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Precipitation of lysozyme with sodium succinate, sodium tartrate and sodium citrate: Solubility and osmotic second virial coefficient data

José Sebastián López Vélez, Adriano Rodrigues Azzoni, Pedro de Alcantara Pessoa Filho *

Department of Chemical Engineering, Engineering School, University of São Paulo (USP), Caixa Postal 61548, 05424-970 São Paulo, SP, Brazil

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ABSTRACT

Precipitation and crystallization are unit operations widely used to concentrate and purify proteins in biotechnology industry. In this work, the potential use of the biodegradable salts sodium succinate, sodium tartrate and sodium citrate to reduce the solubility of proteins is investigated. Lysozyme was studied as a model protein. The solubility of lysozyme in aqueous solutions of sodium succinate, sodium tartrate and sodium citrate was experimentally determined as a function of the ionic strength and pH at 298.2 K. All these salts induce the precipitation of lysozyme, being sodium succinate the most effective one to reduce the solubility of this protein. The osmotic second virial coefficient of lysozyme as a function of the ionic strength was also determined using self-interaction chromatography at 298.2 K and pH 8.5. The experimental data show that the value of the second virial coefficient is negative at the investigated conditions and lies mostly within or close to the crystallization slot, i.e., the range of values of this coefficient for which the formation of crystals is favored.

1. Introduction

Precipitation and crystallization are unit operations used to concentrate and purify proteins from aqueous solutions. The term precipitation describes the unit operation conducted without control of supersaturation. It yields an amorphous precipitate, and is placed mainly at the beginning of the downstream process, with the aim of concentrating the protein. Conversely, the term crystallization describes the operation conducted with proper control of supersaturation and other system conditions. It yields protein crystals, and is usually placed at the end of the downstream process, as protein crystals can be properly stored without losing the biological activity [1]. Protein crystals are used in many fields of chemical industry, including cosmetic and food industries, as well as in the pharmaceutical industry [2]. In a few cases, adequate purity can even be achieved in a single crystallization step. For example, crystallized ovalbumin, with a mass fraction of 99%, can be obtained from a mixture of this protein with conalbumin and lysozyme [3].

Precipitation and crystallization are the oldest unit operations used to concentrate and purify proteins. Since the maintenance of biological activity is a chief issue in downstream processes, this solid phase formation is not induced by changing temperature. The formation of the solid phase is usually achieved by adding a third compound, such as a salt or an organic solvent, to the aqueous solution containing the target protein. The mechanism of the solid phase formation depends on the nature of this precipitating agent. Organic solvents, for instance, shift the medium permittivity and remove water molecules from the hydration shell, thus promoting the association of hydrophobic regions of the protein molecule [4]. Salts are also common precipitating agents. The effectiveness of salts in decreasing protein solubility is usually related to their chaotropic or kosmotropic nature. Salts that promote the spatial arrangement of water molecules (i.e., the kosmotropic ones) are usually good precipitating agents. Kosmotropic salts are also used to promote liquid-liquid phase separation in aqueous two-phase systems. The lowering of solubility by the addition of salts is known as "salting out." Salts used to promote protein salting out include ammonium sulfate (the most used one) [5], sodium sulfate [6], sodium chloride [7], ammonium carbamate [8], among others.

In this work, the use of biodegradable salts (sodium citrate, sodium succinate and sodium tartrate) for lowering the solubility of lysozyme is investigated. These salts were considered due to their biodegradability. The fact that they are also used to promote liquid-liquid phase separation in mixture with polymers [9–12] shows that they can potentially be used to promote the salting out of proteins. On the other hand, lysozyme was chosen as a model protein as it has been extensively studied in precipitation and crystallization operations [13–16]. This allows a comparison of the effectiveness of these biodegradable salts with salts com-

* Corresponding author.
E-mail address: pedro.pessoa@poli.usp.br (P.A. Pessoa Filho).

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Nomenclature

Latin letters

B_{22}	osmotic second virial coefficient [mol·cm^3·g^{-2}]
B_{222}	osmotic third virial coefficient [mol·cm^6·g^{-3}]
B_{HS}	osmotic second virial coefficient for hard spheres [cm^3]
c_p	protein mass concentration [g·cm^{-3}]
c_S	protein solubility in mass concentration [g·cm^{-3}]
k'	chromatographic parameter, Eq. (3)
K_S	salting-out constant [kg·mol^{-1}]
M_P	protein molar mass [g·mol^{-1}]
N_A	Avogadro's number
r	radius of a protein molecule [cm]
R	gas constant [8.314 J·mol^{-1}·K^{-1}]
T	absolute temperature [K]
V_0	retention volume in the absence of immobilized protein molecules [cm^3]
V_R	retention volume [cm^3]
w	mass fraction
z	coordination factor of protein crystal

