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Phase equilibrium and protein partitioning in aqueous two-phase systems containing ammonium carbamate and block copolymers PEO–PPO–PEO

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Abstract

The liquid–liquid equilibrium of aqueous two-phase systems formed by the PEO–PPO–PEO block copolymers F38 or F68 and ammonium carbamate and the partition of the model proteins bovine serum albmin, lysozyme, α-lactalbumin, β-lactoglobulin and γ-globulin in these systems are presented in this paper. Phase diagrams were determined at 4 °C (F38) and 25 °C (F38 and F68); systems containing F68 present a narrow two-phase region, while systems containing F38 have a suitable working region. Partition coefficients, defined as the ratio of protein concentration in the polymer-rich phase to that in the salt-rich phase, were found to vary in the range of 0.1 to 0.8 for bovine serum albumin, 0.5 to 2.0 for lysozyme, 1.0 to 2.5 for α-lactalbumin, 0.1 to 1.0 for β-lactoglobulin and 0.3 to 1.0 for γ-globulin, depending on the polymer chain-size, temperature and tie-line length. The different trends of protein partition coefficients show that high degrees of separation can be achieved, establishing the system F38 and ammonium carbamate as an alternative to be considered when planning the downstream processing of proteins.

Keywords: Protein; Downstream processing; Aqueous two-phase systems; Salt; Polymer

1. Introduction

The use of aqueous two-phase systems (ATPS) for the separation of mixtures of biological molecules can nowadays be regarded as a well-established technique. The number of applications of these systems has steadily increased since the pioneering work by Albertsson in the mid 1960s, who firstly showed the potentiality of using aqueous two-phase systems as a separating medium of complex mixtures of biomolecules [1]. Some of recently described applications of ATPS include not only industrial-oriented ones (such as the purification of recombinant proteins from corn seeds [2] and of immunoglobulin G from plasma [3], and the isolation of α-toxin from *Clostridium perfringens* fermentation broth for the production of vaccines [4]), but also lab-scale ones (for instance, in proteomic [5] and genomic [6] analyses).

The phase split that results in an ATPS occurs when either two polymers or a polymer and a salt are mixed to water within a certain range of compositions – although it must be recalled that neither all polymers nor all salts lead to this phase separation. Commonly used pairs of substances include polyethylene glycol (PEG) and dextran (the most extensively studied system), polyvinyl alcohol and dextran, PEG and ammonium sulfate, and PEG and sodium sulfate. Besides the research about the uses of ATPS, there is also continuous intense research towards the description of new systems [7,8]. The reason for this interest is comprehensible: increasing the number of described systems means expanding the possibilities of separating a target molecule out of its environment: it may happen that some systems are suitable for some definite separations, but not for others.

A class of synthetic polymers whose properties have recently drawn the attention for the formation of new ATPS is the class of block copolymers PEO–PPO–PEO, i.e., copolymers formed by three different homogeneous chains: a block of polyethylene oxide (PEO), a block of polypropylene oxide (PPO), and a symmetrical block of PEO. These copolymers are usually identified

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by their commercial names, which comprise a letter, indicating their physical state at room conditions (L for liquid, P for paste, and F for flocs), one or two figures standing for the size of PPO chain in a standardized scale, and a last figure representing the mass fraction of EO in the polymer. For instance, the copolymers P104 and P108 are pastes and have PPO chains of similar size (approximately 56 monomer unities), but PEO chains of different size (40 and 80% of the whole molecule, respectively).

The use of block copolymers PEO–PPO–PEO to form ATPS is appealing for it may result in more extreme partition coefficients, therefore increasing the efficiency and the selectivity of this unit operation. Research about this use began with Skuse et al. [9], who studied the partition of proteins in systems formed by hydroxypropyl cellulose and P105, and Kitahara et al. [10], who studied the partition of proteins in ATPS formed by F68 and ammonium sulfate.

