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Isolation of alpha-1-antitrypsin from human plasma by partitioning in aqueous biphasic systems of polyethyleneglycol–phosphate

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Abstract

The partitioning of alpha-1-antitrypsin was assayed in biphasic aqueous systems containing potassium phosphate and two polyethyleneglycols of molecular mass 600 and 1000, respectively. In order to isolate the alpha-1-antitrypsin from serum plasma, the partitioning behaviour of human serum albumin, its principal contaminant, was also studied. Several aqueous two-phase systems with different partitioning properties were obtained by varying the PEG1000/PEG600 mass proportion. In systems with PEG1000/PEG600 mass ratio of 8, the optimal difference between the partition coefficients of both proteins was found. Under such conditions, a satisfactory purification was carried out by a three-step extraction procedure. By applying this method the alpha-1-antitrypsin specific activity increased severalfold (nearly 10 times) with a yield of 43%.

Keywords: Partitioning; Aqueous two-phase systems; α-1-antitrypsin

1. Introduction

In recent years, due to the increasing importance of modern biotechnology such as recombinant DNA technology, there has been a growing interest in alternative efficient methods for isolation and purification of macromolecules from other fermentation products.

Aqueous two-phase systems have been successfully used for separation and purification of macromolecules and cell particles [1,2] because of their multiple advantages with regard to the traditional techniques. They show good resolution, high yield and low cost. They are easy to scale up and the polymers can be recycled [3]. Moreover, the high water content of both phases (80–90% w/w) which implies high biocompatibility and low interfacial tension, minimizes the biomolecule degradation.

The aqueous two-phase systems may be composed of two polymers of flexible chains, e.g. polyethyleneglycol and dextran or one polymer and a high concentration salt, e.g. polyethyleneglycol and potassium phosphate. The proteins partitioning in aqueous two-phase systems can be influenced by pH, temperature, polymer concentration, the kind of polymers and their molecular mass.

Alpha-1-antitrypsin (AAT) is a protein of the family of the serine protease inhibitors (serpins), with a plasma concentration of ~2 g/l, thus representing

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~3% of the total proteins, while albumin, the most abundant plasma protein, is present in ~40 g/l (55% of the total plasma proteins). In this way, albumin may interfere either in quantitative detection of AAT or in its isolation and purification from plasma [4]. Commercial techniques for purifying AAT use precipitation and ion-exchange chromatography based on the Cohn et al. method which requires several steps and a long time. The yield of this process is around 45% and the purity is around 70%. On the other hand, standard laboratory methods use dye affinity chromatography (with triazine or dextran blue) as the first AAT capture step from plasma, followed by ammonium sulfate fractionation and DEAE chromatographies at pH 8.8 and 6.5. After these four steps the purity is nearly 95% and the yield is 60% [5,6]. In both commercial and laboratory techniques the albumin is not completely eliminated from AAT preparations due to various causes: the isoelectric point of AAT varies from 4.9 to 5.1, which is very near the isoelectric point of the serum albumin (5.1); the high concentration ratio in plasma (nearly 20 times); both proteins are hydrophilic, therefore they are very stable because of their low content of hydrophobic residues exposed to the solvent.

In summary, there are no currently available methods that have been demonstrated to produce high purity with high yield on a commercial scale. In this study we chose to evaluate the potential of aqueous two-phase systems as a novel purification procedure because of the mild separation conditions, low operating costs, simplicity of handling and suitability for large scale applications. Due to the AAT and albumin similarities their partition coefficient in systems formed by one PEG and potassium phosphate were not different enough to effect their separation. For this reason, we assayed the AAT and albumin partitioning in systems formed by potassium phosphate and a mixture of two polyethyleneglycols of molecular mass 600 and 1000, respectively, in different mass ratios.

2. Materials and methods

2.1. Chemicals

Polyethyleneglycols of average molecular mass 600 (PEG600) and 1000 (PEG1000), respectively, alpha-1-antitrypsin (AAT), human serum albumin (HSA), α-*N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) were purchased from Sigma (St. Louis, MO, USA) and used without further purification.

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 m*M*, 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 liberated 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 the absence and presence of AAT [7,8].

