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Solid-liquid equilibrium for proteins in solutions with an unconventional salt (ammonium carbamate): Phase behavior analysis

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ABSTRACT

In this manuscript, we report the precipitation and crystallization of three proteins (chicken egg-white lysozyme, porcine insulin and bovine insulin) using ammonium carbamate as salting-out agent. For these proteins, data on solubility, metastability limits, and osmotic second virial coefficient (B_{22}) as a function of salt concentration at constant temperature (25.0 °C) were obtained. The value of osmotic second virial coefficient (B_{22}) can be regarded as a selection criterion for protein crystallization, as it is related to the interaction potential between protein molecules. Negative values of B_{22} and large values of the salting-out constant showed that ammonium carbamate is a good precipitating agent for these proteins. Crystallization trials conducted under specific conditions showed that both insulins form amorphous precipitates in ammonium carbamate solutions, which is compatible with the large negative values of B_{22} measured. Conversely, lysozyme crystals were obtained under all conditions studied, and B_{22} values for this enzyme were within or close to the crystallization slot.

1. Introduction

Crystallization and precipitation are unit operations that are widely used in the chemical, pharmaceutical, and food industries. In both operations, a solid phase mainly containing a target compound is formed from a liquid phase. This solid phase formation occurs by shifting liquid phase conditions, such as temperature, pH, and ionic strength. For the precipitation and crystallization of proteins, this solid phase formation is usually achieved by changing the solution pH or by adding another compound such as a salt, a polymer, or an alcohol. Despite their similarity, precipitation and crystallization differ in the type of solid phase formed, amorphous precipitates or crystals, respectively [1,2]. The characterization of the solid-liquid phase behavior of protein solutions may be difficult due to the complexity inherent to protein molecules, and due to the influence of system conditions on the resulting equilibrium (temperature, pH, ionic strength, and the type of solid formed). However, the description of this phase behavior is necessary both to design more efficient downstream processes and to develop models to correlate and predict this phase behavior.

Two parameters are important to understanding the solid-liquid equilibrium underlying precipitation and crystallization operations. The first one is solubility, which is the concentration of protein in the liquid phase in equilibrium with the solid phase. Protein solubility is a complex function of polar interactions with the aqueous solvent, ionic interactions with salt, and repulsive electrostatic interactions between charged molecules [3,4]. Therefore, this solubility depends on the protein itself and the compound used to reduce the solubility (usually a salt), and its concentration, temperature, and pH. The second important parameter is the osmotic second virial coefficient, B_{22}. The osmotic second virial coefficient is obtained from the Taylor expansion of the osmotic pressure as a function of protein concentration. Its value is a measure of the deviation from the ideality of the solution [5]. The parameter B_{22} can be associated with the potential of mean force, a measurement of intermolecular interactions between solute molecules in a liquid solution. Large negative values of this parameter indicate a strongly attractive protein-protein interaction, whereas positive values indicate a predominantly repulsive interaction [6].

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The relationship between the B_{22} value and the outcome of protein precipitation operations was first studied by George and Wilson [7]. Those authors determined B_{22} values by static light scattering (SLS) for different protein/solvent systems under conditions which did or did not favor the formation of a crystal phase. The formation of crystalline solid phases was observed only in a relatively narrow range of negative B_{22} values, -8×10^{-4}–-1×10^{-4} mL mol/g^2. These authors proposed the use of B_{22} as a tool for predicting conditions under which the formation of amorphous precipitates or crystals occurs. This range was henceforth referred to as the crystallization slot [8]. This hypothesis was also investigated and confirmed by other authors [9–12] using different methods. Velev et al. [11] studied the effects of pH and electrolyte concentration on protein interactions by static light scattering (SLS) and small-angle neutron scattering (SANS). Their results corroborated the hypothesis of the crystallization slot proposed by George and Wilson [7]. Tessier and Lenhoff [10] discussed the relationship between B_{22} values and solution conditions (pH, ionic strength) that may lead to crystallization of some molecules, such as lysozyme, ribonuclease A, and albumin. According to these authors, there are four different patterns of protein-protein interactions as a function of ionic strength and the type of salt. Liu et al. [12] analyzed and measured experimentally, under different solution conditions, the nucleation and crystallization rates, solubility, crystal morphology, and B_{22} values to explore phase behavior and lysozyme crystallization.

Although the definition of a crystallization slot allows the selection of conditions that favor crystallization, the fact that the B_{22} value lies within the crystallization slot does not mean that crystals will always be obtained in a precipitation operation [13]. Crystallization depends on both nucleation and crystal growth rates, which depend on the supersaturation, i.e., the difference between the actual protein concentration and the solubility under the same conditions. Thus, knowledge of protein solubility as a function of system conditions (salt concentration, pH, and temperature) is essential to understanding crystallization processes [14,15].

