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Journal of Chromatography B, 837 (2006) 18–23
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Features of the milk whey protein partitioning in polyethyleneglycol-sodium citrate aqueous two-phase systems with the goal of isolating human alpha-1 antitrypsin expressed in bovine milk

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Received 30 December 2005; accepted 22 March 2006
Available online 27 April 2006

Abstract

Partitioning behaviour of the bovine whey proteins (bovine serum albumin, α-lactoalbumin and β-lactoglobulin) and human alpha-1 antitrypsin in aqueous two-phase systems prepared with polyethyleneglycol (molecular masses: 1000, 1450 and 3350)-sodium citrate was analysed at pH 5.2, 6.2 and 8.2. Alpha lactoalbumin concentrated in the polyethyleneglycol rich-phase, while β-lactoglobulin, bovine serum albumin and alpha-1 antitrypsin showed affinity for the citrate rich-phase. In aqueous two-phase systems of high medium pH and high polyethyleneglycol molecular mass the protein partitioning equilibrium is displaced to the citrate rich-phase. The polyethyleneglycol 1450-pH 5.2 system with a top/bottom phase-volume ratio of 3 showed to have the best capability of recovering the alpha-1 antitrypsin from a mixture prepared with natural milk whey and human alpha-1 antitrypsin. The recovery of this protein in the bottom phase was of 90% and the purity of the obtained product was of 98%. The method appears to be suitable as a starting point to isolate other human proteins expressed in transgenic bovine milk.

Keywords: Alpha-1 antitrypsin; Milk whey proteins; Protein isolation; Transgenic milk

1. Introduction

Several human proteins have been expressed in ovine, rabbit and bovine milk, therefore large scale methods for their isolation and purification from milk whey must be developed. Milk whey is a saline solution which prevalently contains the following proteins: albumin, β-lactoglobulin, α-lactoalbumin and inmunoglobulins, thus representing the 98% of the total soluble proteins in milk whey. Some peptides of low molecular mass and low content of lactoferin and lactoperoxidase are also present. A genetic modification of the mammalian may conduce to the expression of different proteins in the milk whey and a further purification is required. Several times, the target proteins have similar physico chemical properties to the other milk proteins making difficult its isolation with the conventional purification methods, such as saline precipitation, etc. Other authors tried to purify the milk whey proteins by using chromatographic methods. Ye et al. [1] described a preparative ion exchange chromatographic process for the separation and recovery of the principal proteins present in milk whey (bovine serum albumin, β-lactoglobulin, α-lactoalbumin). Although the method is suitable to isolate β-lactoglobulin, the prevalent whey protein, it is expensive and provides low yield when it is applied to isolate a low-concentrated protein.

Partitioning in aqueous two-phase systems (ATPSs) is a good alternative method to be employed as a first purification step. ATPSs can be generated into a homogenate of a natural or genetically modified product, thus enabling the removal of contaminants by a simple and inexpensive process. ATPSs are formed by mixing two flexible chain polymers in water or one polymer and a salt (phosphate, citrate, etc.) [2]. Proteins are partitioned between the two-phases with a partition coefficient that can be modified by changing the experimental medium conditions such as pH, salts, ionic strength, etc. ATPSs have several advantages as regards the conventional methods for the isolation and purifi-

Abbreviations: PEG1000, PEG1450 and PEG3350, polyethyleneglycols of average molecular masses: 1000; 1450 and 3350, respectively; BSA, bovine serum albumin; BLG, β-lactoglobulin; ALA, α-lactoalbumin; AAT, alpha-1 antitrypsin; ATPS, aqueous two-phase system

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doi:10.1016/j.jchromb.2006.03.049

cation of proteins: their low cost, the possibility of applying them on large scale and the short time required for reaching the equilibrium condition.

There are many reports in literature concerning the partitioning of milk proteins in ATPSs using different systems. Harris et al. [3] studied the partitioning of milk proteins using polyethyleneglycol 4000-ammonium sulfate and correlated the partition coefficient with the protein solubility in ammoniun sulfate medium. This system was problematic because the ammoniun sulfate rich-phase induced the aggregation of the protein and favoured its precipitation in the system interface.