2.3. Determination of human serum albumin and total protein concentration

Human serum albumin was measured using the cresolsulphophthalein method [9], the protein was added to the colorimetric reagent in a medium of buffer acetate pH 3.8 and the absorbance at 625 nm was determined. A HSA reference solution was prepared using crystallized protein. The total protein concentration was determined by measuring the absorbance at 230 nm, using human albumin as standard [10].

2.4. Preparation of the biphasic aqueous systems

To prepare phase systems, stock solutions of the phase components: PEG 40% w/w, and potassium phosphate, pH 7.0 (23% w/w) were mixed. The total system compositions of PEG and phosphate are shown in Table 1. In order to speed up phase separation low-speed centrifugation was used after a 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.

2.5. Determination of the partition coefficient (K)

Protein partitioned in both phases was analysed by dissolving increasing amounts of protein (5 to 15 μ*M* of total concentration) in the two-phase preformed systems containing 2 ml of each equilibrated phase. The protein aliquots added to the system varied from 5 to 20 μl, the change of the total volume of each phase being negligible. After mixing through 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 the separated phases, and after dilution, the protein content in each phase was determined by measuring the absorbance at 230 nm. For AAT, the enzymatic activity measurement was also employed. Equally diluted samples from identical phase systems without protein were used as blanks, which were prepared in parallel. The partition coefficient *K* was defined as:

$$K = \frac{[P]_{top}}{[P]_{bottom}}$$

where $[P]_{top}$ and $[P]_{bottom}$ are the equilibrium concentrations of the partitioned protein in the PEG (top) and phosphate (bottom) enriched phases, respectively. In the protein concentration range assayed, a plot of $[P]_{top}$ vs. $[P]_{bottom}$ showed a linear behaviour with a slope equal to *K*.

2.6. Multiple partition procedure

The countercurrent distribution was carried out manually as described by Johansson and Andersson [11] using two transfers. To avoid any unnecessary dilution of AAT, the first aqueous biphasic system was prepared by mixing 3.3 g of human plasma, solid PEG600 and PEG1000, and adequate quantities of potassium phosphate 23% w/w, pH 7.0, in order to reach the total composition of system B as shown in Table 1. The system was gently stirred during 1 h at 8 °C, the phases were separated by low speed centrifugation and the precipitated protein at the interphase was eliminated, the volumes of the top and bottom phases being 4.5 and 6 ml, respectively. No change to the volumes was observed during the partitioning. The main portion of bottom phase (5 ml) was transferred to 5 ml of a pure top phase (from a previously prepared phase system obtained under identical conditions but without human plasma) until it reached the equilibrium again. The second transfer was made in similar conditions but employing 4.5 ml of bottom and pure top phases. Total protein concentration and AAT activity in each phase were determined.

3. Results

3.1. Effect of biphasic system composition on the HSA and AAT partitioning

The partitioning of HSA and AAT was assayed in several biphasic systems of potassium phosphate with: PEG600, PEG1000 and mixtures of both PEGs. Table 1 shows *K* values yielded for the two proteins assayed. AAT showed a great affinity for the phosphate phase in systems A and B (K_{AAT} 0.018 and

Table 1
Partition coefficients obtained for AAT and HSA in two-phase systems of different composition

System	Total concentration (% w/w)			[PEG1000]/[PEG600] mass ratio	K_{HSA}	K_{AAT}	K_{HSA}/K_{AAT}
	Potassium phosphate pH 7.00	PEG1000	PEG600				
A	15	13.0	0.0	–	3.6	0.018	200
B	15	11.7	1.4	8.4	6.3	0.034	184
C	15	10.4	2.8	3.7	10.0	0.432	23
D	15	8.7	4.7	1.9	21.6	0.569	38
E	15	6.4	7.0	0.9	30.7	1.009	30
F	15	4.2	9.3	0.4	52.0	2.125	24
G	15	0.0	14.0	0.0	–	30.81	–

The two-phase systems are prepared as described in Section 2. Temperature 20 °C. pH 7.00.

