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Partition of whey milk proteins in aqueous two-phase systems of polyethylene glycol–phosphate as a starting point to isolate proteins expressed in transgenic milk

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Abstract

Partitioning behaviour of the bovine whey proteins (bovine serum albumin, alpha lactoalbumin and beta lactoglobulin) and alpha-1 antitrypsin in aqueous two-phase systems prepared with polyethyleneglycol (molecular masses: 1000; 1500 and 3350)–potassium phosphate was analysed. Bovine serum albumin and alpha lactoalbumin concentrated in the polyethyleneglycol rich phase with a partition coefficient of 10.0 and 27.0, respectively, while beta lactoglubulin and alpha-1 antitrypsin showed affinity for the phosphate-rich phase with a partition coefficient of 0.07 and 0.01, respectively. An increase of medium pH induced an increase of the partition coefficient of these proteins while the increase in polyethyleneglycol molecular mass induced the opposite behaviour. The system polyethyleneglycol 1500-pH 6.3 showed the best capacity for recovering the alpha-1 antitrypsin with a yield of 80% and a purification factor between 1.5 and 1.8 from an artificial mixture of the milk whey proteins and alpha-1 antitrypsin. The method appears to be suitable as a starting point to isolate proteins expressed in transgenic milk.

Keywords: Whey milk proteins; Protein isolation; Transgenic milk

1. Introduction

The advances in molecular biology and genetic engineering have made the scale up production of labile macromolecules possible; however, the isolation and purification process of such products have poorly been developed. Protein purification process is a great and important problem due to the complexity of the protein mixture and the possible loss of the biomolecule biological activity. The traditional methods for isolation and purification of proteins involve several steps: ammonium sulphate precipitation, ionic and affinity chromatography, dialysis and a final concentration of the product, which requires long time and high cost. This induces an increase of the unfolded fraction of the protein, with a decrease of the biological activity and a poor yield of the process. Diamond and Hsu [1] have postulated that 50–90% of the total production cost for a biological product is determined by the purification steps.

Other authors tried to purify the milk whey proteins by using chromatographic methods. Ye et al. [2] described a preparative ion exchange chromatographic process for the separation and recovery of the principal proteins present in milk whey (bovine serum albumin, beta lactoglobulin, alpha lactoalbumin). Although this method is suitable to isolate beta 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

Abbreviations: PEG1000, PEG1500 and PEG3350, polyethylenglycols of average molecular masses: 1000, 1500 and 3350, respectively; BSA, bovine serum albumin; BLG, beta lactoglobulin; ALA, alpha lactoalbumin; AAT, alpha-1 antitrypsin; ATPS, aqueous two-phase system

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step since ATPSs can be generated into a homogenate of a natural or genetically modified product, thus enabling the removal of contaminants by a simple and economic process. ATPSs are formed by mixing two flexible chain polymers in water or one polymer and a salt (phosphate, citrate, etc.) [3]. Proteins are partitioned between the two phases with a partition coefficient which 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 purification of proteins: their low cost, the possibility of applying them in large-scale and that they reach the equilibrium condition very fast.

Several human proteins, such as alpha-1 antitrypsin, have been expressed in ovine or rabbit milk [4]. Therefore, it is necessary to develop large-scale methods for their isolation and purification from milk whey.

There are many reports in literature concerning the partitioning of milk proteins in ATPSs using different systems. Harris et al. [5] studied the partition of milk proteins using polyethyleneglycol 4000-ammonium sulphate, and correlated the partition coefficient with the protein solubility in ammoniun sulphate medium. This system was problematic since the ammoniun sulphate rich phase induces the aggregation of the protein, which favours its precipitation in the system interface.

Alves et al. [6] 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 alpha lactoalbumin and beta lactoglobulin of about 320, which indicated the feasibility of separating both proteins with ATPSs. da Silva and Meirelles [7] partitioned alpha lactoalbumin and betalactoglobulin in polyethyleneglycol (molecular masses from 1000 to 10,000)–maltodextrin. However, they did not assay the pH and salt concentration effect on protein partitioning.

The aim of this work was to study the partition features of the milk whey proteins from solutions of individual proteins or when they are present in a protein mixture. The extraction 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) in order to simulate a transgenic milk whey. 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

Beta lactoglobulin (BLG), alpha lactoalbumin (ALA), bovine serum albumin (BSA), alpha-1 antitrypsin (AAT), polyethyleneglycol of average molecular weights: 1000; 1500 and 3350 (PEG1000, PEG1500, 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 10 min. The inhibitory capacity of AAT is proportional to the difference between the rate of product formation in absence and presence of AAT [8].

