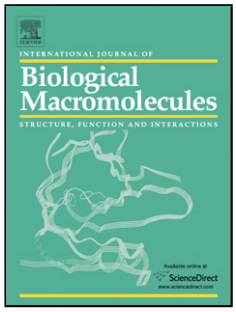

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Effect of counter ions of arginine as an additive for the solubilization of protein and aromatic compounds

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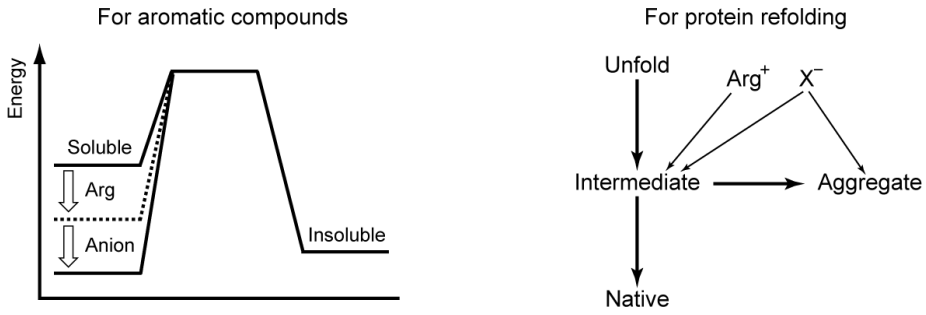
Graphical abstract



Highlights

- Although arginine chloride is widely used, only few studies have been done with other salt forms.
- We examined the effects of six different acids on solubilization of aromatic compounds and unfolded protein by arginine.
- Anions affect solubilization of propyl gallate by arginine.
- Anions also affect the propyl gallate solubility similarly for sodium and guanidine salts.
- Protein solubility and refolding are also affected by anions.

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.

Keywords: arginine, counter ion, solubility

Keywords: arginine, propyl gallate, solubility, counter ion, refolding, protein

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.

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 hour 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:

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

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

$$\begin{cases} x_{\mathrm{a}} = n_{\mathrm{g,a}} / (n_{\mathrm{g,a}} + n_{\mathrm{H_2O,a}} + n_{\mathrm{a,a}} + n_{\mathrm{c,a}}) \\ x_{\mathrm{s}} = n_{\mathrm{g,s}} / (n_{\mathrm{g,s}} + n_{\mathrm{H_2O,s}} + n_{\mathrm{a,s}} + n_{\mathrm{c,s}}) \end{cases} \cdots (3)$$

In the equation (1) to (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 solutions x_{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 $n_{\mathrm{i,s}}$ and $n_{\mathrm{i,a}}$, 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 hour. 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 3h 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 1M 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 ε= 2.37 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 hour 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, 1mM 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 the method section. 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. The effect of ArgHCl, which is commonly used, was moderate, increasing the solubility from 3.5 to 8.8 mg/ml at 1 M. ArgH(acetate) was most effective with the solubility reaching 19.9 mg/ml at 1 M followed by ArgH(formate) and ArgH(citrate), resulting in 13.4 and 10.6 mg/ml at 1 M additives. The latter two Arg salts were still more effective than ArgHCl. On the contrary, ArgH$(sulfate)_{1/2}$ and ArgH(H_2PO_4) were less effective than ArgHCl, resulting in 6.8 mg/ml and 7.5 mg/ml at 1 M. These results indicate that the type of anionic species greatly influence the solubilization effects of arginine. The order of ArgHCl > ArgH$(sulfate)_{1/2}$ = ArgH(H_2PO_4) in increasing the solubility appears to be consistent with the Hofmeister series. Thus, stronger hydration otential of the latter two anions or their stronger Arg clustering proposed by Trout et al. [24] can be used to explain different solubilization effects of these three Arg salts. The stronger solubilization effects of ArgH(acetate), ArgH(formate) and ArgH(citrate) cannot be readily explained from both mechanisms and may be related to the fact that they have caroboxyl groups. We have also attempted the same experiment at pH 9.6, at which Arg itself can serve as a buffer. However, we were unable to determine the propyl gallate solubility at a constant pH of 9.6 due to ionization of phenol group of propyl gallate and thereby pH changes (data

not shown). It should be noted that a similar experiment can be done at or near pH 9.6 using solutes that have no dissociable groups around this pH,

