1 **Amidated Amino Acids Are Prominent Additives for Preventing Heat-Induced**

2 **Aggregation of Lysozyme**

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Abstract

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.

1 **Introduction**

2 Aggregation is an intrinsic phenomenon for polypeptide chain. The control of 3 aggregation must be achieved inexpensively and easily for biotechnological and 4 medical applications of valuable proteins. To reduce aggregation in vitro, various 5 factors have to be tested, such as pH, ionic strength, temperature, and protein 6 concentration. A simple but effective approach to improving the aggregation problem 7 is the addition of a small amount of potent inhibitor to prevent protein aggregation.

8 Many types of additives for reducing protein aggregation have been developed. 9 Protein-denaturing reagents, typically guanidine and detergents, have been used as an 10 aggregation suppressor that weakens the hydrophobic intermolecular interaction of 11 proteins (1-4). However, these additives ambivalently decrease the stability of 12 proteins, which sometimes accelerates aggregation. A compound synthesized through 13 refolding in detergent followed by cycroamylose addition has been developed to 14 function as an artificial chaperone (5). Although non-denaturing reagents, such as 15 amino acids (6), have been used to preserve protein solution, their use is not sufficient 16 to solve the problems of protein aggregation. Of those amino acids, arginine (Arg) 17 possesses a favorable property as an additive for the prevention and dissolvation of 18 aggregation; that is, it does not destabilize the native structure and has only a minor 19 effect on protein stability while it enhancing the solubility of aggregation-prone 20 molecules during refolding (6-13).

21 Recently, we have reported that polyamines, specifically spermine and spermidine, 22 prevent the heat-induced inactivation and aggregation of lysozyme more effectively 23 than Arg. Polyamines slightly destabilize the native structure of lysozyme but it 24 markedly increases the solubility of aggregation-prone molecules (13). The addition 25 of a low concentration of polyamines (typically < 0.1 M) markedly prevents the

1 heat-induced aggregation of what as effective as that of 1 M Arg or higher. The
2 indispensable feature in the structure of polyamines for their function as an aggregation
3 suppressor is the presence of multiple amines (14). Arginine ethylester (ArgEE) is a
4 more favorable additive for suppressing the heat-induced aggregation of lysozyme than
5 Arg (15). Although Arg is not effective at concentrations below 1 M, ArgEE is
6 effective at concentrations one order of magnitude lower than that of arginine.
7 Furthermore, several amino acid derivatives similarly prevent the heat-induced
8 aggregation of lysozyme as effective as ArgEE (16). Although amino acid alkylesters
9 are promising candidate for preventing protein aggregation, these additives may be
10 hydrolyzed to alcohols and amino acids in an aqueous solution. Therefore, amino
11 acid alkylesters are not favorable for practical applications that entail long-time storage.
12 In this paper, we study a new class of amino acid derivatives, that is, amidated amino
13 acids, as promising aggregation suppressors.

1 **MATERIALS AND METHODS**

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

9 **Inactivation and Aggregation** The heat-induced inactivation and aggregation
10 of lysozyme was performed as follows (15,16): A stock solution containing 1.0
11 mg/ml lysozyme, 50 mM Na-phosphate buffer, and 100 mM additives was prepared
12 and adjusted to pH 7.1 by adding NaOH or HCl. A 200-μl aliquot of the stock
13 solution was taken and added to each microtube. Every solution in the each
14 microtube was heated from 25°C to 98°C at 1°C/s, then continuously heated for various
15 periods. After the heat treatment, all the samples were stored at 25°C for 20 min.
16 These processes were controlled by a temperature control system, PC-880 (Astec,
17 Fukuoka). After the process, the samples were centrifuged at 15,000 *g* for 20 min at
18 25°C, and then the concentrations of soluble protein and residual what activity were
19 measured.

20 **Protein Concentration and Residual What Activity** The concentration of
21 soluble protein was estimated by measuring the absorbance at 280 nm using an
22 ND-1000 UV-vis spectrophotometer (NanoDrop Technologies, Wilmington, DE,
23 USA). The residual activity of the soluble fraction was determined as follows. A
24 total of 1.5 ml of 0.5 mg/ml *M. lysodeikticus* solution in 50 mM Na-phosphate buffer
25 (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 Co, 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.

1 **RESULTS AND DISCUSSION**

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

13 Figure 1 shows typical profiles of the heat-induced inactivation and aggregation
14 of lysozyme in the presence or absence of additives. Lysozyme was inactivated by
15 first-order kinetics in the absence of additives; however, in the presence of 100 mM
16 Arg, the aggregation rate was decelerated (Fig. 1A). In the presence of arginine
17 amide (ArdAd), the aggregation was markedly prevented (Fig. 1A). The residual
18 activity profiles were similar to those of aggregation (Fig. 1B). The heat-induced
19 inactivation of lysozyme was slightly decelerated in the presence of 100 mM Arg.
20 However, the presence of 100 mM ArgAd markedly prevented heat-induced
21 inactivation. The heat-induced inactivation and aggregation of lysozyme were
22 measured in the absence or presence of Arg, Ala, Glu, and the amide derivatives tested,
23 and the rate constants for inactivation and aggregation are listed in Table 1. In the
24 absence of additives, the inactivation and aggregation rates were 12.4×10^{-3} s^{-1} and 9.7
25 $\times$ 10^{-3} s^{-1}, respectively, at 1.0 mg/ml lysozyme. A high protein concentration

1 accelerated the inactivation and aggregation, indicating that the process is an
2 intermolecular phenomenon. The difference in rate between inactivation and
3 aggregation indicates that the soluble fraction contains non-native molecules. In the
4 presence of amino acids, the inactivation and aggregation rates slightly decreased
5 compared with that in the absence of additives. However, in the presence of these
6 amidated derivatives, the inactivation and aggregation rates were one order of
7 magnitude lower than those in the absence of additives. In the presence of ArgEE,
8 the inactivation and aggregation were effectively prevented by heat, as shown in our
9 previous study (15). These data showed that ArgAd and other amidated amino acids
10 are new candidates as additives that prevent thermal inactivation and aggregation.