0.034, respectively), while HSA showed more affinity for top phases (K_{HSA} 3.6 and 6.3, respectively). For both proteins the K values obtained increased when the PEG1000/PEG600 mass ratio decreased. The K_{HSA} in system G was impossible to determine because this protein is nearly completely transferred to the top phase; its concentration in the bottom phase being negligible. Table 1 also shows the ratio between the partition coefficients of the two proteins for the totality of systems assayed. The major ratio is observed for systems A and B, suggesting that they have the best capacity of separating both proteins. Fig. 1 depicts a hypothetical three partition step experience conducted in countercurrent fashion in systems A and B [3]. The phases in the different transfers were named as B_{11}, T_{11}, B_{21}, T_{21}, etc.; and the bottom (V_B) and top (V_T) phase volumes were considered to be equal to 1 l. The HSA and AAT total concentrations in the first system were considered to be 40 g/l and 2.5 g/l, respectively (normal plasma concentrations). The mass of each protein in the lower phase (m_B) after equilibrium was calculated according to the following expression [12]:

$$m_B = \frac{1}{1 + K\,V_T/V_B} m_{Total}$$

where m_{Total} is the total mass of protein in the system. The mass of protein in the top phase was calculated by the difference between m_{Total} and m_B.

The percent recovery (R) and purity (P) of AAT after two transfers in the B_{31} phase for systems A

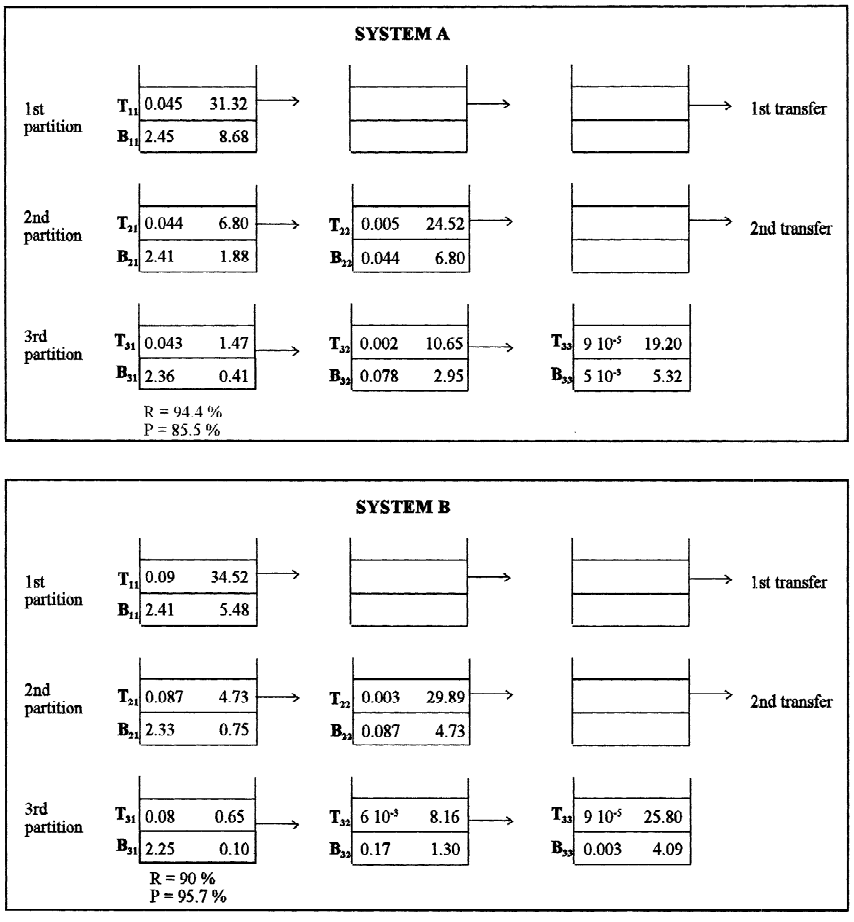

Fig. 1. Theoretical three partition step experience conducted in countercurrent mode for AAT and HSA (taking into account their partition coefficients) in systems with A and B composition. For each system the numbers on the left and on the right indicate the AAT and HSA calculated mass after reaching partition equilibrium. R and P are the percent recovery and purity of AAT in the B_{31} phase, respectively.

and B are shown in Fig. 1 and were calculated as follows:

$$R = \frac{m_{AAT}}{m^{o}_{AAT}}100 \quad P = \frac{m_{AAT}}{m_{AAT} + m_{HSA}}100$$

where m^{o}_{AAT} is the initial mass of AAT, 2.5 g and m_{AAT} is the AAT mass calculated for B_{31} phase. We selected system B to isolate AAT from plasma because, although both systems A and B yielded similar recoveries of AAT, system B yielded a protein with major purity, assuming that HSA is the only contaminant present.

