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Self-interaction of native and denatured lysozyme in the presence of osmolytes, L-arginine and guanidine hydrochloride

Xiao-Yan Dong, Jian-Hua Liu, Fu-Feng Liu, Yan Sun*

Department of Biochemical Engineering, School of Chemical Engineering and Technology, Tianjin University, No. 92 Weijin Road, Nankai District, Tianjin 300072, China

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ABSTRACT

Osmolyte molecules such as betaine and trehalose are protein stabilizers while L-arginine (Arg) and guanidine hydrochloride (GdnHCl) are the most widely used aggregation suppressor in protein refolding. We have herein studied the effects of the osmolyte molecules and L-arginine together with GdnHCl (0–6 mol/L) on the intermolecular interaction of native and denatured lysozyme by self-interaction chromatography. The self-interaction is characterized in terms of the osmotic second virial coefficient (B) of the protein, the increase of which represents the decrease of intermolecular attraction of the protein. It is found that the effect of Arg on the self-interaction of lysozyme is similar with GdnHCl, but its competence is much weaker than the denaturant. At higher GdnHCl concentrations (>0.5 mol/L), Arg can be used to suppress the self-association of lysozyme. In contrast to Arg, B increases with increasing betaine or trehalose concentration at the GdnHCl concentration range studied. The results indicate the cooperativity of each osmolyte with GdnHCl, and the different mechanisms of their effects from Arg on the B values. The work confirms that the osmolytes are not only protein stabilizers, but also protein aggregation suppressors for both native and denatured protein molecules.

1. Introduction

Protein renaturation or refolding is an important unit operation in pharmaceutical protein recovery because lots of proteins produced by recombinant bacteria such as *Escherichia coli* often form inactive aggregates known as inclusion bodies [1–3]. However, difficulties are often encountered in protein renaturation since the solubilized and unfolded protein tends to re-aggregate by intermolecular interactions in the renaturation process. Consequently, suppression of the aggregating reaction is the key point in enhancing protein renaturation yield.

To date, many substances have been found to help protein refolding when they are added to the refolding buffer in a dilution refolding process [2–7]. Generally, these substances can be categorized into two groups, i.e., protein stabilizers and aggregation inhibitors [6–9]. Protein stabilizers, such as polyols, sugars, and betaine, stabilize folded state and increase folding rate, while aggregation inhibitors, such as guanidine hydrochloride (GdnHCl), urea, L-arginine, acetamide, acetone, thiourea and detergents, stabilize unfolded protein or folding intermediates, thus lowering aggregation rate of polypeptide chains [6]. Currently, the effectiveness of these substances for improving the refolding efficiency of different proteins have been widely recognized, but their effect on the self-interaction of protein molecules are not yet clear [8], especially for protein stabilizers [10–13]. Hence, we have herein characterized the molecular interaction of lysozyme in both native and denatured states in terms of the osmotic second virial coefficient (B) determined by self-interaction chromatography (SIC) [14,15]. In an SIC of protein, protein is immobilized on chromatographic particles and the retention of the same protein is measured in isocratic elution.

The osmotic second virial coefficient is a thermodynamic parameter that characterizes two body interactions in dilute solutions that cannot be characterized in terms of quantities such as association constants [15–17]. Positive B values indicate predominantly repulsive intermolecular interactions, whereas negative values reflect predominantly attractive interactions [16,17]. The self-interactions of protein molecules are such a weak interaction that can be characterized in terms of B. Recently, Tessier et al. [15] related the relative protein retention in SIC to the B value via statistical mechanics, and verified the validity of the method by comparison of their experimental data with the literature data obtained by using other light scattering methods. It has proved that the method holds significant promise for the characterization of protein interactions requiring only commonly available laboratory equipment, little specialized expertise, and relatively small investments of both time and protein sample. So, the method has proved very efficient over the other methods for the measurement of B values [15–20]. Based on the established SIC method, Valente et al.

* Corresponding author. Tel.: +86 22 27404981; fax: +86 22 27406590.
E-mail address: ysun@tju.edu.cn (Y. Sun).

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[17] measured B values of native lysozyme in the presence of various additives including sucrose, trehalose, mannitol, glycine, arginine, and combinations of arginine and glutamic acid and arginine and sucrose. They found that all of the additives, alone or in combination, increased B. However, no data of B for a denatured protein in the presence of the additives have been reported.

Hence, this work was conducted to elucidate the effects of protein stabilizers and aggregation suppressors on the self-association of both native and denatured proteins in a protein refolding process by using the SIC method. Two typical protein stabilizers (protecting osmolytes), trehalose and betaine [10,11,13], and the most widely used protein aggregation suppressors, arginine and GdnHCl [9,21–23], were evaluated for their effects on B. The denaturant GdnHCl was also used to create a denaturing condition in the SIC. The research is expected to compare the two types of protein refolding enhancers at the same platform and to find their relationship with GdnHCl in affecting intermolecular protein interactions in protein refolding.

2. Materials and methods

2.1. Materials

Chicken egg white lysozyme (L6876), betaine, trehalose, L-arginine hydrochloride (Arg), GdnHCl, tris(hydroxymethyl) aminomethane hydrochloride (Tris) and sodium cyanoborohydride were obtained from Sigma (St. Louis, MO, USA). Ethanolamine, sodium acetate, sodium chloride, dibasic sodium phosphate and monobasic sodium phosphate were of analytical grade from Kewei Chemicals (Tianjin, China). Glacial acetic acid was also from Kewei Chemicals. Toyopearl AF-Formyl-650M was provided by Tosoh Biosep (Montgomeryville, PA, USA). Deionized water was used to prepare all buffers and solutions. Other chemicals were all commercially available reagents of analytical grade. All chemicals and reagents were used as received.

