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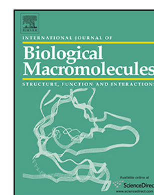

Effect of counter ions of arginine as an additive for the solubilization of protein and aromatic compounds

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ABSTRACT

Arginine is widely used in biotechnological application, but mostly with chloride counter ion. Here, we examined the effects of various anions on solubilization of aromatic compounds and reduced lysozyme and on refolding of the lysozyme. All arginine salts tested increased the solubility of propyl gallate with acetate much more effectively than chloride. The effects of arginine salts were compared with those of sodium or guanidine salts, indicating that the ability of anions to modulate the propyl gallate solubility is independent of the cation. Comparison of transfer free energy of propyl gallate between sodium and arginine salts indicates that the interaction of propyl gallate is more favorable with arginine than sodium. On the contrary, the solubility of aromatic amino acids is only slightly modulated by anions, implying that there is specific interaction between acetic acid and propyl gallate. Unlike their effects on the solubility of small aromatic compounds, the solubility of reduced lysozyme was much higher in arginine chloride than in arginine acetate or sulfate. Consistent with high solubility, refolding of reduced lysozyme was most effective in arginine chloride. These results suggest potential broader applications of arginine modulated by different anions.

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1. Introduction

Arginine (Arg) is one of the versatile co-solvents (additives) in development of therapeutic or reagent proteins due to its ability to suppress protein aggregation without altering or destabilizing the tertiary structure of the protein [1]. It has been used for many applications including refolding enhancement [2–4], suppression of heat induced aggregation [5], reduction of the viscosity of concentrated protein solutions [6,7] and solubilization of aromatic compounds [8–12]. Molecular mechanisms underlying these effects have been proposed, e.g., the cation-π interaction between the guanidium group of Arg and aromatic groups of proteins or small organic solutes [10,11] and weak binding of Arg ions to the protein surface [13,14]. In these applications and analyses, Arg has been used at neutral or acidic pH and hence as a salt form, primarily chloride salt. Few studies were done with other salt forms [15,16].

Ions, specially anions, have specific effects on stability, solubility and aggregation of proteins in aqueous solution, known as Hofmeister series [17] or also as attraction pressure [18,19] and later developed into cavity theory [20]. Such ion-specific effects exist even on guanidium ion, as its denaturation effect differs between chloride and sulfate salts [21]. Molecular mechanisms governing ion-specific effects have been related to the strength of ionic hydration [22,23], different density of water molecules [24], and accumulation or exclusion of the ions from the protein surface [25]. It is thus highly likely that the effects of Arg can be modulated by anions. Recently, Izutsu et al., examined the effects of counter ions on the ability of Arg to stabilize proteins in frozen solutions and freeze-dried solid [26,23]. It was suggested that the interaction between multivalent counter ion and Arg plays an important role in protein stabilization [23]. Although the physical state in consideration is different (solid state vs. liquid state), this argument is consistent with the observation by Trout et al. [27] that multivalent counter ion facilitates clustering of Arg, crowding out the protein–protein interaction and thereby suppressing aggregation. Here, we have initiated a systematic study on the effects of anions using small organic compounds. Previously, we investigated the effects of ArgHCl on the solubility of aromatic compounds [8–11]. In this study, we examined the effects of various Arg salts on the solubility of such aromatic compounds as propyl gallate, phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) and reduce lysozyme and on refolding of the reduced lysozyme.

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2. Materials and methods

2.1. Materials

L-Arginine (Arg) was kindly provided by Ajinomoto Co., Inc. (Tokyo, Japan). Hydrochloric acid, sulfuric acid, phosphoric acid, acetic acid, formic acid, citric acid (anhydrous), sodium hydroxide, sodium sulfate, sodium acetate, guanidium hydrochloride, guanidine sulfate, tyrosine, phenylalanine and tryptophan were purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). Sodium chloride was purchased from Nacalai Tesque Inc. (Kyoto, Japan). Propyl gallate was purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). Hen egg white lysozyme and guanidium acetate were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). All chemicals used were of reagent grade and used as received. All arginine salt forms were prepared by titrating an aqueous solution containing Arg base with the above acids. Although addition of water to Arg base resulted in suspension, acid titration of the suspension lead to clear solution due to protonation of basic groups of Arg. It should be noted that $ArgH(H_2PO_4)$ slowly phase-separated due to low solubility of monovalent phosphate

2.2. Solubility measurement of aromatic compounds

The solubility of aromatic compounds, i.e., propyl gallate and aromatic amino acids, in the absence and presence of additives at pH 4.8 was measured as described in the previous studies [9,10]. Higher pHs were also tested with difficulty in maintaining a constant pH: dissolution of propyl galate resulted in significant pH reduction at such higher pH values. Propyl gallate and aromatic amino acids were transferred into test tubes, to which 0.5 ml of test solvents were added. The suspension was heated at 50 °C for 1 h with frequent vortexing to completely dissolve the solute compounds. The solutions were incubated at 25 °C for 3 days with frequent vortexing, leading to development of suspension. Subsequently, the suspension was centrifuged at 25 °C and 18,800*g* for 30 min to obtain a supernatant saturated with the solutes. After appropriate dilution of the supernatant with 10 mM citrate buffer (pH 4.8), the absorbance of the supernatant was determined at 273 nm, 275 nm, 257 nm and 279 nm for propyl gallate, tyrosine, phenylalanine, tryptophan, respectively. The absorbance spectrum was recorded using an ultraviolet-visible (UV–Vis) spectrophotometer (ND-1000; NanoDrop Technologies Inc., Wilmington, DE, USA) and converted to the concentration on the basis of the standard curve determined for each solute compound. Solubility was determined in triplicate from which the averages and standard errors were obtained.

