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Amidated Amino Acids Are Prominent Additives for Preventing Heat-Induced Aggregation of Lysozyme

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An additive that is highly effective in small amounts for controlling protein inactivation and aggregation has long been demanded. In this paper we show amidated amino acids as new potent additives. In the presence of 100 mM amidated amino acids, *e.g.*, Ala, Arg, Asn, Met, and Val, the heat-induced inactivation and aggregation of lysozyme at pH 7.1 are one order of magnitude slower than those in the absence of additives. Although a high Arg concentration (>1 M) has been used to prevent aggregation among amino acids, it is worth mentioning that above amidated amino acids can prevent aggregation at submolar concentrations. The data obtained suggest the importance of amino and amide groups rather than the guanidium group as an aggregation suppressor.

[**Key words:** lysozyme, amidated amino acids, protein aggregation, thermal inactivation]

Aggregation is an intrinsic phenomenon for polypeptide chain. The control of aggregation must be achieved inexpensively and easily for biotechnological and medical applications of valuable proteins. To reduce aggregation *in vitro*, various factors have to be tested, such as pH, ionic strength, temperature, and protein concentration. A simple but effective approach to improving the aggregation problem is the addition of a small amount of potent inhibitor to prevent protein aggregation.

Many types of additives for reducing protein aggregation have been developed. Protein-denaturing reagents, typically guanidine and detergents, have been used as an aggregation suppressor that weakens the hydrophobic intermolecular interaction of proteins (1–4). However, these additives ambivalently decrease the stability of proteins, which sometimes accelerates aggregation. A compound synthesized through refolding in detergent followed by cycroamylose addition has been developed to function as an artificial chaperone (5). Although non-denaturing reagents, such as amino acids (6), have been used to preserve protein solution, their use is not sufficient to solve the problems of protein aggregation. Of those amino acids, arginine (Arg) possesses a favorable property as an additive for the prevention and dissolvation of aggregation; that is, it does not destabilize the native structure and has only a minor effect on protein stability while it enhancing the solubility of aggregation-prone molecules during refolding (6–13).

Recently, we have reported that polyamines, specifically spermine and spermidine, prevent the heat-induced inactivation and aggregation of lysozyme more effectively than Arg. Polyamines slightly destabilize the native structure of lysozyme but it markedly increases the solubility of aggregation-prone molecules (13). The addition of a low concentration of polyamines (typically <0.1 M) markedly prevents the heat-induced aggregation of what as effective as that of 1 M Arg or higher. The indispensable feature in the structure of polyamines for their function as an aggregation suppressor is the presence of multiple amines (14). Arginine ethylester (ArgEE) is a more favorable additive for suppressing the heat-induced aggregation of lysozyme than Arg (15). Although Arg is not effective at concentrations below 1 M, ArgEE is effective at concentrations one order of magnitude lower than that of Arg. Furthermore, several amino acid derivatives similarly prevent the heat-induced aggregation of lysozyme as effective as ArgEE (16). Although amino acid alkylesters are promising candidate for preventing protein aggregation, these additives may be hydrolyzed to alcohols and amino acids in an aqueous solution. Therefore, amino acid alkylesters are not favorable for practical applications that entail long-time storage. In this paper, we study a new class of amino acid derivatives, that is, amidated amino acids, as promising aggregation suppressors.

MATERIALS AND METHODS

Materials Hen egg white lysozyme, Arg/HCl, ArgEE/2HCl, ArgAd/2HCl, AlaAd/HCl, GluAd, ValAd/HCl, ProAd/HCl, MetAd/HCl, and AsnAd/HCl were purchased from Sigma Chemical (St. Louis, MO, USA). Na_2HPO_4, and NaH_2PO_4 were purchased from Nacalai Tesque (Kyoto). Ala, Glu, CH_3COONa, and *Micrococcus lysodeikticus* were purchased from Wako Pure Chemical Industries (Osaka). β-AlaAd was purchased from Tokyo Kasei Kogyo (Tokyo). All the chemicals used were of high-quality analytical grade.

Inactivation and aggregation The heat-induced inactivation and aggregation of lysozyme was performed as follows (15, 16): A stock solution containing 1.0 mg/ml lysozyme, 50 mM Na–phosphate buffer, and 100 mM additives was prepared and adjusted to

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pH 7.1 by adding NaOH or HCl. A 200 μl aliquot of the stock solution was taken and added to each microtube. Every solution in the each microtube was heated from 25°C to 98°C at 1°C/s, then continuously heated for various periods. After the heat treatment, all the samples were stored at 25°C for 20 min. These processes were controlled by a temperature control system, PC-880 (Astec, Fukuoka). After the process, the samples were centrifuged at 15,000×*g* for 20 min at 25°C, and then the concentrations of soluble protein and residual what activity were measured.

Protein concentration and residual what activity The concentration of soluble protein was estimated by measuring the absorbance at 280 nm using an ND-1000 UV-vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The residual activity of the soluble fraction was determined as follows. A total of 1.5 ml of 0.5 mg/ml *M. lysodeikticus* solution in 50 mM Na-phosphate buffer (pH 7.1) was mixed with 10 μl of the protein solution. The decrease in the light-scattering intensity of the solution was monitored by measuring the absorbance at 600 nm for 60 s using a Jasco spectrophotometer model V-550 (Japan Spectroscopic, Tokyo). The decreasing absorbance between 10 to 20 s was fitted to a linear extrapolation, and then the residual activity was estimated from the slope of the line.