Alves et al. [4] partitioned the whey milk proteins in polyethyleneglycol-potassium phosphate and citrate systems. They studied the partitioning of each pure protein and observed a partition coefficient ratio between α-lactoalbumin and β-lactoglobulin close to 320, which indicated the feasibility of separating both proteins with ATPSs. Da Silva and Meirelles [5] partitioned α-lactoalbumin and β-lactoglobulin in polyethyleneglycol (molecular masses from 1000 to 10,000)-maltodextrin. However, they did not assay the pH and salt concentration effect on protein partitioning.

Rodriguez et al. [6] have studied the partitioning behaviour of α-lactoalbumin and β-lactoglobulin in ammonium sulfate-PEG systems and the recovery of the proteose peptone component 3 in Reppal PES100-PEG from whey milk. They obtained a high recovery of the target protein.

At present, there are a very few studies on the recovery of human proteins from genetic modified milk. We have addressed this important problem studying the partitioning features in ATPSs of milk whey proteins alone and when they are forming a mixture. The purification of alpha-1 antitrypsin was assayed in an artificial mixture formed by AAT and milk whey proteins (with similar concentrations to those of the milk whey). Also AAT was added to a natural bovine milk whey (in order to simulate a transgenic milk whey) and the AAT purification factor and the recovery was determined. The obtained information can be very useful as a starting point to be applied in the isolation of any protein expressed in transgenic milk.

2. Materials and methods

2.1. Chemicals

β-Lactoglobulin (BLG), α-lactoalbumin (ALA), bovine serum albumin (BSA), human alpha-1 antitrypsin (AAT), polyethyleneglycols of average molecular masses: 1000, 1450 and 3350 (PEG1000, PEG1450 and PEG3350) were purchased from Sigma Chem. Co. and used without further purification. All the other reagents were of analytical quality. Table 1 summarizes the physico chemical properties of the employed proteins and the milk whey composition.

2.2. AAT enzymatic activity determination

The AAT inhibits the hydrolysis of α-*N*-benzoyl. DL-Arginine-*p*-nitroaniline (BAPNA) by trypsin in Tris buffer, 20 mM, pH 8.2 at 37 °C. One unit of antitryptic activity is defined as the amount of trypsin (in μg) able to be inhibited by the preparation. Specific activity of tested samples is expressed in units of antitryptic activity per mg of total protein. The reaction is followed by measuring the absorbance of the released reaction product, *p*-nitroanilide, which absorbs at 400 nm (molar absorptivity of 10,500 M^{-1} cm^{-1}) for 4 min. The inhibitory capacity of AAT is proportional to the difference between the rate of product formation in absence and presence of AAT [7].

Table 1
Physico chemical properties of AAT, BLG, BSA and ALA

Protein	Molecular mass	Isoelectric point	Concentration in milk whey (g/L)	Percentage in milk whey (%)
BLG	18000	5.2–5.4	3.0–4.0	60
ALA	14200	4.7–5.1	1.2–1.5	30
BSA	67000	4.9–5.1	0.3–0.6	6
AAT	54000	4.8–5.0	–	–

2.3. Preparation of the aqueous biphasic system

To prepare the biphasic aqueous systems, stock solutions of the phase components: PEG 40% (w/w) and sodium citrate 25% (w/w) of a given pH were mixed according to the binodal diagram obtained previously in our laboratory [8]. The final concentrations of PEG and citrate in the different employed systems are showed in Table 2. The desired pH (5.2, 6.2 or 8.2) of the sodium citrate solution was adjusted by the addition of sodium hydroxide. Low-speed centrifugation to speed up phase separation was used after thorough gentle mixing of the system components, then 1 mL of each phase were mixed to reconstitute several two-phase systems in which the protein partition was assayed. The tie line length (TLL) was calculated according to the Eq. (1):

$$\mathrm{TLL} = \sqrt{[\Delta\mathrm{PEG}]^2 + [\Delta\mathrm{Cit}]^2} \quad (1)$$

where [ΔPEG] and [ΔCit] are the differences between the concentration of PEG and Cit in the top and bottom phases expressed in % (w/w).

