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Protein partitioning equilibrium between the aqueous poly(ethylene glycol) and salt phases and the solid protein phase in poly(ethylene glycol)–salt two-phase systems[1]

B.A. Andrews, J.A. Asenjo*

Centre for Biochemical Engineering and Biotechnology, Department of Chemical Engineering, University of Chile, Beauchef 861, Santiago, Chile

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Abstract

True partitioning behaviour, which is independent of the protein concentration in aqueous two-phase systems, only occurs at relatively low protein concentration. The actual concentration limit depends on the properties of the protein. When the concentration of a protein exceeds relatively low values, precipitation at the interface can be observed. This protein precipitate is in equilibrium with the protein solubilized in each of the phases. This paper discusses the effect of protein solubility in view of the equilibrium of the protein concentration between the aqueous poly(ethylene glycol) and salt phases and the solid protein phase using three proteins. It was found that only rarely will the proteins be completely in solution as the concentration is increased until a solubility limit is reached and then the protein precipitates fully out of solution. A behaviour that came close to this was only seen in one case out of six. In virtually all cases, a third phase is formed which represents a solid aggregate phase which is in equilibrium with the other two, largely aqueous, phases. As the overall concentration of protein in the system is increased and the concentration in the top and bottom aqueous phases increases, the pseudo concentration in the solid-phase, C'_S, also increases. This could have interesting implications in terms of the amount of water associated with this phase and it certainly means that in this particular case, the solid phase is not a crystal.

Keywords: Partitioning; Aqueous two-phase systems; Proteins; Poly(ethylene glycol); Salts

1. Introduction

The partition coefficient of a protein in an aqueous two-phase system (ATPS), K, is usually defined as C_T/C_B where C_T and C_B represent the equilibrium concentrations of the partitioned protein in the top and bottom phases, respectively. This "true" partitioning behaviour, which is independent of the protein concentration, occurs only at low protein concentration. The actual concentration limit depends on the properties of the protein. When the concentration of a protein exceeds relatively low values, precipitation at the interface can be observed. This protein in a "solid" or "dense" phase is in equilibrium with the protein solubilized in each of the liquid phases.

In this respect, two extreme cases can be visualized. One would be a behaviour similar to that

*Corresponding author.

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observed in precipitation where a solid is formed [1,2] usually above a point at which the solubility limit in a particular liquid phase has been reached. The other extreme case would be that a solid or dense protein phase coexists with the protein in solution at all concentrations. There is an important conceptual difference between these two cases, which also has an effect on the design of a decanter or other liquid–liquid separator. In the first case, when operating under the solubility limit, there is no need to recover the interphase, whereas in the second case, recovery of the interphase could be important even at low protein concentrations.

The aim of this paper is to discuss the effect of protein solubility as a function of the equilibrium of the protein concentration between the aqueous poly(ethylene glycol) (PEG) and salt phases and the solid or dense protein phase using three proteins with different properties.

2. Experimental

2.1. Materials

PEG with a molecular mass of 4000 (M_r=3500–4000) was purchased from Fluka (Buchs, Switzerland). All other chemicals were of analytical grade.

2.2. Proteins

Amyloglucosidase (1,4-α-D-glucan glucohydrolase, E.C. 3.2.1.3.) (*Aspergillus niger*) and subtilisin (E.C. 3.4.21.14) (*Bacillus subtilis*) were obtained from Boehringer (Mannheim, Germany). Trypsin inhibitor (Soybean) was purchased from Sigma (St. Louis, MO, USA).

2.3. Preparation of phase systems and partition experiments

Phase systems were prepared from stock solutions of PEG (50%, w/w) and phosphate (40%, w/w). The phosphate stock solution consisted of a mixture of K_2HPO_4 and NaH_2PO_4 at pH 7. All partition experiments were done at 20°C and pH 7. Low-speed centrifugation (1200 *g*, 5 min) was used to speed up phase separation after mixing. Samples of the top and bottom phase were assayed for protein concentration using the modified Lowry method [3].

