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Arginine Increases the Solubility of Coumarin: Comparison with Salting-in and Salting-out Additives

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Poor aqueous solubility of low molecular weight drug substances hampers their development as pharmacological agents. Here, we have examined the effects of arginine on the solubility of organic compounds, coumarin, caffeine and benzyl alcohol, in aqueous solution. Arginine increased the solubility of aromatic coumarin, but not non-aromatic caffeine, concentration dependently, suggesting the favourable interaction of arginine with the aromatic structure. Consistent with this, arginine also increased the solubility of aromatic benzyl alcohol. Guanidine hydrochloride, urea and salting-in salts increased both coumarin and caffeine solubilities, while salting-out salts decreased them. These results suggest the specific interaction of arginine with aromatic groups, leading to increased solubility of coumarin. However, the effect of 1 M arginine on coumarin solubility was at most ~2-fold, which may limit its applications as a solubility enhancing agent.

Key words: arginine, caffeine, coumarin, solubility, surface tension.

Abbreviation: Gdn, guanidine hydrochloride.

Aqueous solubility is one of the major problems in the development of pharmaceutical drug substances (*1*, *2*). Poor aqueous solubility of drug substances causes problems in drug screening and pharmacological bioavailability. Additives, which increase the aqueous solubility but have little cell toxicity, would be a valuable resource for pharmaceutical industries. We have examined here the effects of arginine on the solubility of small organic molecules as a model compound and compared with the results of the known salting-in and salting-out solvent additives.

Arginine has shown a variety of practical applications in protein refolding, purification and formulation (*3*–*11*). The ability of arginine to suppress protein–protein and protein–surface interaction plays a major role in these applications (*12*–*21*). No solubility data of small organic molecules in aqueous arginine solution have been reported until recently (*22*). As arginine is a natural biological metabolite, its safety and negligible cell toxicity make this compound ideal as a solubilizing agent, provided that arginine in fact increases the solubility of small organic compounds as it does on proteins. Here, we have examined the effects of arginine and other salting-out or salting-in compounds on the solubility of anticoagulant, coumarin and a major ingredient of coffee, caffeine, which has been shown to have a potent antiviral activity (*23*, *24*).

MATERIALS AND METHODS

Chemicals—Guanidine hydrochloride (Gdn), urea, arginine (Arg), proline (Pro), serine (Ser), glycine (Gly), lysine (Lys), NH_4SCN, NaSCN, NH_4I, NaI, LiCl, NH_4Cl, KCl, $(NH_4)_2SO_4$, Na_2SO_4 and citrate were from Wako Pure Chemical Industries (Osaka, Japan). Coumarin and caffeine were from Sigma Chemical Co. (St Louis, MO, USA). Benzyl alcohol was from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Sodium dihydrogenphosphate dehydrate and NaCl were from Nacalai Tesque (Kyoto, Japan). All the compounds used were of the highest grade commercially available and used as received.

Solubility of Coumarin and Caffeine in the Presence of Additives at Different pH—The solubility of coumarin and caffeine in the presence of 1.0 M additives (amino acids, denaturants and salts as indicated in each figure) at pH 3.0, 5.0 and 7.5 were measured as follows. LiCl was not used at pH 7.5 because of low solubility. Stock solutions of the additives at 1.0 M were prepared in 50 mM citrate–phosphate buffer at pH 3.0, 5.0 and 7.5. The excess amounts of powder of coumarin or caffeine were added into the stock aqueous additive solutions. Coumarin or caffeine powders were completely dissolved in test solvents in boiling water. Coumarin or caffeine solution was then cooled to 25°C and incubated at this temperature for 2 h (for coumarin) and 12 h (for caffeine) to reach equilibrium. Coumarin consistently formed amorphous precipitates during incubation at 25°C, while caffeine formed different crystal structures from one case to another, which could not be readily controlled. As different crystal forms would have different solubilities, the needle-shape crystal was induced by seeding the precipitates with the pre-formed needle crystal of caffeine. After the incubation, the samples were centrifuged at 15,000g for 20 min at 25°C. The supernatants of the sample was diluted 20-fold with distilled water, and then the concentrations of soluble coumarin and caffeine in the supernatant were measured by absorbance at 278 and 273 nm, respectively, with ND-1000 UV-Vis

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spectrophotometer (NanoDrop Technologies, Inc. Wilmington, Del, USA). The solubilities were then calculated from the standard curves. All the measurements were performed three times and the standard errors are given in Figs 1, 2, 5 and 6 by error bars.