Table 2
Composition of the assayed ATPSs

System[a]	PEG (%, w/w)	Citrate (%, w/w)
PEG1000-8.2	10.50	13.13
PEG1450-8.2	10.37	11.56
PEG3350-8.2	9.70	8.82
PEG1000-6.2	15.92	13.97
PEG1450-6.2	15.75	11.70
PEG3350-6.2	10.78	11.22
PEG1000-5.2	15.92	13.97
PEG1450-5.2	15.75	11.70
PEG3350-5.2	10.78	11.22

[a] Each system is named with the PEG molecular weight followed by the mean pH value.

2.4. Determination of the partition coefficient (Kp)

Partitioning behaviour of milk whey proteins was analysed by dissolving increasing amounts of protein (2–6 μM of total system concentration) in the two-phase systems containing 1 mL of each equilibrated phase. Aliquots of the protein stock solution (1500 μM) added to the systems varied from 5 to 15 μL, 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 the two-phase separation. Samples were withdrawn from separated phases and after dilution, the protein content in each phase was determined by measuring the absorption at 280 nm on a Spekol 1200 spectrophotometer. The partition coefficient was defined as:

$$\mathrm{Kp} = \frac{[P]_\mathrm{T}}{[P]_\mathrm{B}} \tag{2}$$

where $[P]_\mathrm{T}$ and $[P]_\mathrm{B}$ are equilibrium concentrations of the partitioned protein in the PEG and citrate rich-phases, respectively. For AAT, the Kp was calculated by the ratio of the enzyme activities in each phase. Temperature was maintained constant at 20 °C and controlled to 0.1 °C by immersing the glass tubes in a thermostatic bath. All the measurements were developed by triplicate.

In order to monitor the purification process of AAT in both the artificial mixture and in the milk whey, the AAT activity and the total protein concentration in each phase were measured and therefore, the AAT and the total protein partition coefficients ($\mathrm{Kp_{AAT}}$, $\mathrm{Kp_{prot}}$) were calculated. The recovery percentage (y) in the bottom phase for any protein after one extraction step can be calculated according to:

$$y = \frac{100}{1 + R\mathrm{Kp}} \tag{3}$$

where R is $V_\mathrm{T}/V_\mathrm{B}$, V_B and V_T are the bottom and top phase volumes, respectively. In this way the recovery percentages (y_AAT and y_prot) were calculated according to:

$$y_\mathrm{AAT} = \frac{100}{1 + (R\mathrm{Kp_{AAT}})}, \qquad y_\mathrm{prot} = \frac{100}{1 + (R\mathrm{Kp_{prot}})} \tag{4}$$

The AAT purity percentage in the bottom phase was calculated with the following expression:

$$P_\mathrm{AAT} = \frac{m_\mathrm{AAT} y_\mathrm{AAT}}{m_\mathrm{prot} y_\mathrm{prot}} \tag{5}$$

where m_AAT and m_prot are the AAT and total protein content in the milk whey or artificial mixture, respectively.

3. Results and discussion

3.1. Influence of PEG concentration, molecular mass and pH on the protein partitioning

Fig. 1 shows the effect of the tie line length (TLL) on the protein partitioning in PEG 1000-sodium citrate ATPSs at pH 8.2. When increasing the TLL a decrease in the Kp value of all the proteins is observed. Similar behaviour was observed for the other assayed PEGs and pHs. It has been demonstrated that the change in TLL affects the free volume available for a different solute to accommodate in a given phase [9]. The protein transfer to the salt rich-phase may be due to a decrease in the free volume available in the top phase as a consequence of the increased PEG concentration.