Phase diagrams of ATPS formed by block copolymers L64, F68 or P105 and dextran were obtained by Svensson et al. [11]. These authors observed a pronounced shift in the tie-line slope when changing the temperature, which indicated that the aggregation behavior of the block copolymer changes accordingly. The partition of amino acids and peptides in systems formed by dextran T500 and the copolymer P105 was studied by Svensson et al. [12]; these authors noticed that, while the partition coefficient of amino acids is close to 1.0, the partition coefficient of oligopeptides increases considerably when the number of tryptophan residues in the molecule increases. The partition of proteins in systems formed by F68 or P105 and dextran T500 was studied by Svensson et al. [13]: the authors obtained more one-sided values of partition coefficient when compared to similar systems containing polyethylene glycol. The partition of insulin in ATPS formed by L62, L64 or F68 and potassium phosphate was studied by Haraguchi et al. [14]: as an example of the flexibility of these systems, by changing the PEO chain, the authors observed that the partition coefficient shifts from 5.0 to more than 50.0, which constitutes a considerably large amplitude. Phase diagrams for L35 or F68 and potassium phosphate [15] and sulfate salts [16], and of F38, F68, F108, P105 or P103 and dextran [17] also show phase behaviors that differ from those of PEG-containing ATPS.

When a polymer and a salt are used to form an ATPS, both the phase diagram and the behavior of proteins depend on the polymer and on the salt. Salts extensively used to form ATPS are ammonium and sodium sulfate, potassium and sodium phosphate, and sodium citrate; besides them, ammonium carbamate is an alterative recently considered. The use of this salt to form aqueous two-phase systems was firstly presented by van Berlo et al. [18,19], who determined phase diagrams of systems containing ammonium carbamate and PEG of chain sizes 2000, 4000 and 10000 g mol^{-1}. These authors studied also the partitioning of some amino acids in these systems, and observed that the partition coefficient follows a general trend related to their hydrophobicity. Dallora et al. [20] studied the partitioning of bovine serum albumin (BSA), lysozyme and trypsin in ATPS formed by PEG and ammonium carbamate, and observed that the partition coefficients are more one-sided than usual for ATPS containing other salts.

The chemical equilibria occurring in an aqueous solution of ammonium carbamate comprise the ionization of ammonia and carbon dioxide (yielding ammonium and bicarbonate ions), the formation of the carbonate ion (from bicarbonate) and of the carbamate ion (from ammonia and bicarbonate). Therefore, ionic and molecular species co-exist in equilibrium in these solutions, and once the molecular species are volatile, the salt could be removed from the solution by decreasing the pressure or increasing the temperature, which reduces the solubility of molecular species in equilibria (1) and (2). The attractiveness of using ammonium carbamate in downstream processing is often associated to this character as a "volatile salt", even though environment and corrosion problems may be severe obstacles to effectively taking advantage of this characteristic. A single process using volatile electrolytes including recycling has been shown to be technically and economically feasible hitherto: the precipitation of casein using carbon dioxide as volatile acid [21]. However, as shown by Dallora et al. [20], the use of ammonium carbamate as a phase-forming salt can be attractive regardless of its volatile character.

In this paper, the phase equilibrium and the partitioning of some model proteins (bovine serum albumin, lysozyme, α-lactalbumin, β-lactoglobulin and γ-globulin) in aqueous two-phase systems formed by ammonium carbamate and the block copolymers F38 and F68 are presented. The experimental results show that, while it is very difficult to work with the system containing F68, the system formed by F38 and ammonium carbamate allows a good degree of separation between proteins, and therefore is a promising system to be considered when planning the downstream processing of proteins.

2. Materials and methods

2.1. Materials

Block copolymers F38 (copolymer of average structure $EO_{40}PO_{16}EO_{40}$) and F68 ($EO_{76}PO_{29}EO_{76}$) were donated by BASF (Sao Bernardo do Campo, Brazil). Ammonium carbamate (NH_4NH_2COO) was obtained from Fluka (Buchs, Switzerland). Lysozyme from chicken egg white, bovine serum albumin, α-lactalbumin, β-lactoglobulin and γ-globulin (actually a mixture of immunoglobulins) were obtained from Sigma-Aldrich (St. Louis, USA). All compounds were used without further purification. Milli-Q grade water was used in all experiments.

2.2. Methods

2.2.1. Phase equilibrium

Aqueous two-phase systems were produced by mixing water and stock solutions of ammonium carbamate (37% w/w) and copolymer (30% w/w for F68 and 40% w/w for F38) produced beforehand. They were left undisturbed for 48 h in a thermostatic bath Lactea-Julabo whose temperature was controlled within ±0.1 °C. Equilibrium compositions were determined by analyzing the polymer content of both phases (through drying) and salt content of the salt-rich phase (through titrating