3.2. Partitioning of HSA and AAT in a mixture of both proteins

The choice of system B to isolate AAT from plasma is supported by the theoretical calculations of Fig. 1, where we assumed that the partitioning behaviour (partition coefficient) of both proteins in a mixture is similar to that of each protein alone. In order to confirm this assumption, a mixture of HSA and AAT in the same proportion of human plasma (HSA–AAT mass ratio: 20) was partitioned in biphasic systems with different mass relation of PEG600 and PEG1000. The *K* values for both proteins were determined using the cresolsulphophthalein and the enzymatic activity methods for albumin and AAT, respectively. The *K* values observed for AAT and HSA were very similar to those found for each protein alone (data not shown) suggesting that the presence of HSA does not modify the partitioning of AAT and vice-versa. Previous reports [4] have suggested that the electrophoretic mobility of AAT decreased in the presence of HSA due to the formation of a complex between both proteins; however, our finding suggests that there is not a specific interaction between HSA and AAT because they do not perturb each other in their partitioning behaviour.

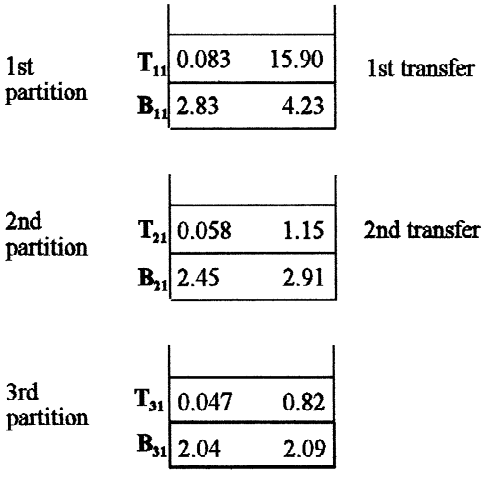

Fig. 2. Three partition step experience applied on human plasma. The numbers on the left and right of each phase indicate the AAT and the protein total concentrations, respectively. The volumes of phases are: T_{11}, 4.5 ml; B_{11}, 6 ml; T_{21} and B_{21}, 5 ml; T_{31} and B_{31}, 4.5 ml. Temperature 20 °C. System B composition according to Table 1.

3.3. Purification of AAT from plasma

Fig. 2 shows the AAT and total protein concentrations in each phase, and Fig. 3 the calculated partition coefficients for AAT and for total plasma proteins for the three partition step procedure. The partition coefficient for AAT for the totality of steps was in the range of 0.024–0.029 which implies a slight decrease with respect to the value obtained for AAT alone. An overall partition coefficient for the plasma proteins in total was also calculated. For the first step, a partition coefficient 10-fold higher (3.75) than the values for the second and third steps indicates that the first partitioning step is very important for the elimination of plasma proteins with non-antitryptic activity, principally those with hydrophobic characteristics. Table 2 shows that the antitryptic activity decreases from 1658 to 1330 units only in the first step while the total protein concentration goes from 207.2 to 25.4. In each purification step, the antitryptic activity decreases ~25% with respect to the value obtained in the previous step, while the percent loss of total protein is maximum from plasma to B_{11} phase and decreases in the B_{21} to B_{31} passage, which implies that the last steps do not contribute significantly to AAT purification.

4. Discussion

Classical methods for the isolation and purification of AAT involve two sulphate ammonium precipitations, two affinity chromatographies and two ion-exchange chromatographies, which limits their scale-

