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Article

The effects of N-acetyltryptophan and caprylic acid on protein aggregation

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N-acetyltryptophan (AT) and caprylic acid (Cap) are known to suppress the heat-induced aggregation of serum albumins. In this study, we examined the commonality of AT and Cap on the protein aggregation using hen egg-white lysozyme, ovalbumin, and bovine γ-globulin (BGG), as well as bovine and human serum albumin. AT and Cap as additives increased the unfolding temperatures of human and bovine albumins analyzed by far-UV circular dichroism. Both AT and Cap highly suppressed the heat-induced aggregation of BGG as well as albumins, while they enhanced the aggregation of lysozyme. In all cases, the addition of 100 mM ArgHCl showed no synergistic effects with AT or Cap in suppressing the aggregation of BGG. The small chemicals of AT and Cap will be useful additives toward the thermal stabilization of albumins and γ-globulin.

Key words: N-acetyltryptophan, caprylic acid, aggregation, ArgHCl, protein.

1. Introduction

Both N-acetyltryptophan (AT) and caprylic acid (Cap) have been used to suppress the heat-induced formation of soluble oligomers of serum albumins [1–3]. Fig.1 shows the chemical structures of AT and Cap. Cap is a type of fatty acid with a carboxyl end, found in coconut, babassu, and milk fat [4]. AT is a tryptophan derivative modified in the amino group by acetyl group. The effects of these additives are derived from their bindings to the albumins [5], which are known to bind various drug substances and fatty acids [6, 7].

Proteins in general can bind ions, organic compounds, and detergents [8, 9]. Binding of these compounds can alter solution properties of the proteins, e.g., stability, aggregation tendency, and solubility. In this short note, we examined the commonality of the effects of AT and Cap on the aggregation of five proteins that have different chemical and physical properties: human and bovine serum albumin, hen egg-white lysozyme, ovalbumin, and bovine γ-globulin. The effects of AT and Cap on the thermal stability were also examined, since it is related to protein aggregation [10]. In addition, we investigated whether the synergetic effects exist with arginine (see Fig.1 for chemical structure), which is a well-known aggregation suppressor of proteins [11–13].

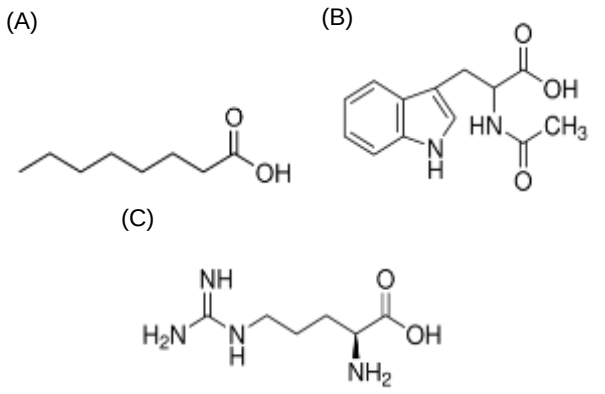

Fig. 1 Chemical structures of additives. (A) Caprylic acid; Cap. (B) N-acetyltryptophan; AT. (C) Arginine hydrochloride; ArgHCl.

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2. Materials and method

2.1 Materials

Human serum albumin (HSA), bovine serum albumin (BSA), hen egg-white lysozyme (lysozyme), ovalbumin, γ-globulin from bovine blood (BGG), N-acetyl-DL-tryptophan (AT), and caprylic acid (Cap) were obtained from Sigma-Aldrich Corp. (St Louis, Mo., USA) Sodium hydroxide (NaOH), sodium dihydrogenphosphate, and L–Arginine hydrochloride (ArgHCl) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All chemicals used were of reagent grade and used as received.