Effect of Additive Concentration on Solubility of Coumarin and Caffeine—The solubility of coumarin and caffeine at pH 7.5 were measured as a function of additive concentration, i.e. 0.1, 0.2, 0.5 and 1.0 M. The stock solutions containing various additives described above were prepared in 50 mM citrate–phosphate buffer at pH 7.5. The coumarin or caffeine samples were similarly prepared, i.e. suspension of excess powders in the test solvents, dissolution at 100°C and incubation at 25°C. The concentrations of soluble coumarin and caffeine were similarly determined.

Solubility of Benzyl Alcohol—The solubility of benzyl alcohol, another model compound, was determined only at pH 7.5 in the absence and presence of 1 M arginine or Gdn. Stock solutions of the additives at 1.0 M were prepared in 50 mM citrate–phosphate buffer at pH 7.5. The excess amount of benzyl alcohol was added into the stock aqueous additive solutions. Benzyl alcohol was completely dissolved in test solvents in boiling water. Benzyl alcohol solution was then cooled to 25°C and incubated at this temperature for 1 h to reach equilibrium solubility. After the incubation, the samples were centrifuged at 15,000g for 20 min at 25°C. The supernatant of the samples was diluted 10-fold with distilled water and then measured for benzyl alcohol concentration by absorbance at 256 nm. The solubilities were then calculated from the standard curves. All the measurements were performed three times and the standard errors are given in Figs 5 and 6 by error bars.

RESULTS

Solubility of Coumarin and Caffeine in Aqueous Solution—Coumarin is used as an anti-coagulant and has a poor aqueous solubility. It is a small organic compound with a molecular weight of 146.15 and contains an aromatic structure. In Fig. 1A is plotted the solubility of coumarin in two protein denaturants (Gdn and urea) and two protein refolding additives (arginine and proline) as well as the control, i.e. solubility in 50 mM buffer alone. There appears to be little pH dependence of the solubility, as expected in part from the uncharged coumarin structure. However, the charged state of amino acids depends on the pH. No apparent pH dependence of coumarin solubility for aqueous arginine and proline solutions suggests that these