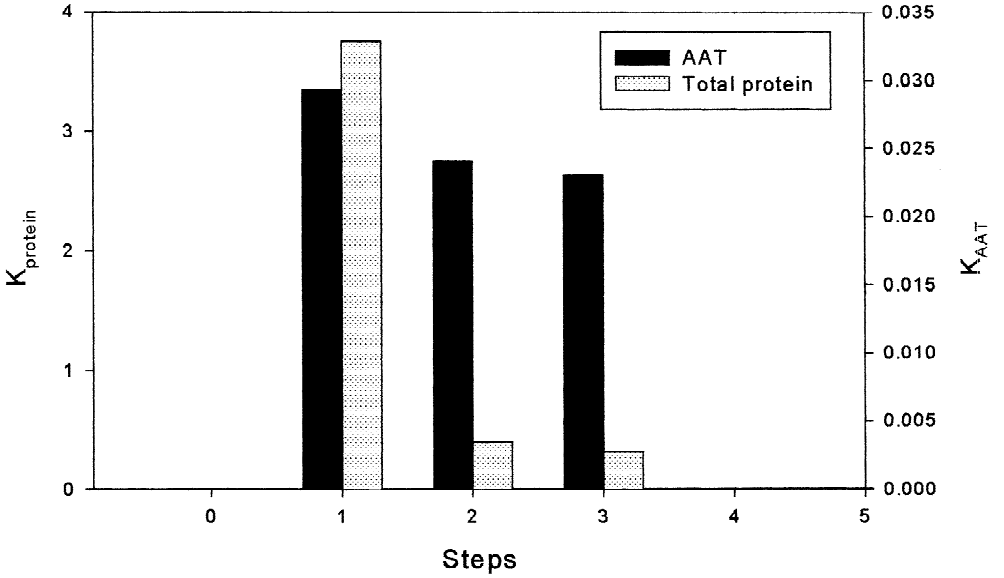

Fig. 3. Partition coefficient of AAT and total protein for the three steps of the multiple partition procedure in the purification of AAT from human plasma. Temperature 20 °C, pH 7.00.

up application. Moreover, a long time is required, the recovery of AAT activity being 25% with a purification of 40-fold [13].

Production of concentrated plasma AAT presents several problems as do other plasma derivatives: (i) purification methods must be compatible with the clinical use of the final product; (ii) the purification factor must be high to avoid the presence of any undesirable protein in the final product; (iii) as AAT has a similar isoelectric point to albumin, the separation of these two proteins is difficult.

The AAT employed with therapeutic purposes must have a high specific antitrypsin activity and a significant grade of purity.

The concentration of AAT by using an aqueous biphasic system is simple, cheap, suitable for large scale application and able to be carried out in a short time (1–2 h) even with large quantities of protein solution. If the partition coefficients (or ratios) of two substances differ by a factor of 10 or more, their separation can be satisfactorily carried out [3]. When a single component must be extracted from a mixture (such as plasma), phase system compositions are often manipulated in such a manner that the component partitions into one of the phases, while the other components of the mixture partition into the other phase [14,15]. The choice of adequate partitioning conditions (pH, polymer concentrations, etc.) is relatively simple when the proteins to be separated differ significantly in their structural properties (such

Table 2
Purification of AAT by a multiple partition procedure

Purification step	Protein content (mg)	Antitryptic activity (units)	Specific activity (units/mg)	Purification (fold)	Recovery of inhibitory activity (%)
Plasma (3.3 g)	207.2	1658	8	1	100
B_{11}	25.4	1330	52	6.6	80
B_{21}	14.5	958	65.8	8.3	58
B_{31}	9.4	718	76.4	9.6	43

as molecular mass, hydrophobic surface) but it becomes more complicated when those differences are minor. AAT and HSA have similar surface hydrophobicities [16], isoelectric points and, although their molecular masses differ by 10 kDa, they have the same mobility both in denaturing and non-denaturing PAGES [4]. In a previous work [16] we concluded that the partitioning of AAT and HSA in systems of polyethyleneglycol–dextran is governed by the combination of several factors. The electrostatic interactions are similar for both proteins while the preferential interaction of the polyethyleneglycol with the protein domain and the structure of water, which determines the strength of hydrophobic interactions, plays an important role in the mechanism of partitioning. In this way, we tried to obtain optimal separation biphasic systems not by changing pH or ionic strength of the medium but employing a two-phase system formed by potassium phosphate and two PEGs of different molecular mass.

Most proteins partition more to the top phase in phase systems with low molecular weight PEG [17]. Table 1 shows that the partition coefficient of AAT and HSA increases when system composition varies from PEG1000 to PEG600 alone, the change being more significant for AAT whose partition equilibrium is displaced to the top phase in the system with PEG600 and to the bottom phase in the system with PEG1000 alone. The influence of the molecular mass of PEG on protein partitioning can be explained on the basis of Flory Huggins theory for polymers in solution [18,19], which takes into account the Flory coefficient of interaction between the protein and the polymer.