2.2 Circular dichroism spectroscopy

Denaturation temperatures (T_m) were determined following signal changes in the far-UV circular dichroism (CD) on a Jasco CD spectropolarimeter model J-720W with a Peltier cell holder model PTC-348W (Japan Spectroscopic Co., Ltd., Tokyo, Japan). Sample solutions containing 0.4 mg/ml protein (HSA, BSA, and lysozyme) or 0.8 mg/ml protein (ovalbumin) in 20 mM sodium phosphate, pH 7.0, in the absence and presence of additives, were prepared and adjusted to pH 7.0 by the addition of NaOH. CD signals were monitored at 224 nm for Cap-containing samples and at 238 nm for AT- or arginine hydrochloride (ArgHCl)-containing samples with a heating rate of 1°C/min using a 0.1-cm path-length cell. A lower wavelength could not be used for AT- or

ArgHCl-containing samples due to high absorbance. The data obtained were fitted to a conventional two-state equation, from which the apparent T_m was determined denaturation temperatures were not determined for BGG, as it is a mixture of many kinds of antibody molecules that generate a broad transition pattern for thermal unfolding.

2.3 Turbidity measurements

Thermal aggregation of proteins was measured as follows. Protein solutions at 1.0 mg/ml in 20 mM sodium phosphate, pH 7.0, in the absence and presence of additives, were prepared and adjusted to pH 7.0 by the addition of NaOH. A 2.0 mL aliquot of the solutions was introduced into a 1-cm path-length glass cell. The sample solutions were incubated at 70°C, 80°C, or 90°C for BGG and at 75°C or 90°C for lysozyme, and the turbidity at 400 nm was monitored using a Jasco spectrophotometer model V-550 (Japan Spectroscopic Co., Ltd., Tokyo, Japan).

3. Results and discussion

3.1 Influence of AT and Cap on thermal denaturation of proteins

The effects of AT and Cap on thermal stability were examined using far-UV CD signal at 238 nm for AT and at 224 nm for Cap. These wavelengths were selected as the lowest possible wavelength due to strong UV absorbance of AT and Cap. In addition, the concentration of AT and Cap above 3 mM could not be tested for the same reason, i.e., strong UV absorbance. CD intensity of 0.4 mg/ml protein solution was followed with increasing temperature from 45°C to 90°C, from which the mid-transition temperature, T_m, was determined by curve fitting to a two-state transition model. The results are summarized in Table 1. Control experiments, i.e., in the absence of additives, showed that the melting curves gave an identical T_m values within experimental errors, whether it was followed at 238 or 224 nm for HSA, BSA, lysozyme, and ovalbumin. As shown in Table 1, 3 mM AT increased the T_m of HSA and BSA by approximately 8°C and approximately 6°C, while 3 mM Cap increased it by approximately 15°C–16°C, demonstrating stronger stabilization effects of Cap. These results indicate preferential binding of AT and Cap to the native structure, leading to stabilization of the native proteins. On the contrary, both 3 mM AT and Cap exhibited negligible effects on the T_m of lysozyme and ovalbumin. There may be reduction of T_m (−2.8°C) for lysozyme by 3 mM AT. It is thus evident that AT and Cap have no stabilization effects on lysozyme and ovalbumin, unlike their effects on HSA and BSA. This in turn suggests that AT and Cap do not bind to these two proteins or bind to them in both the native and denatured states. That is, unlike HSA and BSA, they may bind to lysozyme and ovalbumin, but not preferentially to the native structure.

3.2 Influence of AT and Cap on thermal aggregation of proteins

Then, we examined the effects of heating on aggregation of the above four proteins and BGG. Thermal aggregation was examined by following turbidity at 400 nm. HSA, BSA, and ovalbumin showed no apparent increase in turbidity upon heating up to 90°C, indicating that these proteins do not generate large aggregates, the sizes sufficiently large to cause light scattering at 400 nm: it is possible that they may form small oligomers. No apparent aggregation was also observed upon heating for these proteins in the presence of 20 mM AT and Cap, implying that these additives do not enhance aggregation: note that 20 mM AT and Cap could be used in this experiment, which used 400 nm for turbidity measurements.

