Quina and Chaimovich, 1979).

Therefore, varying both salt composition and pH can be used as a strategy in order to improve the purification of proteins since the effect of electrostatic potential difference has a strong dependence on the protein net charge. Thus, understanding the behavior of coexistence curves of a nonionic micellar system (such as the $C_{10}E_4$/buffer system) in the presence of salts may allow better planning of protein partitioning experiments. In this paper, in particular, we investigated G6PD partitioning in the presence of different salts, keeping constant the excluded-volume effect.

G6PD Partitioning in $C_{10}E_4$ ATPMS in the Presence and Absence of Salts

Initially, we investigated the effect of different $C_{10}E_4$ concentrations on the enzymatic activity of G6PD, which is presented in the form of an activity balance (Figure 2). As can be seen, G6PD stability at surfactant concentrations up to 6% (w/w) was higher than 90%. However, for surfactant concentrations from 8 to 10% (w/w) the G6PD activity balance was approximately 85%, as a consequence of enzyme-surfactant interactions. Usually, an activity balance of 85% is considered to be acceptable in terms of purification processes and we can consider the nonionic surfactant $C_{10}E_4$ to be mild to G6PD and suitable for ATPMS purification. The G6PD partitioning in $C_{10}E_4$/buffer system was investigated in the presence of the salts and the results are presented in Table 2.

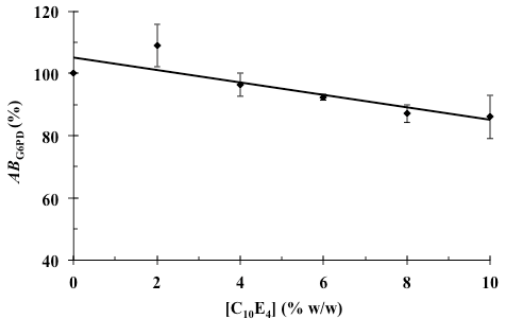

Figure 2: G6PD enzyme activity (%AB_{G6PD}) after one hour of exposure to different concentrations of $C_{10}E_4$ at 15 °C. The error bars represent 95% confidence levels for the measurements.

According to Table 2, the results of the activity balance (%AB_{G6PD}) were approximately 100%. Thus, there was no loss of enzyme activity during partitioning process and the $C_{10}E_4$ system can be employed for the purification of this enzyme with no significant denaturing effect. This confirms that enzyme stability in the presence of $C_{10}E_4$, as presented in Figure 2.

The protein partition coefficient (K_{G6PD}) is a sensitive parameter, useful to evaluate the balance of repulsive and attractive interactions between proteins and surfactant aggregates in ATPMS (Machado *et al*., 2010). Proteins that aggregate with the surfactant molecules are expected to show a preference for the micellar phase, whereas proteins with no attractive interactions with the surfactant or depleted from the aggregates surface generally remain in the less crowded aqueous phase. Here, for all the conditions studied, the K_{G6PD} values were smaller than 1.0, demonstrating the hydrophilic character of the enzyme. In other words, G6PD is not hydrophobically attracted to the micelle-rich, concentrated phase of the system. Moreover, in the dilute, micelle-poor phase, the enzyme can sample a larger available volume. This volume exclusion effect increases with the increase in phase separation temperature and can be estimated based on the coexistence curve (Equation (1)).

Table 2: Experimental results for G6PD partitioning in a $C_{10}E_4$/buffer, for different temperatures, $C_{10}E_4$ concentrations, salts and salt concentrations, but keeping the excluded-volume constant at 0.062. *R* corresponds to the volume ratio between the phases, K_{G6PD} is the partition coefficient of G6PD and $\%AB_{G6PD}$ is the activity balance. The error bars represent 95% confidence levels for the measurements.