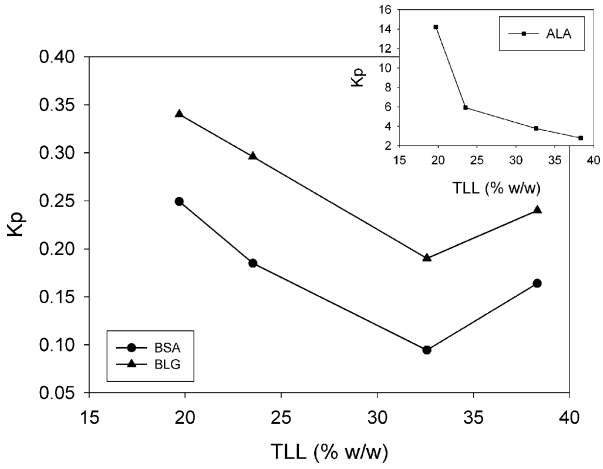

Fig. 1. Dependence of the BLG, BSA and ALA (inserted figure) partition coefficients on the TLL for the PEG1000-citrate at pH 8.2 ATPSs. Temperature 20 °C.

Kp for ALA, whose partition equilibrium is displaced to the top phase, decreased four times with TLL increment showing to be the most affected while BSA and BLG did not change their partition coefficients in a significant manner because both proteins have preference for the bottom phase.

Fig. 2 depicts the PEG molecular mass effect on the ALA, BLG, BSA and AAT partition coefficient (Kp). All the proteins showed the same behaviour, a decrease in their partition coefficient while increasing the PEG molecular mass as it was found for other proteins [10,11]. This behaviour is in agreement with an exclusion effect owing to the diminution of the free volume available in the top phase. Most of the assayed proteins showed a high affinity for the citrate rich phase with Kp values minor than the unity, however, ALA partitioning equilibrium was displaced

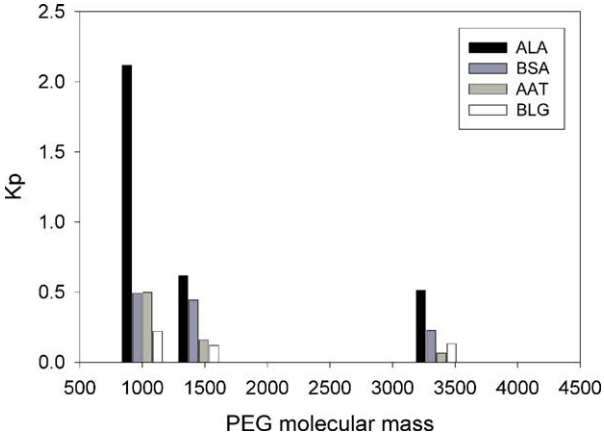

Fig. 2. Dependence of the partition coefficient with PEG molecular weight for ALA, BSA, AAT and BLG at pH 6.2. Temperature 20 °C.

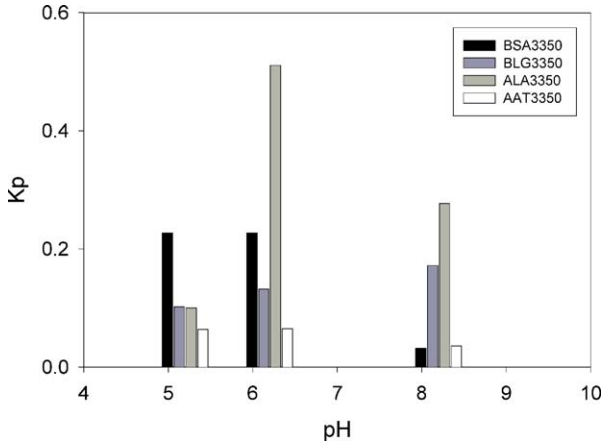

Fig. 3. Effect of the medium pH on the BSA, BLG, ALA and AAT partition behaviours in PEG3350-citrate ATPSs. Temperature 20 °C.

to the top phase in PEG 1000-sodium citrate ATPSs owing to both its low molecular mass and the low exclusion effect of this PEG. Kp of this protein showed to be the most affected by the increase in the PEG molecular mass.