The effects of 20 mM AT and Cap were examined on thermal aggregation of lysozyme. Fig. 2 shows the turbidity increase when incubated at 75°C (A) and 90°C (B). At 75°C, 20 mM AT (squares) and Cap (triangles) accelerated turbidity increase, more strongly for Cap relative to their absence (circles). The onset of turbidity increase occurred at shorter time, and the turbidity increase was sharper in the presence of 20 mM AT and more with 20 mM Cap. Turbidity increase occurred much more rapidly at 90°C (circles). At this temperature as well, 20 mM AT and Cap shortened the onset of turbidity increase and enhanced the rate of aggregation. It is thus evident that AT and Cap enhanced the aggregation of lysozyme. In thermal denaturation experiments, 3 mM AT and Cap showed marginal effects on lysozyme denaturation. The observed enhancement of aggregation suggests that AT and Cap most likely bind to protein and enhance aggregation.

Table 1 Denaturation temperature of proteins in the presence and absence of 3 mM AT or 3 mM Cap

protein		HSA		BSA		lysozyme		ovalbumin	
denaturation temperature		T_m (°C)	ΔT_m (°C)	T_m (°C)	ΔT_m (°C)	T_m (°C)	ΔT_m (°C)	T_m (°C)	ΔT_m (°C)
no additive	(measured at 238 nm)	61.2	—	60.3	—	73.7	—	74.4	—
3 mM AT	(at 238 nm)	69.3	8.1	66.2	5.9	70.9	-2.8	75.1	3.9
no additive	(at 224 nm)	62.0	—	61.6	—	73.0	—	73.8	—
3 mM Cap	(at 224 nm)	77.4	15.4	77.8	16.1	72.1	-0.9	73.5	-0.3

T_m shows the denaturation temperature, while ΔT_m shows the difference in the denaturation temperature in the absence and presence of additive.

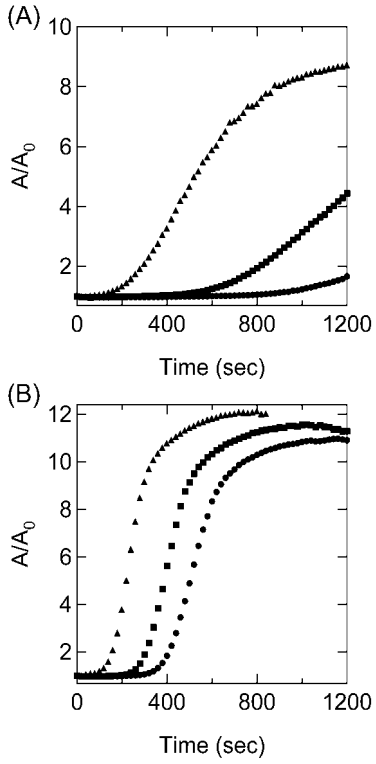

Fig. 2 Thermal aggregation of lysozyme with no additives (circles), 20 mM AT (squares), and 20 mM Cap (triangles). Samples containing 1.0 mg/ml lysozyme and 20 mM phosphate buffer prepared at pH 7.0 and heated to 75°C (A) and 90°C (B). A and A_0 show the measured and the starting absorbance at 400 nm, respectively.

Fig. 3 shows turbidity increase of BGG at 70°C (A), 80°C (B), and 90°C (C). At 70°C, the turbidity increase occurred at ~200 s followed by a sharp increase in the absence of the additives (circles). In the presence of 20 mM AT (squares), the turbidity increase was significantly reduced, although the onset time appeared to be unchanged. Cap at 20 mM further reduced the rate of turbidity increase (triangles). These results demonstrate that both AT and Cap can suppress the aggregation of BGG, Cap being more effective. At 80°C (Fig. 3B), there appeared to be a small delay in the onset of turbidity increase by 20 mM AT (squares) and 20 mM Cap (triangles) compared to their absence, although the rates appeared to be similar with and without AT or Cap. At 90°C (Fig. 3C), three curves overlapped on top of each other. It thus appears that AT and Cap can exert suppressive effects on aggregation of BGG at lower temperatures, at which aggregation is slower, but lose their effectiveness when aggregation becomes faster at 90°C. Because BGG is a mixture of widely different antibodies, its melting properties could not be measured. The observed aggregation suppression of BGG by AT and Cap may be due to their binding to BGG in different way than they bind to lysozyme, whose thermal aggregation was enhanced. They may bind to BGG, so that they mask the hydrophobic regions of BGG and thereby suppress its aggregation. Since the effects at 20 mM are rather weak in particular at higher temperatures, it would be of great interest to see if higher AT and Cap concentrations can increase the aggregation suppressive effects. This could not be done, however, due to the solubility limit of AT. In any case, the results suggest that binding of AT and Cap to BGG is weak, in the order of mM range.