3. Results and discussion

Recently we have reported experimental results on the effect of overall protein concentration on the partition coefficient of the protein between a top, PEG phase, and a bottom, salt phase [4,5]. The results were plotted as a concentration in the phase against overall calculated protein concentration in the system. It was clearly shown that a solid or dense phase appears at concentrations well below saturation of the phases, but no overall material balance was carried out to evaluate the presence and the role of the solid protein phase in equilibrium in the system.

In this paper we report two alternative ways of plotting and analysing the formation of a solid aggregate or dense protein phase in aqueous two-phase systems. In the first case, concentration in the top and bottom phases and also in the solid phase is plotted as a function of overall protein present in the system (Figs. 2 and 3). Alternatively, the concentration in each of the liquid phases is plotted as a function of concentration in the solid phase (Figs. 4 and 5). Although these two ways of plotting the data may appear very similar, Fig. 4 and Fig. 5 actually show the equilibrium between each of the liquid phases and the solid or dense protein phase formed. In an aqueous two-phase system (ATPS) where a single protein is present, and where a solid protein phase has formed, a situation such as that shown in Fig. 1 occurs, where the solid may be present either as a suspension in the top or bottom phases, or as a

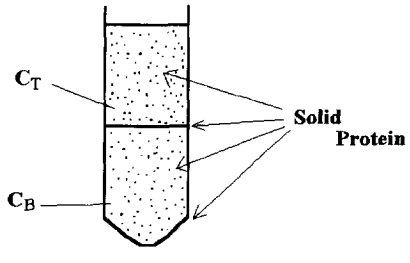

Fig. 1. Diagram showing an aqueous two-phase system with a single protein present where a solid phase has formed.

layer at the interphase or below the bottom phase. After applying a settling force, the solid protein, which is in equilibrium with the protein present in the top and bottom liquid phases, will remain in suspension or settle at the interphase or at the bottom depending on the actual density of the liquid phases and that of the solid aggregate.

In this case, if we express the protein concentrations per unit volume, the protein concentrations in the top and bottom liquid phases and the solid phase are in equilibrium.

$$C_T \rightleftarrows C_S \rightleftarrows C_B \rightleftarrows C_T \quad (1)$$

C_T, C_B and C_S are the protein concentrations in the top, bottom and solid or aggregate phases, respectively. If the concentrations are mole fractions and the solid phase is a crystal, C_S would be a constant.

An overall protein balance in the system is

$$P_O = P_T + P_B + P_S \quad (2)$$

where P_T, P_B, and P_S are total protein in the top, bottom and solid phases, and $P_T = C_T V_T$ and $P_B = C_B V_B$, where V_T and V_B are the volumes of the top and bottom phases, respectively. The exact value of V_S (volume of solid or dense phase) is difficult to ascertain, particularly if C_S is in equilibrium at all concentrations, as shown by Eq. (1). For practical purposes we will use the parameter C'_S, which is a pseudo solid concentration in the system, which can be measured (observable) or calculated and has been defined as P_S/V_O, where $V_O = V_T + V_B + V_S$ represents the overall volume of the two (three) phase system.

Results for C_T, C_B and C'_S are shown in Fig. 2 and Fig. 3 for the three proteins that have shown different behaviour in terms of their solubility in the PEG–salt systems. Fig. 2 represents a PEG–phosphate two-phase system and Fig. 3 represents a similar system with a high concentration of NaCl.