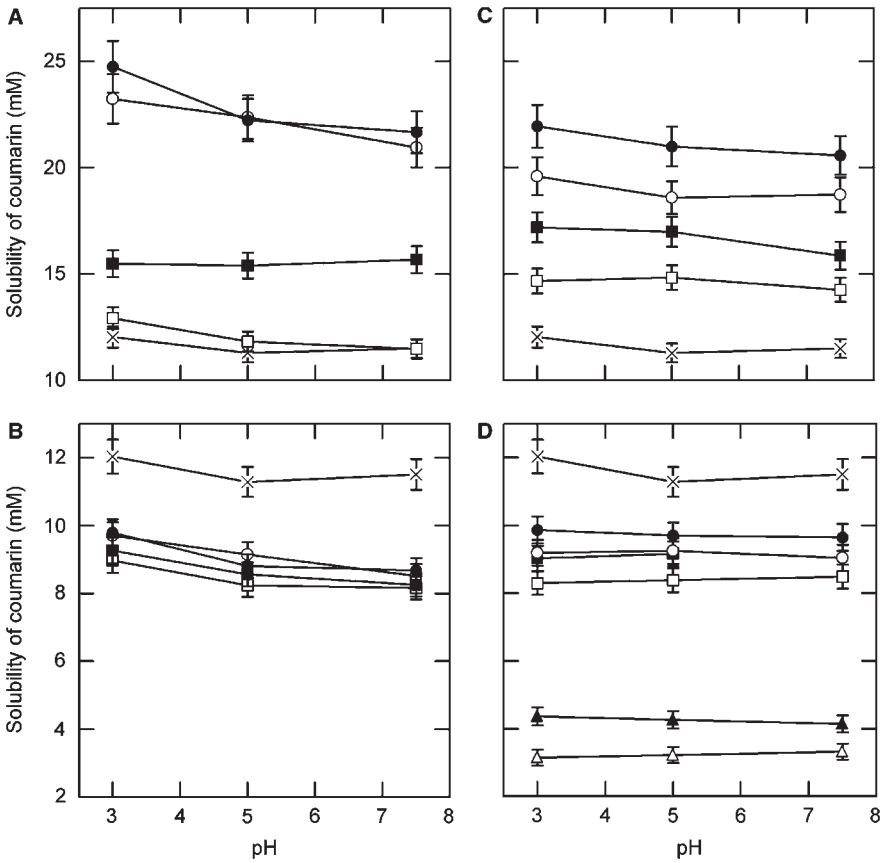

Fig. 1. **Solubility of coumarin in the presence of amino acids, denaturants and salts at 1.0 M and pH 3.0, 5.0 and 7.5, respectively.** Crosses in all figures show no additive. (A) Closed circles, Arg; open circles, Gdn; closed squares, urea; open squares, Pro. (B) Closed circles, Ala; open circles, Gly; closed squares, Ser; open squares, Lys. (C) Closed circles, NH_4SCN; open circles, NaSCN; closed squares, NH_4I; open squares, NaI. (D) Closed circles, NH_4Cl; open circles, KCl; closed squares, LiCl; open squares, NaCl; closed triangles, $(NH_4)_2SO_4$, open triangles, Na_2SO_4.

amino acids affect the solubility of coumarin independent of the charged state. It is evident that arginine and Gdn at 1.0 M significantly increase the coumarin solubility; the solubility increased by about 2-fold in the presence of these two additives. Considering the non-denaturing property of arginine and its safety, 1.0 M arginine should be a better additive. A protein folding assisting additive, proline (*25–27*), has essentially no effect on coumarin solubility at 1.0 M. Urea, a protein denaturant, is significantly less effective than Gdn at the same concentration, consistent with its weaker denaturing potency for proteins.

Figure 1B compares different amino acids at 1.0 M; note that the scale is different. All the amino acids tested, i.e. alanine, glycine, serine and lysine, decreased the coumarin solubility. These four amino acids are similar, within experimental errors, to each other, decreasing the coumarin solubility by about 0.7- to 0.8-fold independent of the pH. It is interesting to point out that a basic amino acid, lysine, decreased the coumarin activity, in contrast to the effect of basic arginine. This demonstrates that the basic nature is not the factor responsible for the observed effectiveness of arginine.

Figure 1C and D compare the effects of salting-in and salting-out salts on coumarin solubility. With these salts as well, there is no apparent pH dependence. Thiocyantates, salting-in salts, effectively increased the coumarin solubility, ammonium salt being more effective, consistent with their salting-in effects on proteins (*28*). Arginine appears to be even stronger, although slightly, than ammonium thiocyanate, indicating its effectiveness as a solubilizing agent. Two iodide salts were also effective, with the magnitude similar to the effect observed for urea (Fig. 1A). As in thiocyanate salts, ammonium salt of iodide was more effective.

Figure 1D plots the results for salting-out salts. All the salting out salts tested, i.e. sulfate salts and chloride salts, decreased the coumarin solubility, consistent with their known effects on proteins. Sulfate salts were most effective, consistent with the Hofmeister series of salts (*29*). With these salts, sodium salts are stronger than the ammonium salts in reducing the coumarin solubility. Within the group of chloride salts, the salting-out effect increased in the order of $NH_4 < K < Li < Na$.

A similar study was carried out on caffeine. Caffeine is a major component of coffee and relatively soluble in water; it is an alkaloid with a molecular weight of 194.19. Figure 2A plots the solubility of caffeine in the presence of arginine and two protein denaturants.