3.3 Protein stabilizing or destabilizing effect of AT and Cap in the presence of ArgHCl

We demonstrated above that AT and Cap can enhance the stability of HSA and BSA and can suppress the aggregation of BGG. Synergistic effects of AT and Cap with ArgHCl, a well-known aggregation suppressor [11–13], were then examined. Table 2 shows the effects of 100 mM ArgHCl on the T_m of HSA and BSA. It slightly increased the T_m by about 4°C for both proteins, far less than the increase observed for 3 mM AT and Cap. When

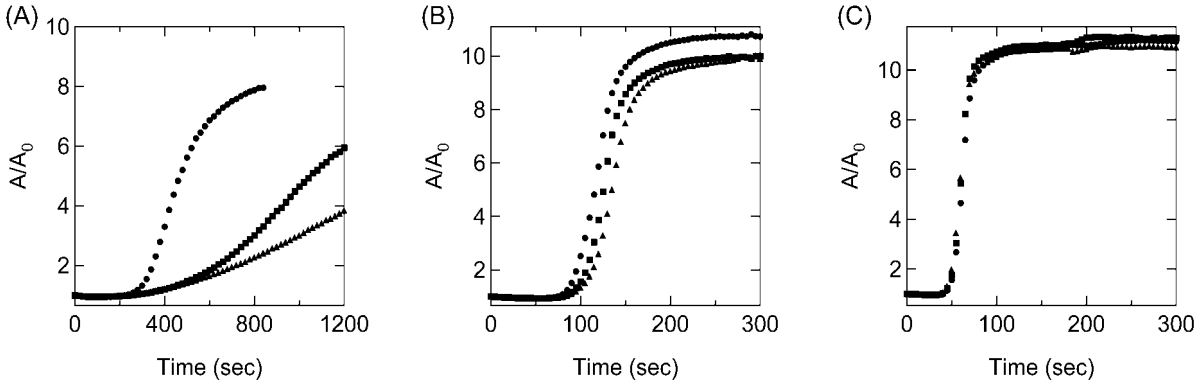

Fig. 3 Thermal aggregation of BGG with no additives (circles), 20 mM AT (squares), and 20 mM Cap (triangles). Samples containing 1.0 mg/ml BGG and 20 mM phosphate buffer prepared at pH 7.0 and heated at 70°C (A), 80°C (B), and 90°C (C). A and A_0 show the measured and starting absorbances at 400 nm, respectively.

100 mM ArgHCl was combined with 3 mM AT, the T_m was slightly changed from the value at 3 mM AT, indicating no effects of ArgHCl. An identical trend was observed for Cap. That is, the addition of 100 mM

Table 2 Denaturation temperature of serum albumin in the presence and absence of 3 mM AT, 3 mM Cap, or 0.1 M ArgHCl

Protein	HSA		BSA	
Denaturation temperature	T_m (°C)	ΔT_m (°C)	T_m (°C)	ΔT_m (°C)
No additive	61.2	—	60.3	—
100 mM ArgHCl	65.6	4.4	64.6	4.3
3 mM AT	69.3	8.1	66.2	5.9
3 mM Cap	81.1	19.9	79.3	19.0
100 mM ArgHCl + 3 mM AT	70.1	8.9	67.5	7.2
100 mM ArgHCl + 3 mM Cap	79.0	17.8	78.4	18.1

T_m shows the denaturation temperature, while ΔT_m shows the difference in the denaturation temperature in the absence and presence of additive.