Fig. 2a shows the concentrations of amyloglucosidase in the top, bottom and solid phases of a PEG–phosphate system with no NaCl. The concentration of the protein in the PEG phase is almost constant, and is very low throughout the range of protein concentrations used. The concentration in the salt phase increases linearly with increasing overall protein concentration. The solid protein concentration, C'_S, also increases linearly with overall protein concentration and there is protein in the solid phase at all concentrations. Fig. 2b shows the same ATPS with subtilisin. Here the protein concentrations in the

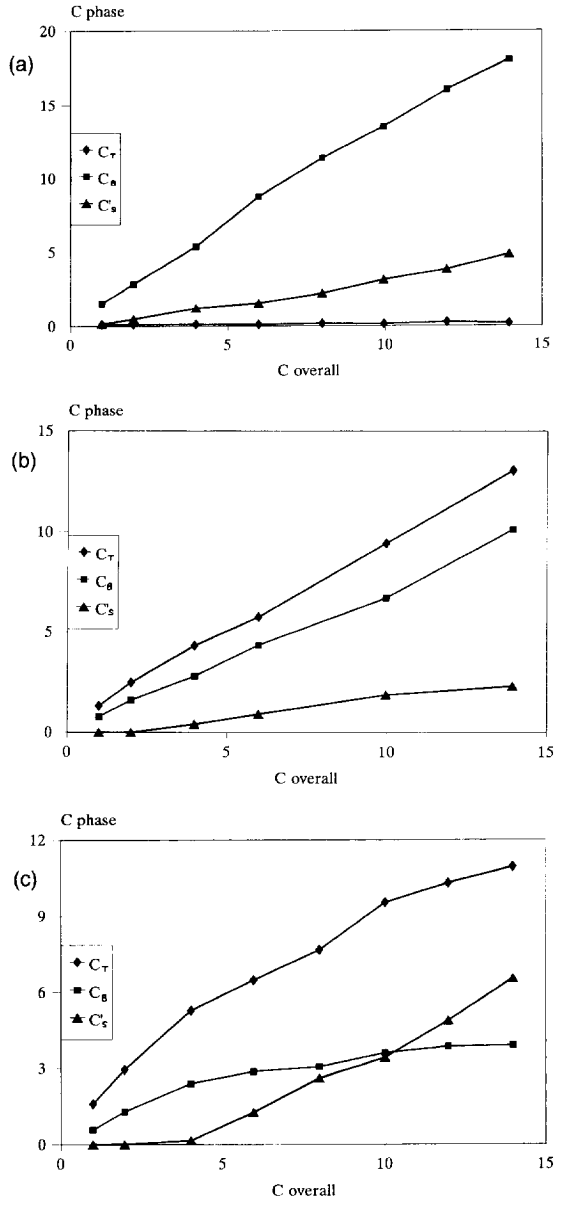

Fig. 2. Effect of overall protein concentration in PEG 4000 (13%, w/w)–phosphate (10.7%, w/w) systems at pH 7 with no NaCl on the protein concentration in the top (C_T), bottom (C_B) and solid (C'_S) phases, with three different proteins. All protein concentrations are given in mg/ml. (a) Amyloglucosidase; (b) subtilisin; (c) trypsin inhibitor.