3.2. Comparison the solubilization effect of other salts with Arg salt

Whether the observed effects of anionic species on propyl gallate solubility is specific to Arg salt or universal was tested using sodium salt and guanidium salt. We chose the acetic, hydrochloric and sulfuric ion as weak, monovalent and multivalent anion, respectively. Fig.2A shows the solubility of propyl gallate in the presence of each sodium salt. Sodium chloride and sodium sulfate decreased the solubility of propyl gallate concentration dependently with the sulfate salt slightly more strongly. On the contrary, sodium acetate increased the solubility of propyl gallate. The observed order, acetate > chloride > sulfate, is identical to the order for their Arg counterpart, suggesting an identical mechanism operating on their effects on propyl gallate solubility. Regardless of cation species (sodium vs. Arg), their anionic species affects the propyl gallate solubility similarly. Fig.2B shows the effects of guanidine salt on the solubility of propyl gallate. All guanidine salts increased the solubility of propyl gallate. The magnitude of solubilization effect of guanidine salts increased in the order of acetate > chloride > sulfate, again consistent with the order observed for Arg and sodium salts.

We next compared the effects of Arg and sodium salts of the same anionic species by dividing the propyl gallate solubility in Arg salt solution by the corresponding value in sodium salt solution. Namely, it measures the ratio of solubility increase by replacing sodium with Arg as a cation. Fig.3 shows the results of such a calculation. Regardless of anionic species, the ratio is greater than 1, indicating that the solubility of propyl gallate is universally greater with Arg than sodium, consistent with the established ability of Arg to increase the solubility of proteins and small organic compounds. The solubility ratios nearly fall on the

same curve for these three anions, meaning that the enhanced solubility by Arg over sodium is independent of the anionic species. It thus appears that the solubilization effects of Arg ion is independent of the anionic species.

Transfer free energy of propyl gallate from sodium salt solution to Arg salt solution was calculated from the solubility ratio as described in the Method section. The transfer free energy, shown in Fig.4, was negative for all cases. For example, the free energy of the propyl gallate decreased ~3 kJ/mol by transferring 1 mol/L of propyl gallate from 1 M sodium salt solution to 1 M arginine salt solution independent of the anionic counter ions. At any concentration, the transfer free energy is negative, meaning that the interaction of propyl gallate is more favorable with Arg than sodium ion and is not affected by the anionic species.

3.3. The effect of counter ion on the solubility aromatic amino acid

It was suggested above that there may be specific interactions between acetate and propyl gallate, as this anion was highly effective in increasing the propyl gallate regardless of the cationic species, i.e., whether Arg, Gdn or sodium. Thus, whether acetate effect is universal or specific to propyl gallate was tested with aromatic amino acids, namely Tyr, Phe and Trp, using Arg salts. Fig.5 shows the solubility change of these amino acids when transferred from buffer solution to Arg salt solutions at 1 M. It is evident from Fig.5 that ArgH(acetate) is more or less comparable with ArgHCl, demonstrating a unique nature of propyl gallate with regard to the interaction with acetate anion. In fact, with Tyr and Phe, the solubilization effects of 1 M ArgHCl, ArgH$(sulfate)_{1/2}$ and ArgH(acetate) were comparable. With Trp, the order was ArgHCl > ArgH$(sulfate)_{1/2}$ > ArgH(acetate), qualitatively different from the order observed for propyl gallate. Interestingly, all these Arg salts were marginally effective on the Phe solubility, suggesting that possible favorable interaction of Arg is weak against Phe.