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 potassium phosphate 25% (w/w) of a given pH were mixed according to the binodal diagram reported by Zaslavsky [9]. The desired pH (6.3 or 8.0) of the KH_2PO_4 solution was adjusted by the addition of potassium hidroxide. The total concentrations were 17% and 8.8% for PEG and phosphate, respectively. Low-speed centrifugation to speed up phase separation was used after through gentle mixing of the system components, then 2 mL of each phase were mixed to reconstitute several two-phase systems in which the protein partition was assayed.

2.4. Determination of the partition coefficient (Kr)

Protein partitioning in both phases was analysed by dissolving increasing amounts of protein (2–6 uM of total system concentration) in the two-phase systems containing 2 mL

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	–	–

Protein composition of milk whey.

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:

$$\mathrm{Kr} = \frac{[P]_\mathrm{T}}{[P]_\mathrm{B}} \quad (1)$$

where $[P]_\mathrm{T}$ and $[P]_\mathrm{B}$ are equilibrium concentrations of the partitioned protein in the PEG and phosphate-rich phases, respectively. In the protein concentration range assayed, a plot of $[P]_\mathrm{T}$ versus $[P]_\mathrm{B}$ showed a linear behavior, Kr being its slope.

In order to evaluate the progress of the purification process the two-phase systems were placed in graduated capped glass tubes (5 mL of total volume, with an accuracy of 0.05 mL) and the top and bottom phase-volumes were measured after each partitioning step. The specific enzyme activity (SA) was calculated as the ratio between the enzyme activity (units) and the protein content (mg), the purification factor (PF) and the enzyme yield recovery in the bottom phase (y) were calculated according to:

$$\mathrm{PF} = \frac{\mathrm{SA}}{\mathrm{SA}^\circ} \quad (2)$$

where SA and SA° are the specific enzyme activities in the bottom phase and in the initial sample, respectively, and:

$$y\,(\%) = \frac{100}{1 + R\mathrm{Kr}} \quad (3)$$

where R is $V_\mathrm{T}/V_\mathrm{B}$, V_B and V_T are the bottom and top phase-volumes, respectively. The system temperature was maintained constant and controlled to within ±0.1 °C by immersing the glass tubes in a thermostatic bath.

3. Results and discussion

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

Figs. 1 and 2 show the PEG molecular mass and pH effect on the ALA, BLG, BSA and AAT partition coefficient (Kr). All the proteins showed the same behaviour, a decrease in their partition coefficient when increasing the PEG molecular mass. For the ATPS of PEG1000/Pi, the mean estimated error was ±0.06 for the partition coefficient of BLG; ±0.7 for ALA; ±0.5 for BSA and ±0.004 for AAT. For ATPSs of PEG1500/Pi and PEG3350/Pi, the mean estimated error was ±0.005 for the partition coefficient of BLG; ±0.08 for ALA; ±0.005 for BSA and ±0.004 for AAT.

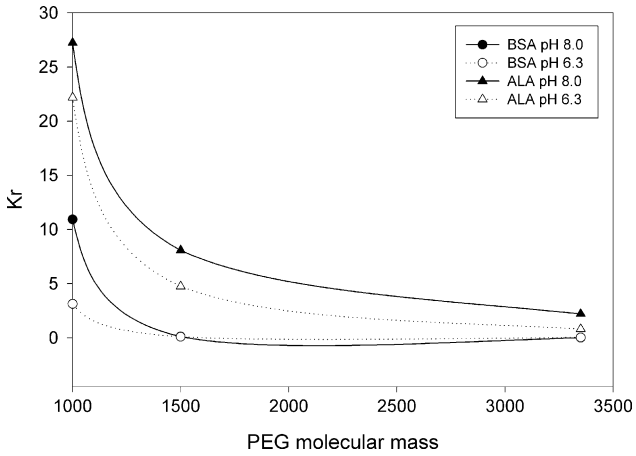

Fig. 1. Dependence of partition coefficient with PEG molecular weight for BSA and ALA at pH 6.3 and 8.0. Temperature: 8 °C.