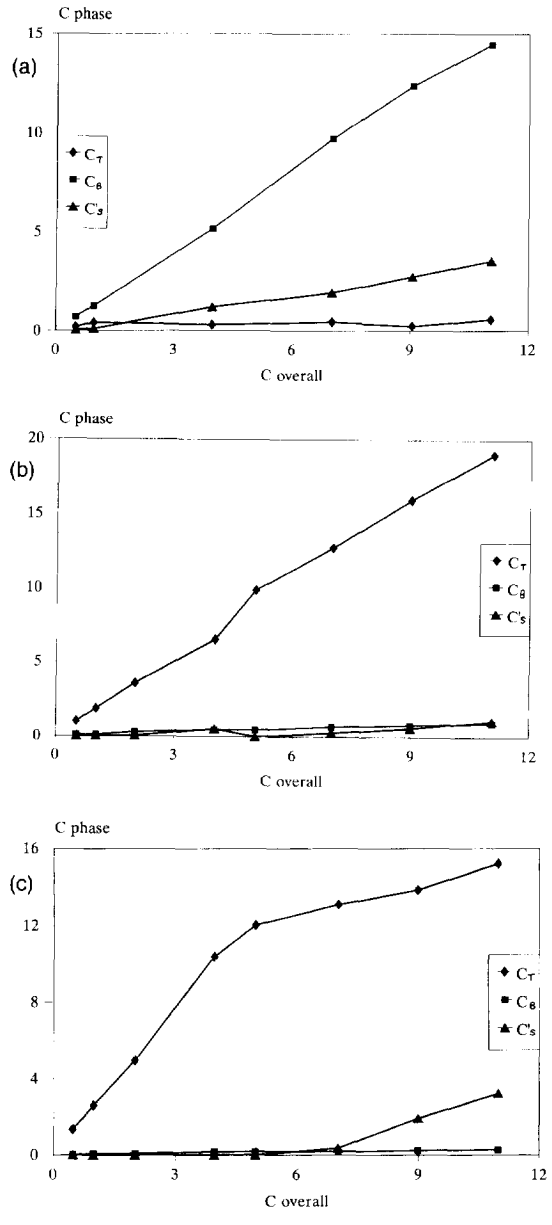

Fig. 3. Effect of overall protein concentration in PEG 4000 (13%, w/w)–phosphate (10.7%, w/w) systems at pH 7 with 8.8% NaCl on the protein concentration in the top (C_T), bottom (C_B) and solid (C'_S) phases, with three different proteins. All protein concentrations are given in mg/ml. (a) Amyloglucosidase; (b) subtilisin; (c) trypsin inhibitor.

top and bottom phases increase linearly with overall protein concentration. There is no solid phase below an overall protein concentration of around 4 g/l, and at higher concentrations the concentration in the solid phase increases linearly. This figure demonstrates that subtilisin has a high solubility in the PEG and salt phases and that there is little protein in the solid phase in this system. Fig. 2c shows the results for the same system with trypsin inhibitor. In this case, the results show that in both the top and bottom phases, the concentration curves tend to saturation values and there is no protein present as a solid, C'_S, at low overall protein concentrations. Only when the PEG and salt phases approach saturation does a solid phase appear. This type of behaviour, observed with trypsin inhibitor, is what is expected if solubility in the phases shows a behaviour similar to precipitation [1,2]. The curves for the other two proteins clearly show an equilibrium between the protein concentrations in the two aqueous phases. The solid or dense phase is formed at almost all concentrations and certainly well before saturation is even approached.

Fig. 3 shows the concentrations of protein in the top, bottom and solid phases of PEG–phosphate systems with 8.8% NaCl. In Fig. 3a amyloglucosidase is used and it shows very similar results to Fig. 2a (without NaCl); the protein concentration in the PEG phase is consistently low (ca. 0.4 g/l) throughout the range of overall protein concentrations used, and the concentrations in both the salt and solid phases increase linearly with increasing overall concentration. Protein is present as a solid even at the lowest concentrations used (0.5 g/l overall concentration). Fig. 3b shows the results for the protein subtilisin. In this system, the addition of NaCl can be seen to have a clear effect when compared with Fig. 2b. The concentration of protein in the top PEG phase increases linearly with increasing overall protein concentration. The concentrations in the bottom and solid phases are both very low throughout the range of concentrations used, which is in contrast with Fig. 2b (no NaCl) where the protein concentration in the bottom salt phase also increased linearly. In the presence of 8.8% NaCl, the protein is thus salted out of the bottom phase and salted into the top PEG phase. Fig. 3c shows the results using trypsin inhibitor, which are similar to Fig. 3b but show saturation type behaviour which, as in Fig. 2c, is more similar to precipitation than to the equilibrium type behaviour where solid protein exists

at all concentrations as seen in Fig. 3a and Fig. 3b. The curve showing protein concentration in the PEG phase is certainly not linear and only after the top phase has begun to approach saturation does solid protein appear. There is no solid phase until an overall protein concentration of ca. 7 g/l. When compared with Fig. 2c, it is clear that, similar to the behaviour in Fig. 3b, the protein is salted out of the bottom phase when NaCl is present in the system.