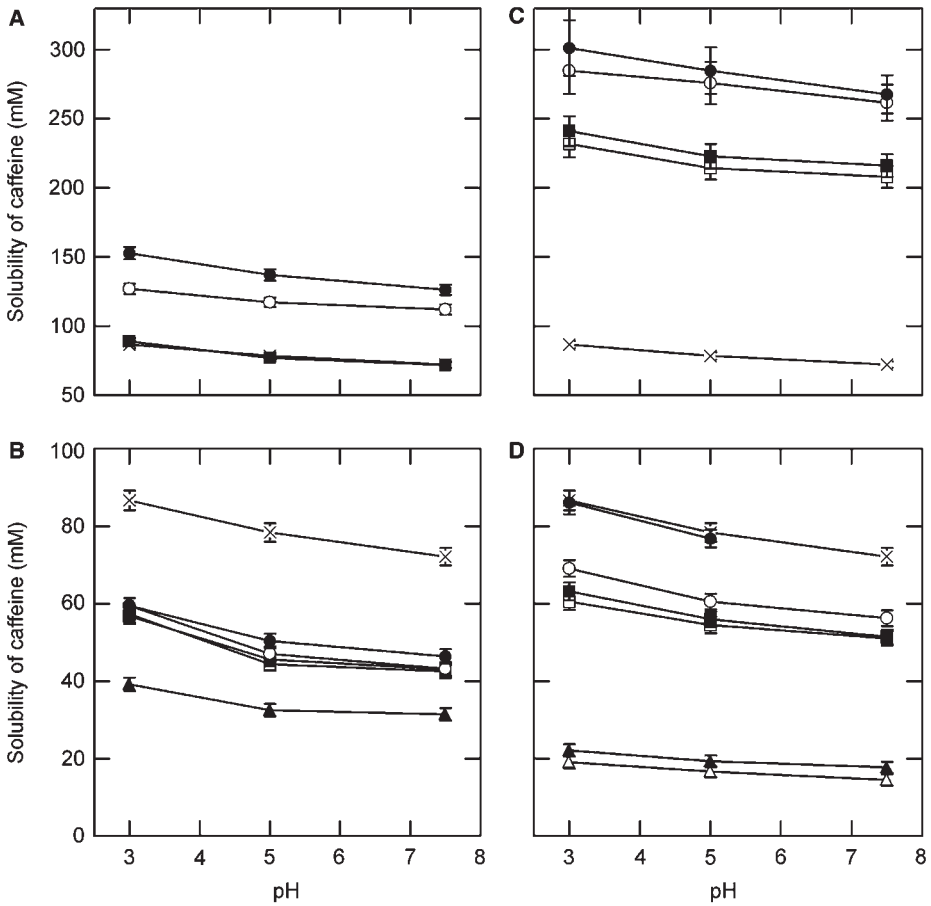

Fig. 2. **Solubility of caffeine in the presence of amino acids, denaturants and salts at 1.0 M and pH 3.0, 5.0 and 7.5, respectively.** Crosses in all figures show no additive. (A) Closed circles, Gdn; open circles, urea; closed squares, Arg. (B) Closed circles, Pro; open circles, Ala; closed squares, Ser; open squares, Gly; closed triangles, Lys. (C) Closed circles, NH_4SCN; open circles, NaSCN; closed squares, NH_4I; open squares, NaI. (D) Closed circles, LiCl; open circles, NH_4Cl; closed squares, NaCl; open squares, KCl; closed triangles, $(NH_4)_2SO_4$, open triangles, Na_2SO_4.