Nevertheless, optimal conditions for the crystallization of proteins are usually determined by trial-and-error procedures [16]. The main reason for this is that systematic studies that present data on B_{22}, protein solubility, and solid phase analysis under the same conditions are scarce. Models relating the B_{22} and the solubility of proteins have been developed [14,15], but they are not predictive: reliable experimental data are needed to adjust model parameters.

This study investigated the solubility, metastability and osmotic second virial coefficient of chicken egg-white lysozyme, porcine insulin, and bovine insulin in aqueous solutions containing ammonium carbamate. This volatile electrolyte dissociates in aqueous solution. The concentration of the ionic species in solution depends on the system temperature and pressure [17]. The electrolyte can be reused without additional purification steps, as it can be converted to the volatile form by changing the system conditions. This salt is an alternative to conventional salts used as precipitant agents, such as ammonium sulfate and sodium chloride [18–20].

To understand the effects of protein-protein interaction, aiming at control of outcome of the solid-liquid equilibrium [21], B_{22} values for these proteins were determined as a function of the salt concentration. B_{22} values were determined both by self-interaction chromatography (SIC) and static light scattering (SLS). While SLS can be seen as a conventional method to determine B_{22}, the use of SIC has received increasing attention in recent years as a tool for understanding the phase behavior of proteins [22–27].

2. Experimental

2.1. Materials

Porcine (96.6% m/m containing 0.5% m/m of zinc) and bovine (95% m/m containing 0.5% m/m of zinc) insulins were donated by Biobrás (Brazil). Chicken egg-white lysozyme (CAS number 12650-88-33) was obtained from Sigma-Aldrich (USA, catalog number L6876, purity $\geq$ 90%). The proteins were used without further purification. Reagent grade ammonium carbamate (NH_4NH_2COO, CASRN 1111-78-0) was obtained from Sigma-Aldrich (USA, 99% pure). Ultra pure water was obtained with a Milli-Q system (Millipore, USA). The activated agarose matrix (CNBr-activated Sepharose™ matrix 4 Fast Flow) and Tricorn 5/50 column were from GE Healthcare Life Sciences (Sweden). Hydrochloric acid P.A. (36.5–38.0%) and glacial acetic acid P.A. (99.7%) were from Êxodo Científica (Brazil); ammonium bicarbonate (99–100%), from J.T. Baker (Mexico); sodium chloride (grade Ph Eur) and tris(hydroxymethyl)aminomethane (Tris, grade Ph Eur), from Merck (Germany); anhydrous sodium acetate P.A. (99%), from Vetec (Brazil); and ethanol P.A. (99.5%) and toluene P.A. (100%), from Synth (Brazil). All the other reagents were of analytical grade. The purities and sources of these compounds are also presented in Table 1.

2.2. Methods

2.2.1. Solubility measurements

Solubility measurements were carried out by dissolution runs conducted in 2.0 mL Eppendorf tubes [28]. Stock solutions of protein and ammonium carbamate had been produced prior to the runs and added to the Eppendorf tubes. The resulting mixture was stirred and placed in a thermostatic bath (model TE-2000 from Tecnal, Piracicaba, Brazil) at 25.0 °C. At different times the liquid and solid phases from a tube were separated by centrifugation at 7500*g* for 15 min. A supernatant phase aliquot was withdrawn with a syringe and filtered (Millex filter, pore size 0.45 μm, Millipore, USA) and its protein concentration determined. Equilibrium was considered to be attained when protein concentration remained constant for 24 h. Porcine insulin and bovine insulin concentrations in solution were determined through the Bradford method [29] using the Coomassie Plus reagent (Pierce, USA). Lysozyme concentration was determined by measuring absorbance at 280 nm. These measurements were made using a DU 640 spectrophotometer (Beckman Instruments, USA). Assays were performed in triplicate and the average protein concentration was considered to be the solubility at the system temperature and salt concentration.

2.2.2. Cloud-point measurements

The metastability limit was estimated by cloud-point

Table 1
Sources and purities of compounds used in the experiments.

Chemical name	Source	Mass fraction purity
Porcine insulin	Biobrás	>96.6
Bovine Insulin	Biobrás	>95.0
Chicken egg-white lysozyme	Sigma Aldrich	≥90.0
Ammonium carbamate	Sigma Aldrich	>99.0
Hydrochloric acid P.A.	Êxodo Científica	P.A.
Acetic acid	Êxodo Científica	P.A.
Ammonium bicarbonate	J.T. Baker	99.0–100.0
Sodium chloride	Merck	grade Ph Eur
Tris(hydroxymethyl)aminomethane	Merck	grade Ph Eur
Anhydrous sodium acetate P.A.	Vetec	P.A.
Ethanol	Synth	P.A.
Toluene	Synth	P.A.