Fig. 3 shows the pH effect on the partitioning behaviour of the four proteins in a PEG3350/citrate ATPS. Practically no change in the partitioning equilibria was observed for BSA and AAT at the time that pH increased from 5.2 to 6.2. When pH was raised to 8.2 a drastic diminution in the Kp value could be seen for both the proteins. This similar partitioning pattern is in agreement with the similar physicochemical properties of BSA and AAT, such as their isoelectric points and their low hydrophobicities [12]. By contrast, ALA showed first an increase of Kp value and then a significant decrease while BLG partitioning equilibrium continuously displaced to the PEG-riched phase when the pH increased. Similar behaviour was observed at the other pHs. According to Albertsson equation the Kp value depends on an electrostatic and a non-electrostatic contribution:

$$\ln \mathrm{Kp} = \ln \mathrm{Kp}^{\circ} + \frac{Zp\Delta\Psi}{RT} \quad (6)$$

where Zp is the net protein charge, $\Delta\Psi$ the interfacial potential (difference between the electrical potential in the top and the bottom phases, $\Psi_{top} - \Psi_{bottom}$) and Kp° is the non-electrostatic term defined as the protein partition coefficient in the absence of electostatic effect ($Zp = 0$ or $\Delta\Psi = 0$). At medium pHs above 5.4 all the assayed proteins are negatively charged (see isoelectrical points in Table 1) and the $\Delta\Psi$ assumes positive values (because the bottom phase is enriched in the citrate anion), therefore the electrostatic term of Albertsson equation will be negative ($Zp\Delta\Psi/RT < 0$). The magnitude of both the net protein charge and the interfacial potential increase as the medium pH increases, therefore the electrostatic term becomes more negative and the Kp value decreases. This effect is more pronounced at pH above 7 where the trivalent citrate anion prevails due to the complete citric acid dissociation. However, BLG exhibited an anomalous behaviour because its Kp increased with the increase in pH. BLG conformation is very sensitive to a change in pH. Except at pH around 4.5, where BLG octamerises, it exists only in the form of dimmer which dissociates into its monomers at extreme values of pH. At pH around 7.5, the BLG undergoes a conformational change which seems to involve an exposure of apolar amino acids and induces an increase in the interaction with hydrophobic solutes. When pH increases the protein conformational change and its molecular mass decrease (owing to the dissociation into monomers) would induce an increase in Kp°. In this way the non-electrostatic term would overcome the electrostatic one thus increasing the Kp value [13].

3.2. Recovery and purity of AAT after an extraction process from an artificial mixture of BSA, BLG and ALA

In order to select the ATPS with the best capability of purifying AAT from a genetic modified milk we calculated the theoretical recovery and purity of AAT in the bottom phase after one extraction process (see Table 3). We considered that the mixture to be hypothetically partitioned in the different ATPSs would be formed by AAT (50% of the total protein mass) and BSA, BLG and ALA with a composition similar to that of the milk whey. The expected recoveries for ALA, AAT, BSA and BLG in the bottom phase were calculated by applying the Eq. (3) and employing the measured Kp values at the different ATPSs. Because BLG, the prevalent whey protein and AAT showed to have the lowest Kp values at the different assayed ATPSs, they exhibited the highest recoveries in the bottom phase. The total protein mass that would be present in the lower phase after partitioning was calculated by considering the individual recoveries (%) and the mixture composition. Therefore, the AAT purity in the bottom phase was calculated with Eqs. (4) and (5). From a visual inspection of Table 3, two ATPSs (PEG1000-pH 8.2 and PEG1450-pH 5.2) showed to have the best capability of separating AAT from BLG, its major contaminant. A high AAT theoretical recovery was obtained in both systems, between 88 and 96% but with low purity of approximately 56%.