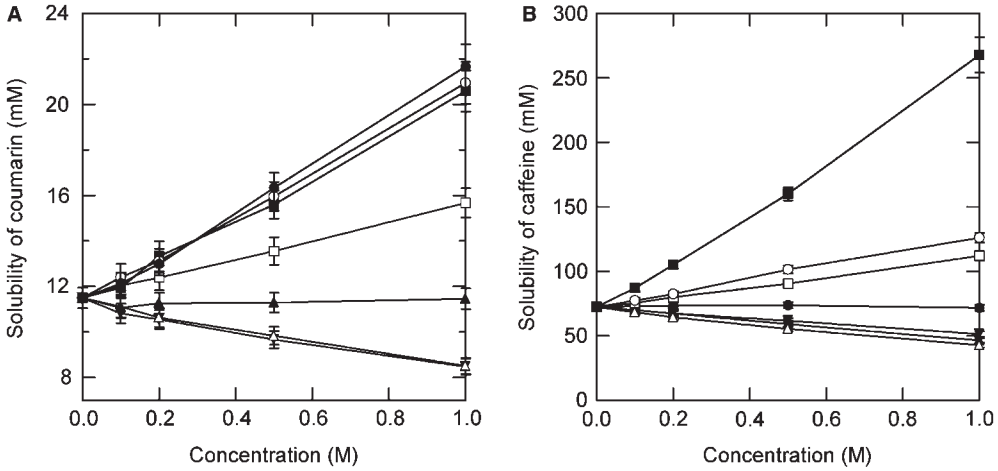

Fig. 3. **Solubility of coumarin (A) and caffeine (B) in the presence of various concentrations of amino acids, denaturants and salts at pH 7.5.** Closed circles, Arg; open circles, Gdn; closed squares, NH_4SCN; open squares, urea; closed triangles, Pro; open triangles, Gly; closed inverse triangles, NaCl.

High solubility in buffer alone is evident in this figure (compare the scale of this figure with Fig. 1). There was little dependence on pH, consistent with the uncharged structure of caffeine. In this case, 1.0 M arginine showed no effect; i.e. it did not affect the solubility of caffeine. Both urea and Gdn increased the solubility, Gdn being slightly more effective. Figure 2B compares several amino acids at 1.0 M. All the amino acids decreased the caffeine solubility. Lysine was most effective, leading to the solubility by less than half the level of buffer control. Other amino acids (proline, alanine, serine and glycine) at 1.0 M decreased the solubility by about 30–40%. While proline showed no effect on coumarin solubility, it significantly reduced the caffeine solubility (by about 35%).

Figure 2C shows the data for salting-in salts. All the salting-in salts increased the solubility of caffeine, indicating that their effects do not require the presence of aromatic rings, similar to urea and Gdn but different from arginine. The salting-out salts decreased the caffeine solubility (Fig. 2D). Both the trend and the relative magnitude of the effects are similar to those observed for coumarin.

The solubility of proteins in general follows a complex dependence on salt concentration. The solubility first increases with salt concentration at low salt concentration (e.g. below 0.2 M) and then decreases in the presence of salting-out salts or continues to increase in the presence of salting-in salts as the salt concentration is increased. This is due to two different factors involved in the salt effects. The initial increase in solubility is due to electrostatic stabilization of charged proteins by salt ions and the following change in solubility is due to salt-specific effects. Such a complex behaviour in salt effects is due to heterogeneous chemical properties of the protein surface. As both coumarin and caffeine have a much simpler structure than the proteins, it is expected to follow simple concentration dependence. Figure 3A and B shows the effects of the additives on the coumarin and caffeine solubility, respectively as a function of additive concentration. As expected, the effect is monotone; for example, arginine increased the solubility of coumarin concentration-dependently, while it showed no effect on caffeine solubility regardless of the concentration.

The solubility of benzyl alcohol, another model aromatic compound, was also determined at pH 7.5 and compared with the solubility of coumarin and caffeine. Since these compounds greatly differ in aqueous solubility, the results are expressed as the solubility ratio. As shown in Fig. 5 (see also inset in the expanded scale), the solubility of benzyl alcohol significantly increased, although to a much lesser extent, by 1 M arginine and Gdn, similarly to the increased solubility for coumarin. However, the solubility increase is much smaller for benzyl alcohol than for coumarin and caffeine (Fig. 5), which may reflect the fact that the aqueous solubility of benzyl alcohol is much higher than coumarin and caffeine (Fig. 6). Thus, benzyl alcohol is highly solvated already in water and additional solvation energy due to 1 M arginine or Gdn would make less contribution to the solubility of this compound.