SALT [Molar]	$[C_{10}E_4]$ (%w/w)	T(°C)	R (V_c/V_d)	K_{G6PD}	$\%AB_{G6PD}$
Without salt	4.00	19.70	0.90	0.25 ± 0.04	92.4 ± 3.00
0.5 M NaCl	3.10	13.85	1.00	0.47 ± 0.04	105.5 ± 5.00
0.2 M Li_2SO_4	3.10	13.85	1.00	0.46 ± 0.03	102.9 ± 2.00
0.5 M Li_2SO_4	3.10	8.11	1.20	0.50 ± 0.03	99.5 ± 3.00
0.5 M KI	3.00	23.40	0.30	0.12 ± 0.03	120 ± 5.00

The G6PD partitioning in the system without salt at 19.7 °C resulted in K_{G6PD} = 0.25. However, the addition of NaCl and Li_2SO_4 promoted an increase in K_{G6PD}, which means that the enzyme partitioned comparatively less to the dilute phase (micelle-poor phase). Probaby, there was a salting out effect on the protein from the micelle-poor to the micelle-rich phase. The experiments with 0.5 M NaCl and 0.2 M Li_2SO_4 at 13.85 °C showed similar results, with K_{G6PD} ~ 0.46. The lowest partition coefficient was obtained in the presence of 0.5 M KI at 23.40°C (K_{G6PD} = 0.12), with major recovery of the enzyme in one of the phases (micelle-dilute, %Recovery = 90%). Initially, we were expecting similar K_{G6PD} results in the presence of 0.5 M KI, 0.5 M NaCl and 0.2 M Li_2SO_4, since both the excluded-volume parameter and the initial surfactant concentration were similar in all cases. The significant difference observed for KI might be related to an error in cloud point values for the higher concentrations of $C_{10}E_4$ in the presence of KI. If we slightly overestimated the T_{CLOUD} in the presence of KI, the excluded-volume of the experimental partitioning condition would be much higher and *R* would be smaller, as observed.

Our group also investigated G6PD partition in ATPMS composed of the nonionic surfactant Triton X-114 (TX-114), which is also a PEO surfactant type, employing 1.5% of TX-114 at 27.5 °C and 2.5% of TX-114 at 30 °C and either NaCl or KI at different concentrations (0.25 and 0.4 M). In this study, we observed the same tendency of G6PD to partition to the micelle-dilute phase in the absence of salts and an increase in partitioning to the micelle rich phase when salts were added to the system. Furthermore, the migration trend to the micelle-rich phase was more pronounced in the presence of NaCl than KI, which was also in agreement with the present work (unpublished data).

Jozala *et al*. (2012) studied the addition of salts in Triton X-114 ATPMS in order to investigate nisin partitioning behavior. In the presence of only buffer, partition coefficient (*K*) values were around 3. In the presence of $MgSO_4$ and $(NH_4)_2SO_4$, nisin showed *K* values of 5.6 and 5.4, respectively. Comparing our results with these related ones, we observe that the addition of salts to aqueous solutions of $C_{10}E_4$ influences the coexistence curve, but no major changes in G6PD partitioning behavior are observed. For a less hydrophilic and/or smaller protein, more significant changes in partition coefficient could be observed. Nonetheless, the addition of salts can be an interesting artifact when purifying proteins in ATPMS since it allows one to play with temperature while keeping the excluded-volume effect constant. This might have importance for very thermo-sensitive and very thermo-resistant target proteins. In the first case, a chaotropic ion should be employed in order to lower T_{CLOUD} and favor the target protein stability. In the second and perhaps more interesting case, a kosmotropic ion might be used to increase T_{CLOUD} and, consequently, the chances of denaturing contaminant proteins that could then be removed in the micelle-rich phase.

CONCLUSION

The influence of salts on both the cloud point of $C_{10}E_4$ and the partitioning of G6PD was studied. In this context, we observed that both NaCl and Li_2SO_4 promoted an increase of enzyme partitioning to the micelle-rich phase, whereas KI resulted in the highest G6PD recovery in the micelle-dilute phase. These results demonstrate that the presence of electrolytes influences the cloud point of the $C_{10}E_4$ system and that knowledge of this parameter is of paramount importance before starting partitioning studies. The addition of salts allows one to play with the partitioning temperature while keeping the excluded-volume effect constant, and this can be useful to purify thermo-sensitive, as well as thermo-resistant proteins. In a complex medium, i.e., fermented broth, this methodology can promote separation of a target biomolecule from other contaminants such as pro-

teins. Moreover, the results support the applicability of ATPMS to extract G6PD.

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