Both BSA and ALA (Fig. 1) showed the highest affinity for the top PEG rich phase. In the case of ALA, Kr changed from 27 to 2 when the PEG molecular mass increased, while the variation was from 11 to 0.02 for BSA. Moreover, the partition equilibrium of both proteins displaced to the bottom phase when the pH decreased from 8 to 6.3, the most pronounced effect being at the low PEG molecular mass. BLG and AAT (Fig. 2) showed a partition coefficient lower than the unity. Fig. 2 (inserted figure) shows the ratio between the partition coefficients of only these two proteins ($\mathrm{Kr_{BLG}/Kr_{AAT}}$) versus PEG molecular mass because AAT is our target protein and BLG is its main contaminant in the transgenic milk whey. At pH 6.3, the system with PEG1500 can be seen to have the highest capacity for separating both proteins while, at pH 8.0, the ATPS of PEG1000 shows the most different protein partitioning behaviour. In this way, both conditions appear to be optimal for assaying the BLG and AAT separation.

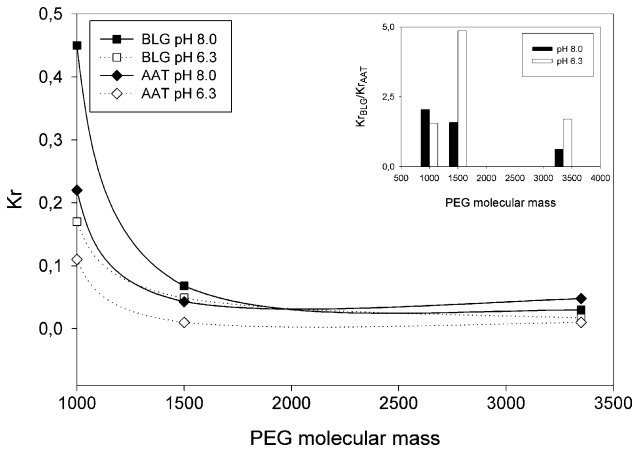

Fig. 2. Dependence of partition coefficient with PEG molecular weight for BLG and AAT at pH 6.3 and 8.0. Temperature: 8 °C. Inserted figure: separation factor ($\mathrm{Kr_{BLG}/Kr_{AAT}}$) in ATPSs formed by phosphate and PEGs of different molecular weight.

Milk whey is principally formed by a mixture of different proteins (see Table 1). BLG, the prevalent whey protein, 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. Other authors have studied the effect of pH on partitioning behaviour of BLG and have demonstrated that the partition coefficient of the dimmer was higher than the one which corresponds to the monomer form [10,11].

ALA is the milk whey protein with the lowest molecular mass; its aminoacid composition and structural characteristics are similar to those of egg lysozyme [12]. Previous studies [7] have shown a partition displacement of ALA in favour of the PEG rich phase. This behaviour can be attributed to the inclusion of this small protein molecule in the available free volume in the PEG rich phase. Moreover, PEGs of low molecular weight such as PEG1000 and PEG1500 can interact with the protein domain. The strength of the protein–PEG interaction and the partition coefficient value will, therefore, be related to the polymer molecule size.

Alves et al. [6] partitioned ALA and BLG in a PEG1500–phosphate system and found an average partition coefficient of 0.031 for BLG which showed a low dependence with respect to system PEG concentration. Our results (0.068 at pH 8.0 and 0.048 at pH 6.3) are in agreement with those obtained by Alves et al. [6] for this protein, indicating thereby that BLG prefers the salt-enriched phase. However, ALA behaviour was different since the partition equilibrium is displaced to the top phase. The high Kr-value obtained for this protein leads to an ALA recovery in the top phase of about 90% for nearly all the assayed systems in the first partition step. In this way, our target protein, the AAT, whose partition coefficient in ATPSs is lower than one, will be present in the bottom phase together with the BLG, which becomes its major impurity.

The Kr-values in ATPS of PEG1500 were lower than those observed in PEG1000 for all the assayed proteins. This finding, which is a general rule in protein partition, indicates that the protein transfer to the bottom phase may be due to an exclusion effect of PEG of higher molecular weight. On the other hand, at pH 8.0, the partition equilibria are more displaced to the PEG-enriched phase (higher Kr) than at pH 6.3 for all the proteins. This fact may be explained taking into account the low affinity of the phosphate anion for the PEG-rich phase which induces a positive electrical potential on this polymer side of the phase (within the requirement of electroneutrality in the phases). Since all the assayed proteins exhibit a p*I* near 5.0 (see Table 1), they are slightly charged at pH 6.3 while they have a significant negative electrical charge (Zp between −10 and −20) at pH 8.0, thus inducing their transfer to the top phase [13].

