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Short communication

Solubility of lysozyme in aqueous solution containing ethanol or acetone: Unexpected dependence on the initial protein concentration

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ABSTRACT

This short communication presents unexpected experimental evidence that the solubility of lysozyme in aqueous solutions containing organic solvents (ethanol or acetone) depends not only on the fraction of the organic solvent, but also on the initial protein concentration (i.e., the concentration of protein before precipitation). The balance of enzymatic activity corroborates this fact. The observed shift in measured solubility is nearly proportional to the shift in initial protein concentration (up to 5-fold in ethanol solutions and up to 3-fold in acetone solutions). The dependence of solubility on the protein initial concentration has already been considered in literature as the formation of a second liquid-like phase instead of a solid precipitate, but calculated partition coefficients indicate that this description does not hold for these systems. While the results here presented do not allow an unequivocal description of the precipitate phase, they show that care must be exercised when describing protein precipitation with organic solvents.

1. Introduction

Precipitation (i.e., the formation of a solid phase by changing the conditions of a liquid solution) is largely employed in lab-scale protocols and in industrial processes to concentrate and/or purify proteins. Precipitation is actually the oldest unit operation used with this aim: the first examples of protein precipitation date back to the XIX century, with the seminal works by Lewith [1] and Hofmeister [2,3]. In these cases, precipitation was promoted by adding salts to protein-containing aqueous solutions. Protein precipitation can also be achieved by using other precipitant agents such as organic solvents. The first large-scale procedure to separate proteins, Cohn's method to fractionate blood plasma components, comprises many steps of precipitation with ethanol at low temperatures [4].

The formation of a protein precipitate, either an amorphous solid or a crystal, depends on system conditions such as temperature, pH, ionic strength and on the precipitant agent used. The conditions that favor precipitation are known for many proteins of industrial importance. However, only recently the phase equilibrium in protein precipitation was investigated. The works by Shih et al. [5], Moretti et al. [6], Popova et al. [7] and Watanabe et al. [8] were the first ones to have special focus on this aspect. These works addressed the precipitation that results from adding a salt to a protein solution. They show that the phase equilibrium underlying protein precipitation may be subtle and counterintuitive. Shih et al. [5] demonstrated that the second phase formation in protein precipitation may sometimes be better described as a liquid-phase split than as a solid-phase precipitation. Moretti et al. [6] showed that amorphous precipitates and crystals of lysozyme may have very different solubilities in salt solutions. Popova et al. [7] and Watanabe et al. [8], by applying a variant of Schreinemaker's method to phase equilibrium data of lysozyme precipitation with salts, showed that the composition of the solid phase may differ even when amorphous solid precipitates are formed.

To be best of the authors' knowledge, the phase equilibrium in the precipitation of single proteins using organic solvents has not been systematically investigated, despite the industrial importance

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of organic solvents as precipitating agents. In this short communication, we briefly describe the phase equilibrium on the precipitation of lysozyme in aqueous solutions using ethanol or acetone. The experimental results show that the solubility of this enzyme apparently depends on its initial concentration (i.e., the overall protein concentration, or its concentration before precipitation). This behavior is opposite to that observed in its salting-out by Shih et al. [5].

2. Materials and methods

The compounds used, along with their purities, are presented in Table 1. They were used as received, and no further purification was carried out. Milli-Q (Millipore, USA) grade water was used in all experiments.

2.1. Precipitation experiments

Stock lysozyme solutions with concentrations 5.0, 15.0, and 25.0 mg mL^{-1} were prepared in 66.0 mmol L^{-1} potassium phosphate buffer at pH 6.20. The pH was measured with a pH-meter model WD-35616 (Oakton, USA). Samples of 5.000 mL of protein solutions were added to polyethylene tubes and kept at 298.2 ± 0.1 K in a thermostatic bath (Tecnal TE-2000, Brazil). The organic solvent (either ethanol or acetone) was added dropwise at a rate of roughly 2 mL min^{-1} using a micropipette, until predetermined final solvent concentrations were reached. The tubes were closed, gently stirred, and maintained in a thermostatic bath at 278.2 K for 60 min for equilibration. This time interval was determined in previous kinetic experiments (data not shown). After equilibration, the tubes were centrifuged at 12500 g at 298.2 K for 30 min in a refrigerated centrifuge (model 5804R, Eppendorf, Germany). Precipitate and supernatant phases were carefully separated. For each phase, the total mass, the protein concentration and the enzymatic activity were determined. Precipitation experiments were conducted with ideal final ethanol volume fractions of 0.40, 0.45 and 0.50 and with acetone volume fractions of 0.35, 0.40 and 0.45.