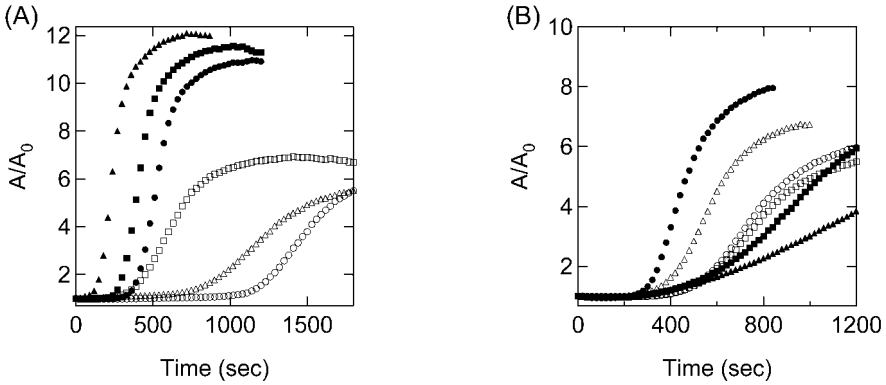

Fig. 4 Thermal aggregation of lysozyme heated at 90°C (A) and BGG heated at 70°C (B) with no additives (closed circles), 20 mM AT (closed squares), 20 mM Cap (closed triangles), 100 mM ArgHCl (open circles), 100 mM ArgHCl + 20 mM AT (open squares), and 100 mM ArgHCl + 20 mM Cap (open triangles). Samples containing 1.0 mg/ml lysozyme or BGG and 20 mM phosphate buffer prepared at pH 7.0. A and A_0 show the measured and starting absorbance at 400 nm, respectively.

ArgHCl slightly changed the T_m of BGG in the presence of 3 mM Cap.

Then, the synergistic effects of 100 mM ArgHCl on lysozyme and BGG aggregation were examined. Fig. 4A shows the results for lysozyme. When heated at 90°C, 100 mM ArgHCl greatly delayed the onset of turbidity increase (open circles vs closed circles). The rate of turbidity increase was also slower in 100 mM ArgHCl. This suppressive effect of ArgHCl was reduced by 20 mM AT (open squares) and 20 mM Cap (open triangles), reflecting the effects of AT or Cap by itself, which was to enhance the aggregation of lysozyme.

Similarly, 100 mM ArgHCl significantly reduced turbidity increase of BGG when heated at 70°C, as shown in Fig. 4B. That is, the onset of turbidity increase was delayed by 100 mM ArgHCl (open circles) relative to the control (closed circles). AT (closed squares) at 20 mM also similarly suppressed the turbidity increase. However, when these two additives were combined, the aggregation suppression was similar to 100 mM ArgHCl alone or 20 mM AT alone (see open squares). Thus, it is clear that there is no synergy between ArgHCl and AT. On the contrary, the suppressive effects of 100 mM ArgHCl or 20 mM Cap were largely lost when they were combined. As described earlier, 20 mM Cap showed strong effects on BGG aggregation (see closed triangles). When combined with 100 mM ArgHCl, the turbidity increase was close to that of the control (see open triangles). It thus appears that ArgHCl and Cap suppress the effect of the other additives. Although this observation cannot be readily explained, it might be that ArgHCl influences the binding of Cap to BGG or alters the way Cap binds to the protein.

4. Conclusion

This study showed the effect of AT and Cap as additives on the thermal stability of proteins. We successfully concluded as follows: 1) AT and Cap highly increased the thermal stability of human and bovine albumins; 2) AT and Cap suppressed the heat-induced aggregation of bovine γ-globulin as well as albumins; 3) In contrast, AT and Cap rather accelerated the hen egg-white lysozyme; and 4) The synergistic effect of AT and Cap with ArgHCl, the well-known aggregation suppressor, was not observed toward heat-induced aggregation of γ-globulin and lysozyme. These findings of AT and Cap as additives in protein solution improve the protein stability toward the thermal stresses, which may use the storage additive in ambient temperature.

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