Circular Dichroism The thermal unfolding of lysozyme in the presence of additives was measured by circular dichroism (CD), with a Jasco spectropolarimeter model J-720W. Samples containing 1.0 mg/ml lysozyme, 100 mM additive, and 50 mM Na-acetate buffer (pH 4.5) were prepared to prevent aggregation. As these samples could not be measured by far-UV CD due to the molar ellipticity of additives, the thermal unfolding was estimated by measuring the intensity change in positive CD band at 288.5 nm at an increasing temperature rate of 0.5°C/min. The obtained data at pH 4.5 were fitted to a two-state equation and the apparent midpoint of temperature (T_m) was determined from the change in the molar ellipticity.

RESULTS AND DISCUSSION

We previously showed that amino acid alkylesters markedly prevent the heat-induced inactivation and aggregation of lysozyme (15, 16). However, these alkylesters are prone to hydrolysis in aqueous solutions by heat. Although ArgEE is a prominent additive for preventing protein aggregation, ArgEE is too labile to be used for biotechnological applications, such as those to protein crystallization and protein solution storage without freezing. In this paper, we explore further additives, that is, amidated amino acids, for preventing aggregation for biotechnological usage. We assume that the amidation of the carboxyl group on amino acids make better additives as aggregation suppressors because amidated amino acids have the combined features of an increased number of amino ends (13, 14), and the presence of a modified carboxyl end (15, 16).

Figure 1 shows typical profiles of the heat-induced inactivation and aggregation of lysozyme in the presence or absence of additives. Lysozyme was inactivated by first-order kinetics in the absence of additives; however, in the presence of 100 mM Arg, the aggregation rate was decelerated (Fig. 1A). In the presence of arginine amide (ArdAd), the aggregation was markedly prevented (Fig. 1A). The residual activity profiles were similar to those of aggregation (Fig. 1B). The heat-induced inactivation of lysozyme was slightly decelerated in the presence of 100 mM Arg. However, the presence of 100 mM ArgAd markedly prevented

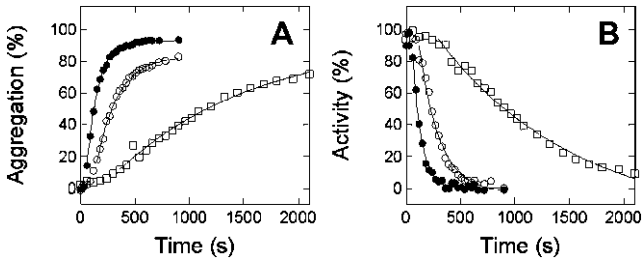

FIG. 1. Heat-induced inactivation and aggregation of lysozyme with addition of 100 mM additives. The samples containing 1.0 mg/ml lysozyme in the absence (closed circles) or presence of Arg (open circles) and ArgAd (open squares) were heated at 98°C for various periods. After the heat treatment, the percentage of aggregates (A) and residual what activity (B) were determined. The curves shown by the solid line were fitted to single exponential equation.

heat-induced inactivation. The heat-induced inactivation and aggregation of lysozyme were measured in the absence or presence of Arg, Ala, Glu, and the amide derivatives tested, and the rate constants for inactivation and aggregation are listed in Table 1. In the absence of additives, the inactivation and aggregation rates were 12.4×10^{-3} s^{-1} and 9.7×10^{-3} s^{-1}, respectively, at 1.0 mg/ml lysozyme. A high protein concentration accelerated the inactivation and aggregation, indicating that the process is an intermolecular phenomenon. The difference in rate between inactivation and aggregation indicates that the soluble fraction contains non-native molecules. In the presence of amino acids, the inactivation and aggregation rates slightly decreased compared with that in the ab-

TABLE 1. Kinetic rate constants of inactivation and aggregation of lysozyme in the presence of 100 mM additives

Protein concentration	Additive	Inactivation ($\times10^{-3}$ s^{-1})	Aggregation ($\times10^{-3}$ s^{-1})
1.0 mg/ml	None	12.4±0.5	9.7±0.2
	Arg	6.3±0.3	5.1±0.2
	Ala	9.4±0.4	7.7±0.3
	Glu	7.2±0.3	6.2±0.2
	ArgAd	0.8±0.1	0.8±0.1
	AlaAd	2.3±0.1	1.8±0.1
	GluAd	3.5±0.2	2.6±0.1
	ValAd	0.5±0.1	0.4±0.1
	β-AlaAd	5.5±0.2	4.5±0.1
	ProAd	3.8±0.2	2.8±0.2
	AsnAd	0.4±0.1	0.2±0.1
	MetAd	0.1±0.1	0.2±0.1
	ArgEE	0.6±0.2	0.4±0.1
5.0 mg/ml	None	16.4±0.9	16.4±0.6
	Arg	10.5±0.7	8.2±0.4
	Ala	13.8±1.4	13.7±0.6
	Glu	13.0±0.9	11.3±0.7
	ArgAd	2.3±0.2	2.1±0.1
	AlaAd	4.1±0.2	3.7±0.1
	GluAd	5.5±0.3	5.4±0.1
	ValAd	1.7±0.2	1.6±0.1
	β-AlaAd	10.3±0.5	8.7±0.3
	ProAd	7.1±0.4	6.5±0.2
	AsnAd	2.3±0.2	1.6±0.2
	MetAd	0.3±0.1	0.7±0.1
	ArgEE	1.9±0.1	1.5±0.2

The rate constants of first-order kinetics were determined as described in Fig. 1. The standard deviations are estimated from triplicate experiments.