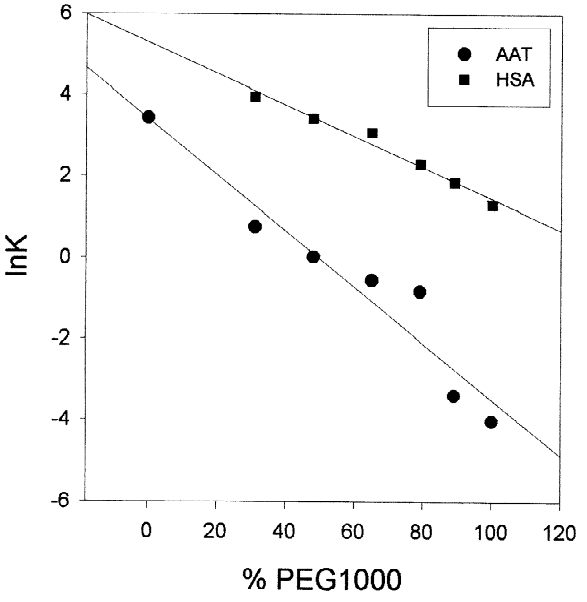

Fig. 4. Experimental relationship between natural logarithm of AAT and HSA partition coefficients and mass percent of PEG1000 in the mixture PEG600–PEG1000. Temperature 20 °C, pH 7.00.

We postulate a preferential interaction between the PEG molecule and the protein domain that decreases when the molecular mass of PEG increases because of its exclusion from the protein domain. When two different molecular mass types of PEG are mixed the preferential interaction adopts an intermediate magnitude depending on the relative composition of both PEGs.

Fig. 4 shows a linear relationship between the natural logarithm of partition coefficient (ln K) and the mass percent of PEG1000 in the mixture PEG600–PEG1000 for both proteins, which suggests that each type of PEG contributes in an independent manner to the Gibbs free energy of partition. These findings are in agreement with previous reports [20] that demonstrated the correlation of ln K with the PEG concentration difference between the phases, assuming that the PEG concentration in the bottom phase is negligible with respect to the top one.

We chose the system of composition B because in theory it has the best capability of separating both proteins (Fig. 1). The composition of both PEGs in each phase after equilibrating was impossible to determine by analytical methods because of similarities in their properties. By comparing the theoretical calculations of Fig. 1 with the experimental data of Fig. 2, there are some differences. Although we have demonstrated that K_{AAT} was independent of HSA presence, Fig. 3 shows that K_{AAT} decreases slightly in the presence of other plasma proteins due to the influence of protein–protein interactions on the activity coefficient contained in the partition coefficient expression [21].

Results in Table 2 suggest that the method can be carried out satisfactorily in only one partitioning step; the addition of one or two more partitioning steps not only increases slightly the purification but induces a significant decrease in the recovery of the

AAT activity. This last finding suggests that only one partition step is necessary to obtain a protein pool with a content of about 80% of the initial AAT activity, but only 12% of the total proteins of the plasma.

Sometimes the application of a purification method to obtain a therapeutical substance introduces new contaminants that must be eliminated in order to avoid adverse effects. In this case AAT is obtained from the phosphate-rich phase, thus the predominant non-protein contaminant present is potassium phosphate with a small amount of PEG. According to the binomial diagram of PEG1000–potassium phosphate system reported by other authors, the bottom phase has a very low PEG concentration, about 1–3% w/w and potassium phosphate concentration about 12–20% w/w. By means of diafiltration both components are potentially removed from the protein.

Finally, although further work needs to be done to optimise partitioning conditions, we concluded that this partition procedure constitutes a viable and potentially useful first step procedure for purification of AAT from plasma. Given the relative ease of scaling up two-phase partition economically, they could form part of future industrial purification protocols.

5. Nomenclature

AAT	alpha-1-antitrypsin
HSA	human serum albumin
PEG600 and PEG1000	polyethyleneglycols of average molecular mass 600 and 1000

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