3.4. Effect of Arg salt on protein solubilization and refolding

As mentioned above, the solubility of small aromatic compounds were influenced not only arginine but also their counter ions. It may be possible that the solubility of proteins is also similarly affected by Arg salts. Therefore, we next examined the solubility of RCM-lyz in Arg salt solutions. ArgHCl, as shown in Fig.6, increased the solubility of RCM-lyz from 0.03 mg/ml to 1.54 mg/ml at 1 M. Other Arg salts, ArgH(sulfate)$_{1/2}$ and ArgH(acetate), were also effective, increasing the solubility of RCM-Lys to 0.45 and 0.4 mg/ml, but which were far less than the solubility in ArgHCl. It is thus evident that for protein solubility, ArgHCl is more effective than ArgH(sulfate)$_{1/2}$ and ArgH(acetate), an observation different from the propyl gallate solubility.

Fig.7 shows the refolding yield of lysozyme at pH 8.0. In the absence of additives, the refolding yield was only 6 % due to the formation of aggregates. ArgHCl increased the refolding yield to 65 % at 1 M. Consistent with the solubility of RCM-Lyz, sulfate ion and acetate ion greatly suppressed the refolding effectiveness of ArgHCl, resulting in 7 and 18 % refolding yield.

4. Discussion

This study examined how counter anions modulate the solubilization effects of arginine and also how the counter cations alter the effects of anionic species in the Arg, sodium and guanidine salts. Arg salts increased the solubility of the propyl gallate in the order of acetate > formate > citrate > chloride > phosphate > sulfate, of which the first three acids do not follow the Hofmeister series (Fig.1). Among these three acids, acetate was particularly effective in increasing the propyl gallate solubility with not only Arg but also sodium and guanidine, suggesting unique interaction between propyl gallate and acetate. This

was supported by the observation that ArgH(acetate) was not special compared with sulfate and chloride against Tyr, Phe and Trp solubilities. Since all these solutes possess aromatic ring structure, the unique interaction between acetate and propyl gallate may be due to propyl group in propyl gallate. However, acetic acid, formic acid and citric acid have all carboxyl groups, of which the latter two were significantly less effective. Previously, molecular dynamics simulation suggested favorable hydrogen bonding interactions between hydroxyl group of phenol and carboxylate anion [31]. If this is an only mechanism of acetate on propyl gallate solubility, then ArgH(acetate) should also increase the Tyr solubility, inconsistent with only small increment of Tyr solubility by acetate over chloride. Thus, there must be additional factor responsible for the acetate effects on propyl gallate solubility. Regardless of the mechanism, ArgH(acetate) greatly increases the solubility of propyl gallate, which is used as an antioxidant, and hence may have a commercial value according to the previously proposed arginine assisted solubility system (AASS) [10].

With regard to ArgHCl and ArgH(sulfate)$_{1/2}$, it appears that the solubility of propyl gallate decreased in that order. The propyl gallate solubility also decreased with sodium or guanidine chloride and sodium or guanidine sulfate, more so for sulfate, meaning that their effects are independent of the cationic species. Such anion-specific effects on protein solubility were first observed by Hofmeister and have been explained by surface tension increment caused by ions [18–20] or ion hydration [24]. When a solute molecule is introduced into an aqueous solution, solvent water generates a cavity to accommodate the solute and creates an interface between bulk water and the solute. If the surface tension of aqueous solution is low, less energy is required to make a cavity, resulting in higher solubility. If the surface tension is high, greater energy is required.

The solubility of RCM-Lyz increased in Arg, whose effects are modulated by anionic species (Fig.6). This effect can be explained by the interaction of Arg with aromatic amino

acids in lysozyme (Fig.5). Hen egg white lysozyme has three tyrosines, three phenylalanines and six tryptophans [32]. Favorable interactions between Arg and these aromatic side chains should play a role in increased solubility of RCM-Lys. Such Arg effects are most favorable with chloride ion, compared with sulfate and acetate ions.