In order to verify experimentally the calculated results, a mixture of the four milk whey proteins and AAT with the composition indicated in the footnote of Table 4 was prepared and then partitioned in the two selected systems. The partition coefficients determined for the AAT (in the mixture and when it was partitioned alone) and for the total protein (Kp_{prot}) are showed in Table 4. The AAT recovery and purity were also calculated.

Table 3
Theoretical recoveries and purities for AAT in the bottom phase in different ATPSs after an extraction step from an artificial mixture[a]

System	Recovery (%)	Purity (%)
PEG1000-8.2	**88.9**	**57.4**
PEG1450-8.2	91.4	54.8
PEG3350-8.2	96.6	54.5
PEG1000-6.2	66.8	50.2
PEG1450-6.2	86.3	52.6
PEG3350-6.2	93.9	54.4
PEG1000-5.2	99.3	55.5
PEG1450-5.2	**96.3**	**56.4**
PEG3350-5.2	94.0	52.2

[a] The artificial mixture was considered to be formed with AAT (50%), BLG (34%). ALA (12%) and BSA (4%).

Table 4
AAT recovery and purity after one extraction step in a PEG-citrate ATPS[a] from an artificial milk whey[b]

System	Kp_{AAT}		Kp_{prot}	y_{AAT} (%)	P (%)
	Alone	In mixture			
PEG1000-pH 8.2	0.125	0.120	0.41	89	63
PEG1450-pH 5.2	0.039	0.053	0.42	95	67

[a] Top/bottom volume-ratio equal to one.
[b] Artificial milk whey composition: AAT, 25 mg/mL; BLG, 17 mg/mL; ALA, 6 mg/mL and BSA, 2 mg/mL.

The Kp values for AAT when it is partitioned alone and in the mixture differ from each other slightly. The presence of other proteins may change the partitioning pattern of a given protein with respect to the obtained behaviour when the protein is alone owing to the presence of a protein–protein interaction. Although the two selected systems showed high recoveries and moderate purities, the system PEG1450-pH 5.2 resulted the most adequate to be applied to the AAT extraction.

3.3. Effect of top–bottom volume ratio (R) on the AAT extraction from its mixture with natural milk whey

In developing a method for isolating a target protein from a natural product several steps must be followed. First, the partition pattern of the pure target protein in different ATPSs (i.e. varying pH, molecular mass of the polymer, salt concentration, etc.) must be described. When the ATPS with the best separating capability is selected the partitioning behaviour of the target protein in the natural product must be studied. The effect of phase volume ratio on the recovery, purity and purification factor of the target protein must also be assayed.

In this way, the capability of isolating AAT by the system PEG1450-pH 5.2 was assayed using a mixture formed by natural milk whey and AAT (in order to simulate a transgenic milk). According to the Eq. (3), the protein recovery depends on the Kp and R values and moreover, the Kp value may also change when the R values changes. Huddleston et al. [11] reported that the protein partition coefficient only remains constant along a given tie line when the ATPS composition is near to the binodial curve. Marcos et al. [14] studied the partition of penicillin acylase in PEG-phosphate system and the influence of the phase-volume ratio. The partition coefficient of this enzyme present in a homogenate decreased while the Kp of the total protein increased when the volume phase ratio increased.

Table 5 shows the results obtained for the partition of AAT in a natural milk whey using the system PEG1450-pH 5.2, at different top/bottom volume-ratios. The partition coefficients of AAT and total proteins varied with respect to those obtained from the artificial milk whey (see Table 4).

The increase in the top/bottom volume-ratio induced a decrease in both the Kp_{AAT} and Kp_{prot} values. For AAT the significant decrease of Kp compensated the R increase (see Eq. (3)), thus resulting in similar AAT recoveries for the three R values. By contrast, the total protein recovey drastically decreased, conducted to an increase in the relative mass of AAT present in the bottom phase and resulted in an increase of this protein purity (98%).