An alternative way of plotting the data, shown in Figs. 2 and 3, is to plot the concentration of both aqueous phases (C_T and C_B) as a function of C'_S, the pseudo solid concentration in the system. This is shown in Fig. 4 and Fig. 5.

Fig. 4a (amyloglucosidase) looks very similar to Fig. 2a except for a shift in the values of the abcissa, since C'_S increases almost linearly. Fig. 4b (subtilisin) looks somewhat different from Fig. 2b since a few values of C'_S equal to zero exist at the lower concentrations of C_0. Fig. 4c, on the contrary, is very different from Fig. 2c (trypsin inhibitor); the bottom phase shows a clear saturation behaviour which is not as pronounced in the top phase. A similar analysis applies when comparing Figs. 5 and 3 (with NaCl). Here though, Fig. 5b is quite different from Fig. 3b (subtilisin), particularly as the top phase shows some indication of saturation type behaviour. Fig. 5c, on the other hand, shows an extremely clear case of saturation type behaviour. At lower protein concentrations, there is no solid phase and after the solubility limit is reached, virtually all protein precipitates as a solid. When analysing the equilibrium between only one liquid phase and a solid protein phase during precipitation using salts [6], it was found that for lysozyme a similar behaviour to that found in this work for trypsin inhibitor could be observed. For α-chymotrypsin and BSA, an equilibrium between the solid and liquid phase similar to that observed here for aqueous two-phase systems was found. In this case, an equilibrium constant, K_c, was defined [6]. This would correspond in our case to the slope of the initial part of the curve in Fig. 4a and Fig. 5a and to some extent Fig. 4b and Fig. 5b.

The analysis presented in this paper shows that in aqueous two-phase systems only rarely will the proteins be completely in solution as the concentration is increased until a solubility limit is reached and then the protein precipitates fully out of solution.