The dependence of refolding yield of lysozyme on counter ion is generally the same as the dependence of RCM-Lyz solubility, i.e., ArgHCl is most effective in increasing the refolding yield (Fig.7). Previously, it is reported that sodium sulfate stabilizes the intermediate state of protein [33]. On the basis of this, we consider that sulfate decreases the refolding yield of lysozyme by stabilization of the aggregates and the intermediate states. Although sulfate also stabilizes the native state by salting-out effect, higher energy barrier between the native state and the intermediate state makes kinetics of refolding slower and facilitates aggregation. Compared with sulfate, acetate has a weaker salting-out effect [25], which may be consistent with a slightly higher refolding yield by ArgH(acetate) than $ArgH(sulfate)_{1/2}$ and Arg can exhibit small effect. Interestingly, Trout et al. reported that $ArgH(sulfate)_{1/2}$ more effectively suppresses thermal aggregation of α-chymotrypsinogen than ArgH(acetate) and ArgHCl [27]. This discrepancy is perhaps due to the different states of the proteins used. Thermal aggregation may be more effectively suppressed by stabilizing the native state, the effect conferred by sulfate salt. Refolding solution, on the contrary, may need not only aggregation suppression but also providing a proper folding pathway, which can be afforded by ArgHCl.

In summary, we showed that the selection of counter ions can modulate the effects of Arg on the solubility of aromatic compounds and reduced protein as well as on refolding of the reduced lysozyme. Such modulation is independent of the interaction between Arg and aromatic compounds. Namely, the anionic species modulate the propyl gallate solubility independently. ArgHCl has been commonly used in many biological and biotechnological

applications [34,35]. Here, we have shown that the effects of Arg may be further enhanced using different anionic counter ions depending on the target solute.

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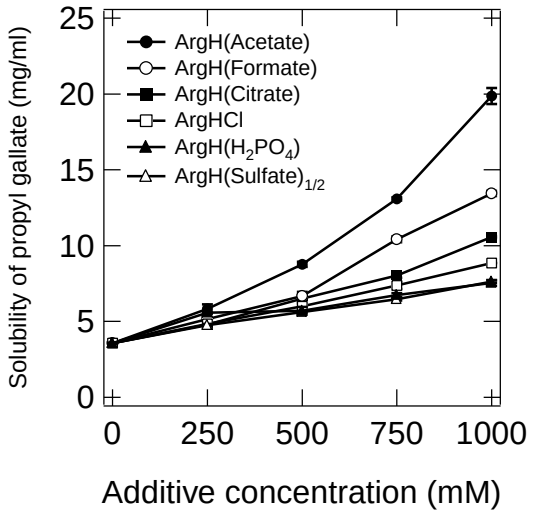
Figure Captions

Fig. 1 Solubility of propyl gallate in the absence and presence of arginine salt as a function of additive concentration. All solutions contained 10 mM citrate buffer (pH 4.8). The measurements were performed three times, and the error bars indicate the standard deviation of the mean throughout the paper.



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

Shunsuke Yoshizawa, Tsutomu Arakawa, Kentaro Shiraki

Fig. 1

Fig. 2 Solubility of propyl gallate in the absence and presence of (A) sodium salt and (B) guanidium salt as a function of additive concentration. All solutions contained 10 mM citrate buffer (pH 4.8).