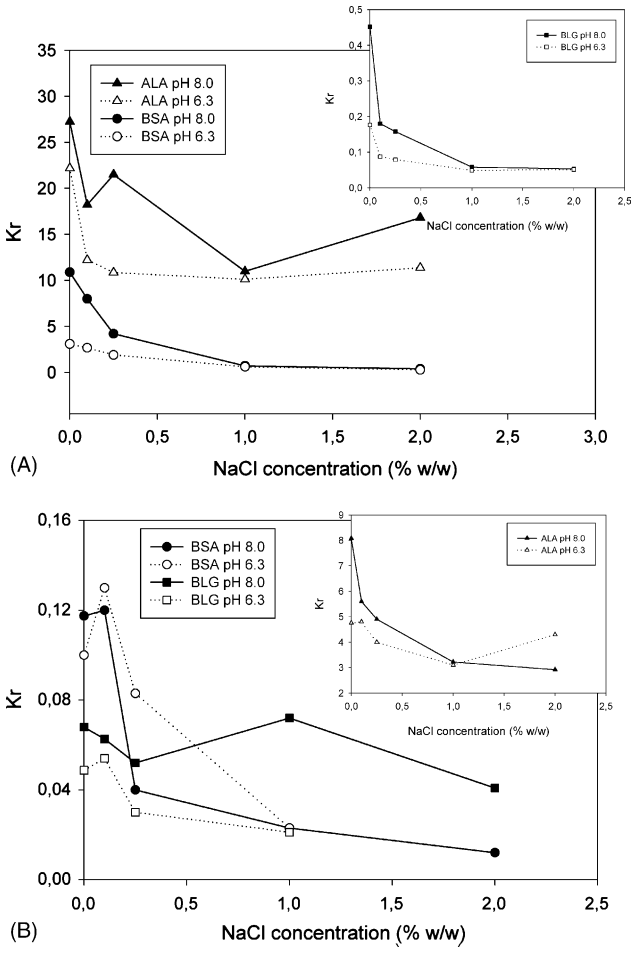

Fig. 3. Effect of the NaCl concentration on the partition behaviour of ALA, BLG and BSA in an ATPS formed by phosphate-PEG1000 (A) and phosphate-PEG1500 (B) at pH 6.3 and 8.0. Temperature: 8 °C.

3.2. *Influence of NaCl on protein partitioning*

In order to displace the partition equilibrium of AAT contaminants to the top phase, the effect of increasing NaCl concentration on Kr was analysed. This salt was selected because it has been used to favour the protein transfer to the top phase by several authors [14]. Fig. 3 shows the partition behaviour of the three contaminant proteins in ATPSs of PEG1000 (Fig. 3A) and PEG1500 (Fig. 3B). The observed trend for nearly all the proteins is a decrease in the partition coefficient when the NaCl concentration increases, in agreement with the results found for other proteins [15]. This effect was more significant in ATPSs of PEG1000 where the protein Kr-value at higher NaCl concentration decreased several times as regards the value in salt absence. These results suggest that the interaction PEG-protein is further affected by NaCl in PEG of low molecular mass (1000) [15]. Moreover, the lower change of Kr observed at pH 6.3 with respect to pH 8.0 is consistent with the more hydrophobic character of all the proteins near their isoelectrical point (see Table 2). For BLG in PEG1500 at pH 8.0, a poor salt effect was observed, while in the other systems NaCl induced a BLG transfer to

Table 2
Effect of NaCl presence on partition behaviour of BSA, BLG and ALA

Protein	pH	Kr/Kr_{NaCl}	
		PEG1000	PEG1500
BSA	8	27	9.2
	6.3	10.5	4.4
BLG	8	8.3	1.6
	6.3	3.4	2.3
ALA	8	2.5	2.8
	6.3	2.2	1.5

Kr partition coefficient in absence of salt; Kr_{NaCl} partition coefficient in presence of the higher assayed NaCl concentration.

the bottom phase. Table 2 also shows the ratio between the Kr-values in absence and presence of the higher salt concentration for all the proteins. The decrease in the Kr due to the salt presence can be seen to depend on both the protein and PEG molecular mass. Thus, the partition of BSA (highest molecular weight) in systems of PEG1000 and PEG1500 proved to be the most affected by the increase in salt concentration. The partition coefficient of BLG was affected to a lesser extent, the Kr change being negligible in the presence of PEG1500. On the other hand, the ALA partition was slightly modified by the salt concentration. Moreover, in all the cases, the diminution of Kr at pH 8.0 was higher than at pH 6.3. Taking into account that NaCl presence affects the electrostatic component of partitioning, these findings agree with the higher protein charge density at the upper pH. These last results, together with the PEG molecular mass effect, allow us to conclude that two types of forces drive the partition of these proteins in PEG–phosphate system: one is of electrostatic nature and the other is due to the interaction of hydrophobic rests of the protein domain with the PEG molecule.