DISCUSSION

A striking picture emerges from the arginine effects on the coumarin and caffeine solubilities. Arginine only increased the coumarin solubility. Coumarin has an aromatic ring structure, which is absent in caffeine, suggesting that the aromatic structure plays an important role in interacting with arginine, consistent with the known interaction between π-electron cloud and guanidinium group (*30*). The observed slight, but significant, increase of benzyl alcohol solubility by 1 M arginine supports this notion. Both Gdn and, to a lesser extent, urea, also enhanced the coumarin solubility. However, they also increased the solubility of caffeine, which contains no aromatic rings. In this sense, arginine is more specific, which may be the reason why it is not a denaturant.

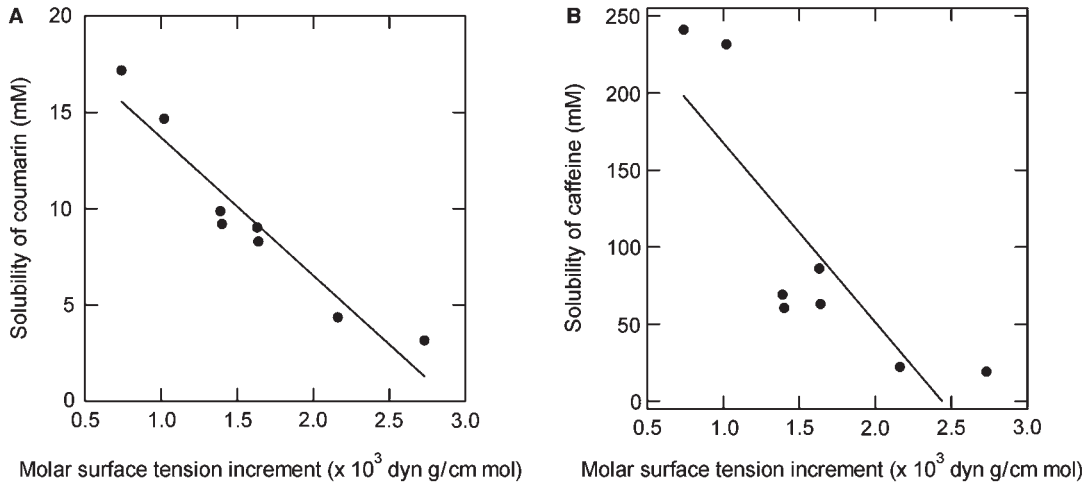

Fig. 4. **Correlation between the solubility of coumarin (A) or caffeine (B), and the molar surface tension increment.** The values of the molar surface tension increment were from the early literature (*38*), except for NaSCN and NH_4SCN that were not obtained from it. The data of the solubility of coumarin or caffeine for 1.0 M salts at pH 3.0, except for NaSCN and NH_4SCN, were used. Linear lines indicate the least-square fitting of the line with correlation coefficients of -0.9573 ± 0.0006 (coumarin) and -0.8315 ± 0.0024 (caffeine).

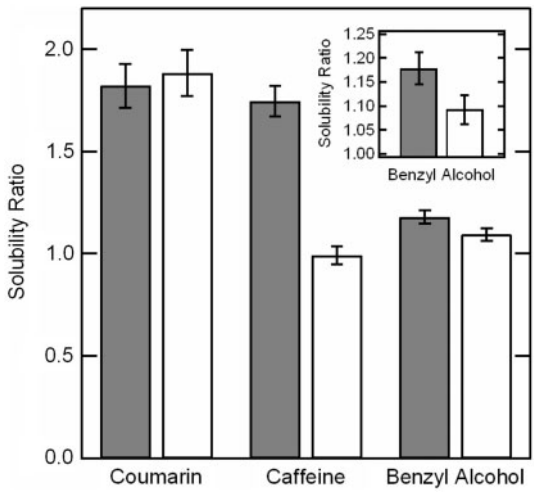

Fig. 5. **The effects of 1 M Gdn and arginine on coumarin, caffeine and benzyl alcohol solubilities at pH 7.5.** Inset, benzyl alcohol data in the expand scale. Solubility ratio was calculated as a ratio of the solubility in 1 M Gdn (grey bars) or arginine (white bars) to the solubility in the buffer solution.