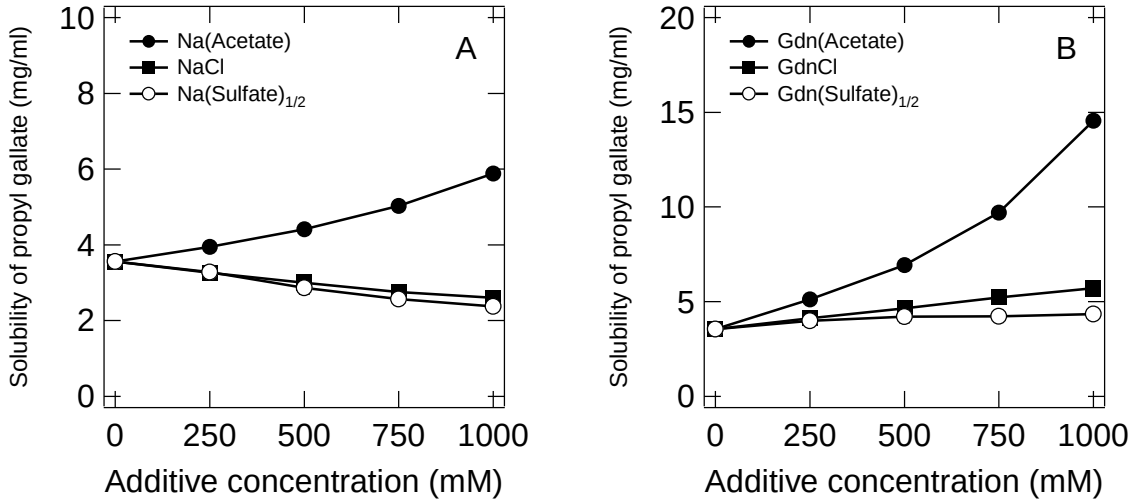


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Fig. 2

Fig. 3 Ratio of the solubility of propyl gallate in Arg salt solution to that in sodium salt solution at a given additive concentration.

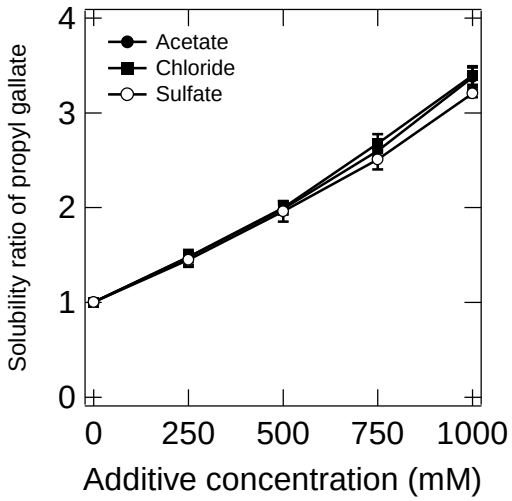

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Fig.

Fig. 4 Transfer free energy of propyl gallate from sodium salt solution to Arg salt solution at a given additive concentration.

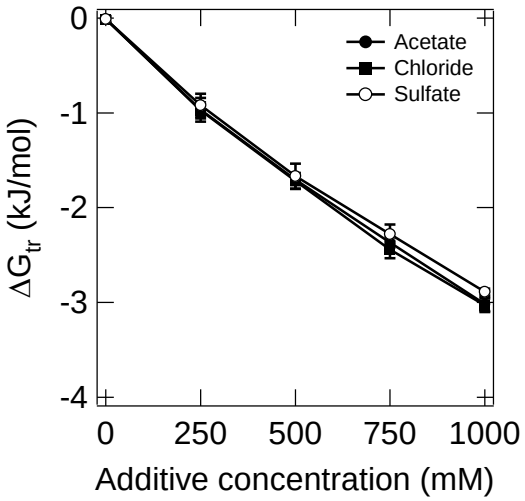

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Fig. 4

Fig. 5 Ratio of the solubility of Tyr, Phe and Trp in 1 M Arg salt solution to that in its absence. All solutions contained 10 mM citrate buffer (pH 4.8).