In resume, salt presence did not favour the AAT and BLG separation, therefore, the next partition experiments were carried out in the absence of this salt.

3.3. Recovery of the AAT from a mixture of BLG, BSA, ALA and AAT

Since BLG and AAT showed Kr-values lower than one, they will be present in a great proportion in the bottom phase in an extraction process. By using the experimentally obtained Kr-values for ALA, AAT, BSA and BLG, the theoretical recovery (or yield) for the four proteins in the bottom phase was calculated as function of the top/bottom volume ratio (R) according to Eq. (3).

Fig. 4 A shows the theoretical yield in an ATPS of PEG1000-pH 8.0. An increase in the R-value can be seen to induce a significant diminution in the recovery of all the proteins. For ALA and BSA a drastic yield decrease is observed at R-values lower than two, while the yield remained constant and low at higher R. In contrast, AAT and BLG yields

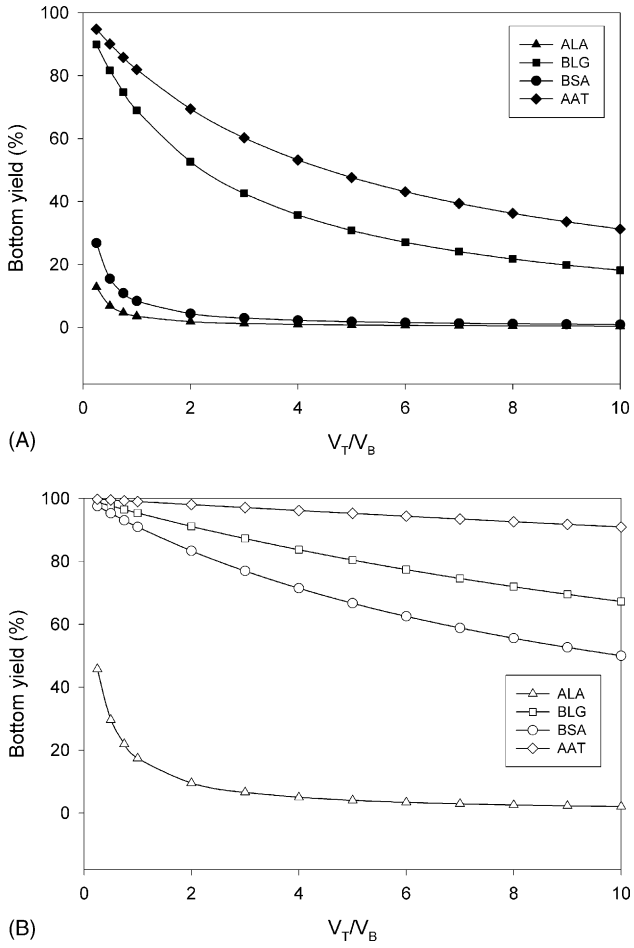

Fig. 4. Theoretical bottom recovery of ALA, BSA, BLG and AAT as function of the top-bottom volume ratio calculated from Eq. (3) in ATPS of PEG1000-pH 8.0 (A) and PEG1500-pH 6.3 (B).

continuously decreased with R. The difference between the recoveries of both proteins showed to be minimal at R-values lower than one and became constant at higher V_T/V_B ratios. The calculated protein recovery in the system of PEG1500-pH 6.3 is shown in Fig. 4B. ALA yield showed an initial diminution followed by a constant low value, while BLG and BSA showed a continuous diminution of their yields. The highest AAT recovery in the bottom phase was practically unaffected by the R variation.

For the two selected systems, low R-values would lead to a high AAT yield with low purity while higher R would produce a lower AAT recovery with higher purity. We selected two extreme R-values: 4:1 and 1:4 to experimentally develop the extraction process of AAT from an artificial protein mixture. Higher R-values were not assayed in order to avoid the target protein dilution.