2.2. Preparation of immobilized lysozyme

Tessier et al. [15] established the method and theory for the determination of B value by SIC with lysozyme as a model protein, so we adopted the same stationary phase (Toyopearl AF-Formyl-650M) and experimental procedures as reported by Tessier et al. to immobilize lysozyme. The immobilized concentration of lysozyme was determined by mass balance to be 16.8 mg/mL (packed-bed volume), similar with that obtained by Tessier et al. (17.3 mg/mL). The stationary phase was also modified with ethanolamine as described by Tessier et al. to prepare blank particles for control experiments (see below).

2.3. Self-interaction chromatography

The solid particles prepared above was packed into HR 5/10 column (5 mm × 100 mm) (GE Healthcare, Uppsala, Sweden), and the SIC was carried out on the ÄKTA FPLC system (GE Healthcare). SIC experiments were performed in 20 mmol/L NaAc-HAc (pH 4.5) and 20 mmol/L Tris–HCl (pH 8.5). NaCl was added at 0–1 mol/L to adjust ionic strength, and the mobile phase contained 0–6 mol/L GdnHCl. Betaine, trehalose, or arginine (0–1 mol/L) was added to the mobile phase to study the effect of each additive together with GdnHCl. The SIC experiments with lower GdnHCl concentrations in the mobile phase were first carried out to keep the immobilized lysozyme in native state, and those with higher GdnHCl (4 and 6 mol/L) were finally performed.

Prior to an SIC run, both the protein sample and column were equilibrated for 4 h at room temperature (23 ± 1 °C) with the mobile phase containing an additive at defined concentration. This period of equilibration led to the complete denaturation of free and immobilized lysozyme when 6 mol/L GdnHCl was present in the mobile phase, as confirmed by activity assay of lysozyme [6]. In the measurement of retention time, 100 μL of the equilibrated lysozyme solution was loaded and the mobile phase was supplied at 0.2 mL/min for isocratic elution. The loaded protein concentration was 20 mg/mL, as suggested by Tessier et al. [15]. Triplicate SIC measurements under each condition were carried out.

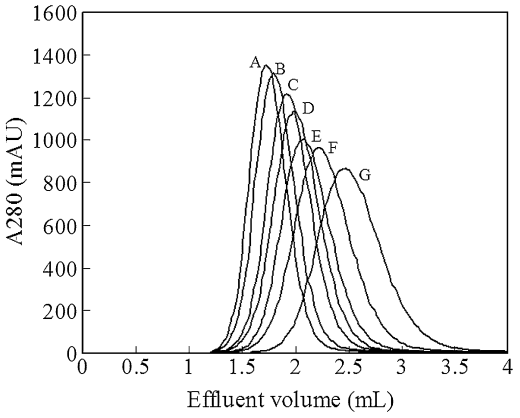

Fig. 1. SIC profiles of lysozyme in acetate buffer (pH 4.5) at NaCl concentrations of 0 mol/L (A), 0.05 mol/L (B), 0.2 mol/L (C), 0.3 mol/L (D), 0.4 mol/L (E), 0.6 mol/L (F) and 1.0 mol/L (G).

2.4. Calculation of second virial coefficient

The value of B was estimated from Eq. (1) derived by Valente et al. [17] according to Tessier et al. [15]:

$$B = \frac{N}{M^2}\left(B_{HS} - \frac{k'}{\rho\phi}\right) \quad (1)$$

where N is the Avogadro's constant, M the molecular mass, B_{HS} the excluded volume of lysozyme, ρ the immobilized density of lysozyme (molecules per accessible surface area), ϕ the phase ratio (accessible surface area per mobile phase volume), and k' is the capacity factor of protein in SIC:

$$k' = \frac{V_R - V_0}{V_0} \quad (2)$$

where V_R is the retention volume of lysozyme on the immobilized lysozyme column, V_0 the retention volume of lysozyme under non-retained condition, determined as Tessier et al. with correction using the column without lysozyme immobilization [15]. The value of V_0 was determined as 1.57 ± 0.08 mL for the 2-mL packed column under different solution conditions including those with GdnHCl and/or any other additives, so the packed-bed porosity for lysozyme was 0.785. The retention volumes were taken as the maximum peak height, because Gaussian peaks were observed (Fig. 1). In addition, because we used the same solid matrix for lysozyme immobilization as that of Tessier et al., B_{HS} and ϕ in Eq. (1) were also took from Tessier et al. [15] as $B_{HS} = 6.24 \times 10^{-20}$ cm^3 and $\phi = 20.9$ m^2/mL. Then, the value of ρ was calculated by dividing the immobilized concentration (16.8 mg/mL) by the packed-bed porosity (0.785) and the phase ratio (20.9 m^2/mL) to be $\rho = 1.02$ mg/m^2 (or 4.3×10^{16} molecules/m^2).

As described above, three retention time values were obtained by triplicate experiments under each condition. The B value at the condition was estimated from Eq. (1) with the average capacity factor. Then, the B values with individual capacity factors were calculated and their standard deviation from the B value estimated