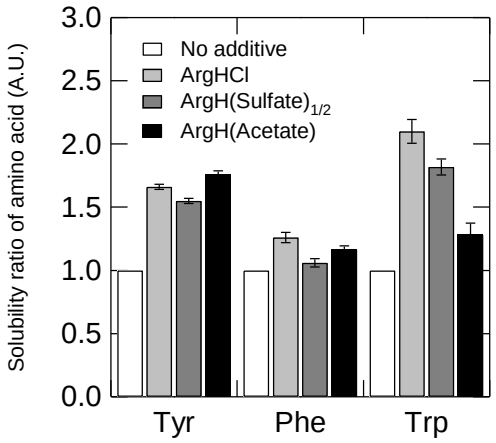

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Fig. 5

Fig. 6 Solubility of RCM-lyz in the absence and presence of 1 M Arg salt. All solutions contained 10 mM Tris buffer (pH 9.5).

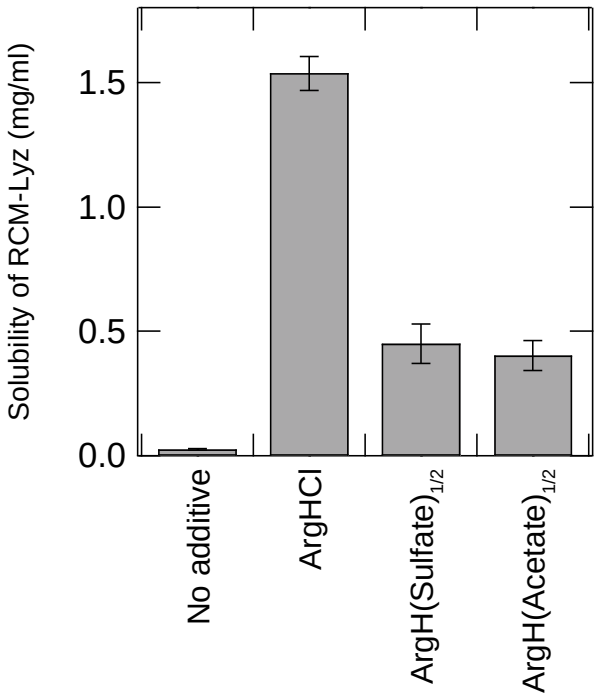

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Fig. 6

Fig. 7 Refolding yield of lysozyme in the absence and presence of 1 M Arg salt. All solutions contained 150 mM Gdn-HCl, 1 mM DTT, 5 mM GSH, 5 mM GSSG, 1mM EDTA and 100 mM Tris (pH 8.0).

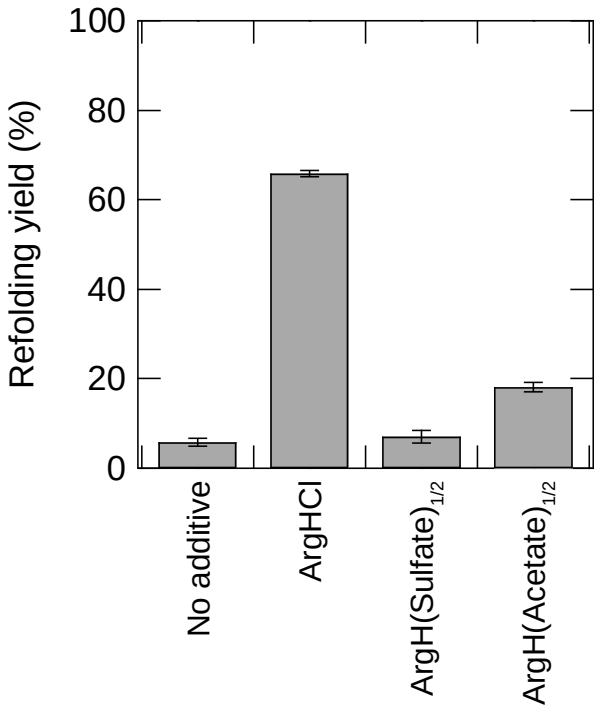

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Fig. 7