The artificial mixture was formed by the three main whey proteins with the same composition of milk whey (BLG 0.32%, w/v; ALA 0.12%, w/v; BSA 0.04%, w/v) and AAT (0.48%, w/v). The extraction process was performed in the two selected ATPSs at R 4:1 and 1:4, respectively, and

Table 3
Purification of AAT from an artificial mixture of milk whey proteins

ATPS	*R*	Purification step	Specific activity (units/mg)	Purification (fold)	Recovery of inhibitory activity (%)	Purity[a] (%)	Theoretical recovery[b] (%)
		Artificial whey milk	22	1.0	100	28	100
PEG1000	4:1	1° Extr.[c]	29	1.3	36	37	53
pH 8.0		2° Extr.[d]	49	2.2	21	62	28
	1:4	1° Extr.	26	1.2	51	33	95
		2° Extr.	35	1.6	43	44	90
PEG1500	4:1	1° Extr.	32	1.5	88	41	96
pH 6.3		2° Extr.	40	1.8	80	51	92
	1:4	1° Extr.	25	1.1	60	32	79
		2° Extr.	28	1.3	57	36	63

[a] Calculated as the ratio between the enzyme mass and the total protein mass.
[b] Calculated according Eq. (3).
[c] Bottom first extraction step.
[d] Bottom second extraction step.

aliquots of the mixture were partitioned in the four systems. Two extraction steps were performed in each case by replacing the first equilibrated top phase by an identical volume of a new proteinless upper phase with the aim of decreasing BLG bottom phase content. Both in the first and second extraction steps, the AAT activity and the total protein concentration were determined.

Table 3 shows the experimental recovery and purity of AAT in the bottom phase for the four systems in the two extractions and the theoretical yields calculated from Eq. (3). The ATPS of PEG1500-pH 6.3 was seen to show the best yields. For *R* 4:1, the recovery was 88% in the first and 80% in the second extraction step. Moreover, the observed purities were 41 and 51%, respectively. Therefore, this system seems to be the most suitable to recover the AAT in an artificial milk whey. On the other hand, the system PEG1000-pH 8.0 at both *R*-values showed lower recoveries of AAT with slightly higher purity values.

Table 3 also shows that there exists a significant difference between the calculated and experimental AAT recovery values. In all cases, the measured yields were lower than the theoretical ones because the recoveries were calculated by assuming that the Kr of AAT in the protein mixture was the same as that of AAT alone. However, a different partitioning behaviour of a protein in a mixture with respect to that of the pure protein has been previously observed [13]. In a mixture formed by different proteins, the protein–protein interaction becomes relevant. Moreover, the presence of membranes and subcelular elements may induce a modification of the medium viscosity and density in a homogenate from a biological tissue. These reasons modify the protein activity coefficient, and therefore, its capability for being partitioned. Certain natural mixtures such as milk whey have been reported to induce [16] a substantial modification of the ATPS binodial diagram, thus changing the polymer and salt composition in both phases, and therefore, the protein Kr also changes.

4. Conclusions

BLG represents about 60% of the total milk whey proteins, while ALA represents only 30%. These proteins have isoelectric points between 4.9 and 5.4, which makes it difficult to isolate them by using the classical methods of ion exchange chromatography. This problem is more complex if another protein, such as AAT, with similar isoelectrical point (4.8) is expressed in trangenic milk whey. Earlier, AAT was isolated from human plasma [17], but since albumin, the principal serum protein, represents about 50% of the total proteins and has similar p*I* to AAT, the traditional ion exchange chromatography did not allow us to separate both proteins efficiently. In a previous work [18], we solved this problem by partitioning human plasma, without previous treatment, in an aqueous two-phase system formed by polyethyleneglycol 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 four assayed experimental situations, the ATPS of PEG1500-pH 6.3, R 4:1 showed to be the most efficient with an AAT recovery of more than 80% in the first and second extractions and a protein purity grade of about 50% in the bottom phase. This implies a purification factor between 1.5 and 1.8. Under experimental conditions, the principal impurity is the BLG due both to its high milk whey concentration and its similar partitioning behaviour with AAT. Although the results from Table 3 suggest that the method can be satisfactorily carried out in only one partitioning step, better purity results would be allowed by performing more extractions without significant loss of protein recovery.

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 from the phosphate-rich phase, thus the predominant non-protein contaminants are the potassium phosphate 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.

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