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

Compound	Source	Mass fraction purity
Chicken egg-white lysozyme	Sigma-Aldrich, USA	≥0.90
Ethanol	Synth, Brazil	≥0.995
Acetone	LS Chemical, Brazil	≥0.995
Sodium phosphate	Synth, Brazil	≥0.98
Coomassie blue dye	Bio-Rad, USA	Not defined
Micrococcus lysodeikticus	Sigma-Aldrich, USA	Not defined

2.2. Protein concentration

Lysozyme concentration was determined through Bradford's method [9] using calibration curves built with lysozyme solutions of known concentration. The equilibrium phases were diluted in 66.0 mmol L^{-1} potassium phosphate buffer (pH 6.20) to avoid interference of the organic solvent in the measurement and to assure that the protein concentration were in the linear range of the calibration curve. Absorbance was measured with a spectrophotometer Q898DRM (Quimis, Brazil).

2.3. Enzymatic activity

Lysozyme activity was determined through the method by Shugar [10]. First, 2.500 mL of a *Micrococcus lysodeikticus* suspension with 15 mg mL^{-1} (in potassium phosphate buffer 66.0 mmol L^{-1}, pH 6.20) was added to a quartz cuvette. Then, 0.100 mL of the protein solution in the same buffer was added to the same cuvette. After gentle stirring, the absorbance at 450 nm was recorded during 5 min in a spectrophotometer coupled to a thermostatic bath kept at 298.2 K. The enzymatic activity was thus calculated through Eq. (1):

$$a\left(UI\cdot mL^{-1}\right) = \frac{\left(\Delta A_{450} - \Delta A_{450}^{ref}\right) \times D}{t \times V_A} \quad (1)$$

in which ΔA_{450} is the change in absorbance at 450 nm during the time interval t (min), D is the dilution factor, V_A is the volume of enzymatic solution expressed in mL (0.100 mL). The reference experiment (that generated the value of ΔA_{450}^{ref}) was conducted through the same procedure, except that 0.100 mL of buffer solution was added to the suspension of *Micrococcus lysodeikticus* instead of 0.100 mL of protein solution.

All experiments and measurements were conducted in triplicate.

3. Results and discussion

Experimental results are summarized in Table 2 (systems with ethanol as precipitant agent) and 3 (systems with acetone as precipitant agent). In these tables and in the text that follows, the term solubility means the protein concentration in the supernatant phase in equilibrium with a solid phase. The solubility of lysozyme in aqueous solutions containing ethanol is presented in Fig. 1A, and the enzymatic activity in the supernatant is presented in Fig. 1B. Fig. 2A and B presents similar data for aqueous solutions containing acetone.

The analysis of Figs. 1A and 2A shows that the solubility of lysozyme in aqueous solutions containing either ethanol or acetone

Table 2
Experimental data for the system lysozyme (1) + water (2) + ethanol (3) at 298.2 K and 91 kPa. Protein concentration (C), mass fraction (w), mass (m) and activity (a) in the stock solution (subscript 0) and in precipitate (subscript P) and supernatant (subscript S) phases as a function ideal ethanol volume fraction (ϕ_3).[a]

ϕ_3	C_0/mg ml^{-1}	C_S/mg ml^{-1}	w_S	w_P	m_0/mg	m_P/mg	m_S/mg	Recovery/%	$a_0/10^3$ UI	$a_P/10^3$ UI	$a_S/10^3$ UI	Recovery/%
0.40	4.8	2.6	0.0030	0.1260	24.1	5.2	21.2	109	157	35	140	111
	15.0	5.1	0.0081	0.2400	74.9	39.4	42.7	109	475	241	276	108
	26.2	11.3	0.0140	0.3152	131.0	38.2	93.9	100	793	219	619	105
0.45	4.9	1.1	0.0013	0.1341	24.4	15.3	10.3	104	165	93	57	91
	15.1	3.4	0.0042	0.1800	75.4	47.6	31.3	104	487	278	231	104
	26.1	6.9	0.0084	0.2621	130.4	74.6	62.5	105	798	423	398	102
0.50	4.8	0.5	0.0006	0.1230	24.1	19.2	5.0	100	157	129	30	100
	14.9	1.1	0.0014	0.1624	74.7	66.9	11.1	104	475	390	74	99
	26.5	2.8	0.0036	0.2048	132.2	107.0	28.3	102	816	637	195	101

[a] - Estimated uncertainties are u(T) = 0.1 K, u(P) = 1 kPa, u(w) = 0.0001, u(m) = 0.1 mg and $u(a) = 10\cdot10^3$ UI.