Table 5
Effect of R on the AAT purification after a natural milk whey[a] partitioning in a PEG-citrate ATPS

R	1	2	3
Volume top/volume bottom (mL/mL)	0.75/0.75	1.5/0.75	2.1/0.7
Partitioned aliquot (μL)	40	45	60
Kp_{AAT}	0.071	0.046	0.034
Kp_{prot}	0.47	0.35	0.36
y_{prot} (%)	68	59	47
y_{AAT} (%)	93	92	90
Protein mass[b] (mg)	1.30	1.27	1.36
AAT mass[b] (mg)	1.01	1.03	1.33
P_{AAT} (%)	77	81	98
Purification factor[c]	1.38	1.45	1.75

[a] Natural milk whey composition: AAT, 27 mg/mL and total protein, 48 mg/mL. AAT purity in the initial sample: 56%.
[b] In bottom phase.
[c] Calculated as the ratio between the purity of AAT in the bottom phase and in the initial partitioned sample.

The difference between the partition coefficients of AAT alone and in an artificial mixture may be due to the presence of a protein–protein interaction. When the partitioned AAT is present in a natural milk whey the problem is more complex. The Kp value for a pure protein differs from that corresponding to the target protein in a natural product [15] owing to several causes. This natural product contains fatty acids, some electrolites, peptides and gamma globulins. These proteins have high molecular mass and precipice in an irreversible way when the milk whey is added to the biphasic system. While preparing an ATPS, the different effects that each protein may experiment according to the system assembly method must be considered. By contrast, it is well known that the binodial diagram of an ATPS may change when a natural product is partitioned thus modifying the protein partition coefficient. The addition of biological suspensions affects the phase formation changing in a significant manner the citrate and PEG concentration equilibrium in both phases. It has been reported that the presence of residual fat in cheese whey caused the upwards shift of the binodal curve [15].

4. Conclusions

BLG represents about 60% of the total milk whey proteins, whereas ALA represents only 30%. These proteins have isoelectric points between 4.9 and 5.4, making difficult their separation by the classical methods of ion exchange chromatography. This problem is even more complex if another protein, such as AAT, with similar isoelectrical point (4.8) is expressed in a transgenic milk whey. Earlier, AAT was isolated from human plasma, but as albumin, the principal serum protein, represents about 50% of the total proteins and has similar pI to AAT, the traditional ion exchange chromatography did not allow us to separate both proteins efficiently. In a previous work [16], we solved this problem by partitioning human plasma, without previous treatment, in an aqueous two-phase system formed by polyethylene glycol and

potassium phosphate. Taking into account the protein acid–base properties, a mixture of AAT, BLG, ALA and BSA should have a similar behaviour to a mixture of serum albumin and AAT. Therefore, in order to isolate AAT from a transgenic milk whey, we followed a similar way to that employed for isolating this protein from human plasma. From the nine assayed ATPSs (see Table 3), the ATPSs of PEG1000-pH 8.2 and PEG1450-pH 5.2 showed to be efficient with a high AAT theoretical recovery (above 80%), but a low purity (about 56%) in the bottom phase was calculated with measured Kp values of the pure protein. The partitioning behaviour of AAT in both the artificial and the natural milk whey indicated that the PEG1450-pH 5.2 ATPS could be satisfactorily employed to purify AAT from a transgenic milk whey in only one extraction step. Moreover the top/bottom phase-volume ratio showed to improve the purification factor.

Sometimes, the application of a purification method to obtain a therapeutical substance introduces new contaminants that must be removed in order to avoid adverse effects. In our case, the AAT is obtained in the citrate-rich phase, thus the prevalent non-protein contaminants are the sodium citrate, a biodegradable and non toxic salt, and a little amount of PEG, which can easily be removed from the target protein by means of ultrafiltration.

Finally, at present, several human proteins have been expressed in bovine milk; therefore, the study about the partition features of the milk whey protein components is necessary as a starting point to isolate and purificate any protein.

Acknowledgements

This work was supported by a grant from FoNCyT no. 06-12476/02. We thank María Robson, Susana Spirandelli and Marcela Culasso for the language correction of the manuscript.

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