Greek letters

σ	standard deviation in the measure of B_{22} [mol·cm^3·g^{-2}]
β	parameter in Cohn's equation, Eq. (2)
Γ	ionic strength [mol·kg^{-1}]
μ	chemical potential [J·mol^{-1}]
Π	osmotic pressure [Pa]
ρ'	number of protein molecules immobilized in the solid matrix per area [cm^{-2}]
φ	ratio between area and volume of the resin [cm^2·cm^{-3}]

monly used to promote salting out. Experimental data on the solubility of lysozyme can be found for aqueous solutions containing sodium sulfate, ammonium sulfate, sodium chloride, ammonium carbamate, among others. However, no experimental data on the solubility of lysozyme in aqueous solutions of these biodegradable salts exist, to the best of the authors' knowledge.

When predicting the outcome of solid-liquid operations involving proteins (either an amorphous precipitate or a crystal), an important parameter to analyze is the osmotic second virial coefficient (B_{22}). This parameter is defined by the virial expansion of the osmotic pressure as a function of protein concentration [17]:

$$\Pi/(c_P RT) = M_p^{-1} + B_{22}c_P + B_{222}c_P^2 + \ldots \quad (1)$$

wherein Π is the osmotic pressure, T is the absolute temperature, R is the gas constant, M_P is the protein molar mass, B_{222} is the osmotic third virial coefficient, and c_p is the protein concentration (in units of mass per volume).

Since the work by George and Wilson [18], a correlation has been established between the value of this coefficient and the structure of the resulting solid phase: the formation of protein crystals is favored if the value of the second virial coefficient lies in a specific range, known as crystallization slot. The value of B_{22} can be measured using different experimental techniques [19], such as self-iteration chromatography (SIC) [20], membrane osmometry [21] and static light scattering [22]. The use of SIC is more recent, and yields reliable results. This technique is based on the effect of immobilized protein molecules (in the stationary phase in a chromatographic column) on the elution profile of a solution containing the same molecule in the mobile phase [23]. Even though the experimental uncertainty of measurements of B_{22} is often large, the analysis of this parameter provides a guide for investigating the possibility of crystal formation.

2. Thermodynamic framework

The equilibrium between a solid protein and an aqueous solution containing this protein can be described by the equality of chemical potential of the protein in both phases. Even though this statement is almost a truism, the equality of chemical potentials is seldom applied to the description of protein precipitation. Issues as the very nature of the solid phase and the dependence of the protein thermodynamic activity in liquid phase on system conditions (temperature, pH and concentration of other compounds) hinder the application of usual solid-liquid equations to describe protein precipitation/crystallization.

The decrease in protein solubility by the addition of a salt is usually described by the so-called Cohn equation [24]:

$$\ln(c_S/\mathrm{g\cdot cm^{-3}}) = \beta + K_S(\Gamma/\mathrm{mol\cdot kg^{-1}}) \quad (2)$$

where c_S is the protein solubility, Γ is the ionic strength, β is the calculated limit for zero ionic strength, and K_S is the so-called salting-out constant. This equation shows that the solubility decreases exponentially with the increase in the ionic strength. It does not describe the whole solubility curve, but can be applied to the salting-out region, the most important one for industrial applications. In practice, both β and K_S depend on the system (the protein and the salt) and its conditions (temperature and pH), although theoretically β should not depend on the salt, and K_S should not depend on the pH [24].

Besides relating the solubility and the ionic strength, relationships between the solubility and the second virial coefficient B_{22} are also necessary in the context of protein precipitation. At least four different approaches can be applied to relate these properties.

The model presented by Haas et al. [25] was developed from the definition of the osmotic second virial coefficient, assuming a square-well potential. The final equation of this model relates B_{22} to the solubility (expressed in mass fraction) to the power of $-2/z$, in which z is the coordination factor of the protein crystal. The proportionality constant between B_{22} and the solubility is a parameter accounting for the anisotropy in the interactions between protein molecules. This parameter can be regarded as an adjustable one.

Another relationship was presented by Guo et al. [26]. These authors assumed the McMillan-Mayer approach and, by using the Gibbs-Duhem equation, obtained an expression for the protein chemical potential, which must be kept constant along the solubility curve. The final expression relates B_{22} to the reciprocal of the solubility, and depends on the difference in the chemical potential of the protein in Henry's standard state and in the solid phase ($\Delta\mu_2$). The value of $\Delta\mu_2$ can be considered an adjustable parameter.

The approach by Ruppert et al. [27] assumes the validity of Henry's law and relates the osmotic second virial coefficient from the McMillan and Mayer framework and the experimentally obtained value of B_{22}. This model requires information on the partial volume of the protein at infinite dilution [28], the refractive index and the change of the refractive index with protein concen-