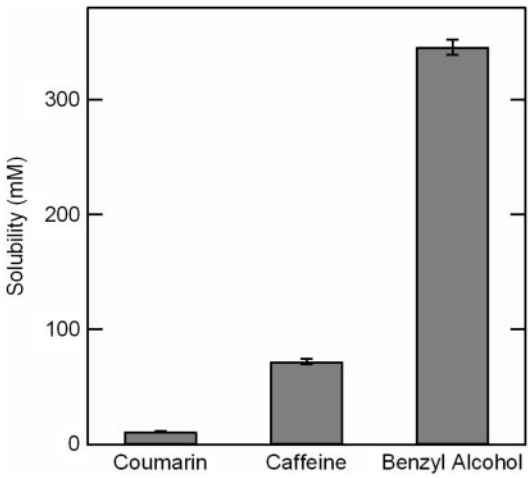

Fig. 6. **Aqueous solubility of coumarin, caffeine and benzyl alcohol at pH 7.5.** Solubility of coumarin, caffeine and benzyl alcohol in the buffer solution was measured at pH 7.5.

It should be pointed out, nevertheless, that arginine did not decrease the solubility of caffeine. This amino acid showed no effect on caffeine solubility at any concentration. This is in contrast to other amino acids tested, which decreased the solubility of caffeine as well as coumarin. Lowering the solubility means unfavourable interactions; *i.e.* these amino acids except arginine interact with caffeine (and coumarin) unfavourably. The origin of such unfavorable interaction could be their large excluded volume (*31*, *32*) or their effect on water structure. As arginine may share these properties with other amino acids, the observed no effect on caffeine solubility suggests a compensatory favourable interaction of arginine with caffeine molecule, whose origin is not clear at this point.

As described earlier, additives at high concentration affect the water structure, as manifested, *e.g.* on the surface tension. In fact, the surface tension increment of salts has been the most useful parameter to explain the salt effects (*33–35*). This effect was successfully used to explain the Hofmeister series (*36*), the solubility of inert gasses (*37*), and the protein solubility (*38*). However, the heterogeneous nature of protein surface has often caused deviation for certain salts from the surface tension effect. Surface tension effect is an interfacial phenomenon between water and hydrophobic air. Melander and Horvath (*38*) formulated this effect as 'cavity theory', in which protein surface was assumed to resemble the property of air. The solubilities of coumarin and caffeine, whose structures are less complex than the protein surface, are expected to more closely follow the surface tension effect than does the protein. Figure 4A and B

plots the coumarin and caffeine solubility, respectively versus the molar surface tension increment of the salts examined here. There appears to be a significant correlation between the solubility and surface tension. Coumarin showed a high correlation coefficient, probably due to its low water solubility. This compound may be characterized simply as 'hydrophobic', just as for air. On the other hand, caffeine showed a much lower correlation coefficient. This compound has considerably high water solubility, meaning that it has a hydrophilic property. Thus, it is expected that certain salts may interact with the hydrophilic surface favorably than do other salts, which compensates for unfavourable interaction due to the surface tension effects.

We have chosen here relatively water-soluble compounds, coumarin and caffeine, and hence were able to observe both salting-in and salting-out effects of various additives relative to the effects of arginine. The solubilizing effects of arginine on coumarin are at most ~2-fold at 1 M, which would probably limit its applications as a solubility enhancing solvent additive. Marginal solubilizing effects of arginine are also evident for a more water-soluble benzyl alcohol (Figs 5 and 6). We are now investigating more effective technologies using arginine to increase the poorly water-soluble compounds and also exploring stronger solubilizing agents with weak cell toxicity.

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