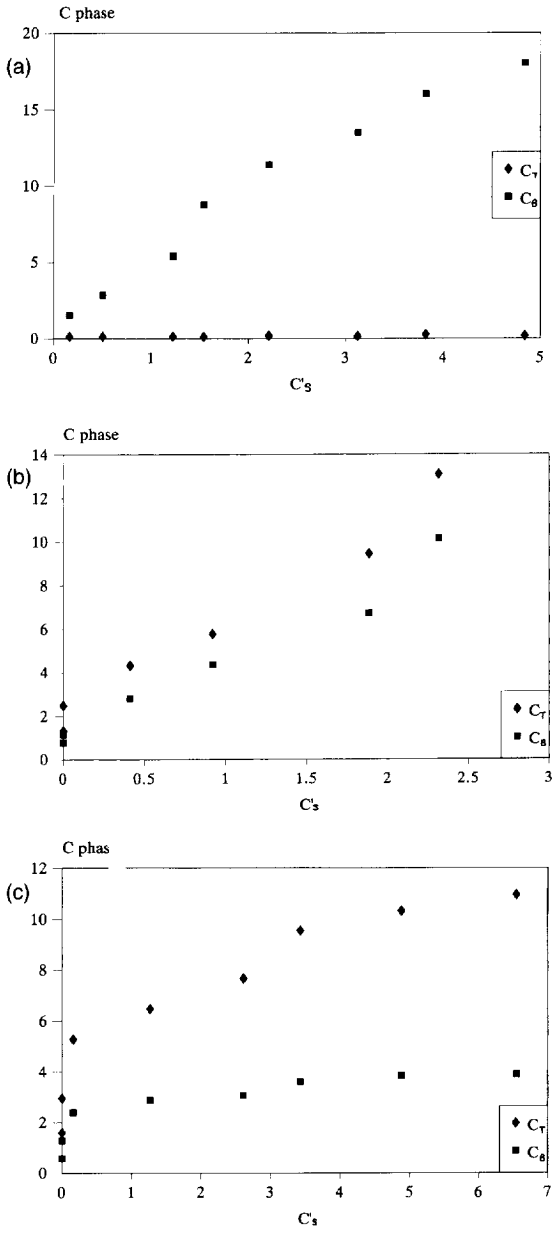

Fig. 4. Effect of pseudo solid protein concentration (C'_S) on the concentration of protein in the top (C_T) and bottom (C_B) phases of a PEG–phosphate system with no NaCl for three different proteins. All protein concentrations are given in mg/ml. (a) Amyloglucosidase; (b) subtilisin; (c) trypsin inhibitor.

This behaviour was only seen in one case (Fig. 5c). In virtually all cases, a third phase is formed which represents a solid aggregate or dense protein phase

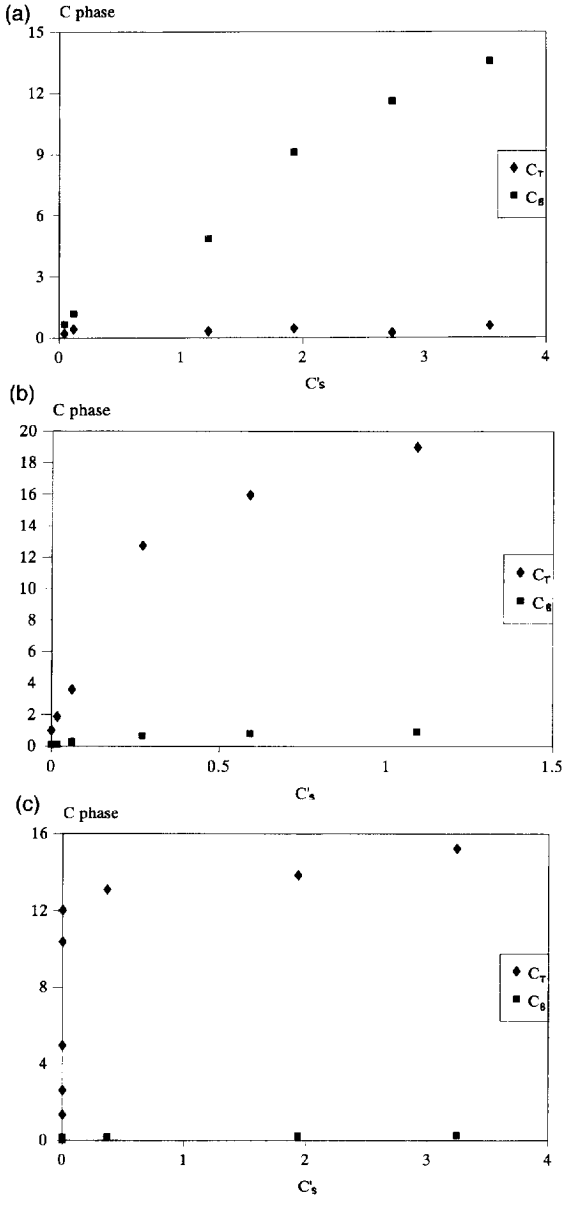

Fig. 5. Effect of pseudo solid protein concentration (C'_S) on the concentration of protein in the top (C_T) and bottom (C_B) phases of a PEG–phosphate system with 8.8% NaCl for three different proteins. All protein concentrations are given in mg/ml. (a) Amyloglucosidase; (b) subtilisin; (c) trypsin inhibitor.

which is in equilibrium with the other two, largely aqueous, phases. As the overall concentration of protein in the system is increased and the concentration in the top and bottom aqueous phases increases, the pseudo concentration in the solid phase, C'_S, also increases. This could have interesting implications in terms of the amount of water associated with this phase, and it certainly means that in this particular case (e.g. Fig. 2a and Fig. 2b, or Fig. 4a and Fig. 4b, and Fig. 3a or Fig. 5a) the solid phase is not a crystal that would have a constant value of C_S.

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