2.3. Estimation of transfer free energy

The transfer free energy ΔG_{tr} of the propyl gallate from sodium salt solution to arginine salt solution at a given additive concentration, e.g. from 1 M sodium chloride solution to 1 M Arg chloride solution, was calculated according to the following equations:

$$\Delta G_{tr} = \mu_a^0 - \mu_s^0 = -RT\ln(x_a/x_s) \quad (1)$$

$$\begin{cases} \mu_a = \mu_a^0 + RT\ln(x_a) \\ \mu_s = \mu_s^0 + RT\ln(x_s) \end{cases} \quad (2)$$

$$\begin{cases} x_a = n_{g,a}/(n_{g,a} + n_{H_2O,a} + n_{a,a} + n_{c,a}) \\ x_s = n_{g,s}/(n_{g,s} + n_{H_2O,s} + n_{a,s} + n_{c,s}) \end{cases} \quad (3)$$

In Equations (1)–(3), μ_a and μ_s are the chemical potentials of the alkyl gallate in the presence of Arg salt (a) and sodium salt (s), respectively, while μ_a^0 and μ_s^0 are the corresponding standard chemical potentials. The transfer free energy of the propyl gallate from the sodium salt solution to the arginine salt solution can be calculated from the solubility of the propyl gallate in the respective solutionsx_a and x_s, expressed as the mole fraction solubility of the propyl gallate in the presence of the additives. The mole fraction concentration is calculated using $n_{i,s}$ and $n_{i,a}$, which correspond to the molarity of the component i at saturation in the presence of the additives. Subscript g, H_2O, a, c are used to express the propyl gallate, water, additive and counter ion, respectedly. The activity coefficient was considered to be close to unity because of poor solubility of the alkyl gallate. R and T correspond to the universal gas constant and absolute temperature, respectively.

2.4. Solubility measurement of unfolded lysozyme

Reduced carboxamidometylated lysozyme (RCM-lyz) was prepared as previous study [28]. Briefly, lysozyme was solubilized at 20 mg/ml in 100 mM Tris-HCl buffer, 8 M guanidine-HCl, 40 mM DTT, 1 mM EDTA and incubated at 37 °C for 3 h. Then, 100 mM iodoacetic acid was added to the solution and adjusted the pH to 8.0 by 5 M NaOH. The mixture was incubated at room temperature for 3 h in the dark and subsequently dialyzed against 10 mM HCl for 1 day. After dialysis, the sample solution was freeze-dried. The resultant RCM-lyz powder was suspended into the test solutions containing 1 M Arg salt(pH 9.5) at room temperature for 3 days. After incubation the solution was centrifuged at 18,800*g* for 30 min and the absorbance of the supernatant was measured. The solubility was calculated from the absorbance at 280 nm by $\varepsilon = 2.37\ \mathrm{ml\,mg^{-1}\,cm^{-1}}$,for denatured lysozyme [29].

2.5. Refolding assay

Refolding experiments were carried out as previously described [30] with slight modifications. Lysozyme was reduced and denatured at 40 mg/ml in a solution containing 6 M Gdn, 1 mM EDTA, 40 mM DTT and 100 mM Tris-HCl buffer (pH 8.0) and incubated for 2 h at 37 °C. The denatured and reduced lysozyme was diluted 40-fold with an appropriate refolding buffer containing Arg salt and GSH and GSSH. The final concentrations of each ingredient in the refolding mixture are 1 mg/ml lysozyme, 150 mM Gdn-HCl, 1 mM DTT, 1 M Arg salt, 5 mM GSH, 5 mM GSSG, 1 mM EDTA and 100 mM Tris (pH 8.0). The diluted solutions were vortexed for 2 s and incubated at 25 °C for 15 h without shaking. After incubation, the sample solutions were centrifuged at 18,800*g* for 30 min to remove the aggregates. After centrifugation, 10 μl of supernatant was mixed with 1490 μl of 0.3 mg/ml *Micrococcus Luteus* solution containing 50 mM phosphate buffer (pH 7.0) and monitored the absorbance at 600 nm using a V-630 UV–vis spectrophotometer (Japan Spectroscopic Co., Ltd., Tokyo, Japan). The refolding yield was determined from the initial velocity of decreasing absorbance, which was compared with 1 mg/ml native lysozyme.

3. Results

3.1. Solubility of propyl gallate in different Arg salt solution as a function of additive concentration

To investigate the effect of counter ion on the cation-π interaction between guanidinium group of arginine and aromatic ring, the solubility of propyl gallate was used as a model aromatic compound was determined as described in Section 2. Fig. 1 shows the solubility of propyl gallate in the presence of various Arg salts, i.e., acetate, formate, sulfate, chloride, citrate and phosphate, as a function of additive concentration at pH 4.8. All arginine salts increased the solubility of propyl gallate concentration dependently. The magnitude of solubilization effect depended on anionic species.

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