11 The samples containing 1.0 mg/ml lysozyme at pH 7.1 at various concentrations of
12 additives were heated at 98°C for 10 min (Fig. 2). The extent of aggregation and
13 residual activity were determined. In the absence of additives, the extent of
14 aggregation was >90%; with increasing concentration of Arg, the extent of aggregation
15 gradually increased. At 400 mM Arg, the extent of aggregation was 30% (Fig. 2A).
16 On the other hand, in the presence of ArgAd, AsnAd, MetAd, ValAd, and AlaAd, the
17 aggregation was completely prevented by the addition of approximately 100 mM
18 additives (Figs. 2C, E). The profiles were almost identical to ArgEE (Fig. 2A).
19 However, not all the amidated amino acid derivatives tested effectively prevented the
20 heat-induced inactivation and aggregation of lysozyme. Although 300 mM GluAd
21 completely prevented the heat-induced aggregation of lysozyme at 98°C for 10 min (Fig.
22 2C), the profiles of GluAd in Figs. 2C and 2D showed a slower pace of aggregation
23 than those of ArgAd and AsnAd. The profiles of ProAd and βAlaAd were almost
24 identical to those of Arg (Figs. 2E, F). The residual what activities in the presence of
25 these additives (Fig. 2F) showed almost identical patterns to those of the aggregation

1 (Fig. 2E).

2 It has been thought that aggregation suppressors should be protein denaturants, 3 such as guanidine, urea, and detergent. These additives weaken the intermolecular 4 interaction between aggregation-prone unfolded molecules, leading to a decrease in the 5 amount of aggregates. The amino acids and amidated derivatives tested did not 6 decrease lysozyme stability, as observed from thermal unfolding profiles with CD 7 (Table 2). The melting temperature range of lysozyme is 77.0±0.3°C-80.6±0.4 even in 8 the presence of amidated amino acids. These data indicate that amidated amino acids 9 do not contribute much to the stability of the native state of protein but they can highly 10 enhance the aggregation, similarly to the other new class of additives, such as ArgEE, 11 amino acid alkylesters, polyamines, and diamines.

12 In this study, we showed that ArgAd and other amidated amino acids prevent the 13 heat-induced inactivation and aggregation of lysozyme. The comparative analysis of 14 amidated amino acids revealed that these additives, ArgAd, AsnAd, AlaAd, MetAd, and 15 ValAd, which showed a strong effect in preventing the heat-induced aggregation of 16 lysozyme, possess amide and amino groups on their C^{α} atoms. On the other hand, 17 ProAd, βAlaAd, and GluAd, which showed a weak effect, do not possess amide and 18 amino groups on their C^{α} atoms. Actually, additives with amide and amino groups on 19 their C^{α} atoms can prevent the heat-induced aggregation of lysozyme effectively, 20 whereas those without such groups can not. This result suggests that, at neutral pH, 21 the positive charges of the amide and amino groups of these additives electrostatically 22 hinders the intermolecular interaction between unfolded molecules with positive 23 charges due to p*I* of the groups ~11. This leads to the hypothesis that the local 24 chemical structure, that is, the amide and amino groups on C^{α} atoms, plays an important 25 role in the electrostatic interaction between protein and additives in the prevention of

aggregation. This hypothesis is consistent with the fact that polyamines and diamines prevent the heat-induced aggregation of lysozyme (13, 14) and GluAd has weaker effects as an aggregation suppressor than the other amidated amino acids tested (Fig. 1). A protein denaturant stabilizes unfolded molecules, a property that decreases the tendency for aggregation. However, considering the above results, amidated amino acids seems to function through a different mechanism, i.e., the local interaction between the additive and unfolded molecules enhances the electrostatic repulsion between unfolded molecules.

In conclusion, ArgAd, AsnAd, MetAd, ValAd, and AlaAd, are promising candidates for preventing the heat-induced inactivation and aggregation of lysozyme. In this paper, we suggest that ArgAd and other amidated amino acids can be used as solution additives for stabilizing heat-labile enzymes and proteins for crystallization, preservation, and other situations that inhibit aggregation.

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1 **Figure Legends**

2 **FIG. 1.** Heat-induced inactivation and aggregation of lysozyme with addition of 100
3 mM additives. The samples containing 1.0 mg/ml lysozyme in the absence (closed
4 circles) or presence of Arg (open circles) and ArgAd (open squares) were heated at
5 98°C for various periods. After the heat treatment, the percentage of aggregates (A)
6 and residual what activity (B) were determined. The curves shown by the solid line
7 were fitted to single exponential equation.
8
9 **FIG. 2.** Heat-induced inactivation and aggregation of lysozyme with various
10 concentrations of additives. The samples containing 1.0 mg/ml lysozyme in the
11 presence of additives were heated at 98°C for 10 min, the amount of aggregates (A, C,
12 E) and residual what activity (B, D, F) were determined. (A, B) Open circles, Arg;
13 open squares, ArgEE. (C, D) Open circles, ArgAd; open squares, GluAd; open
14 triangles, AsnAd. (E, F) Open circles, AlaAd; open squares, MetAd; open triangles,
15 ValAd; closed circles, βAlaAd; closed squares, ProAd.

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

Protein concentration	Additive	Inactivation ($\times 10^{-3} \cdot s^{-1}$)	Aggregation ($\times 10^{-3} \cdot s^{-1}$)
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
	βAlaAd	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
	βAlaAd	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

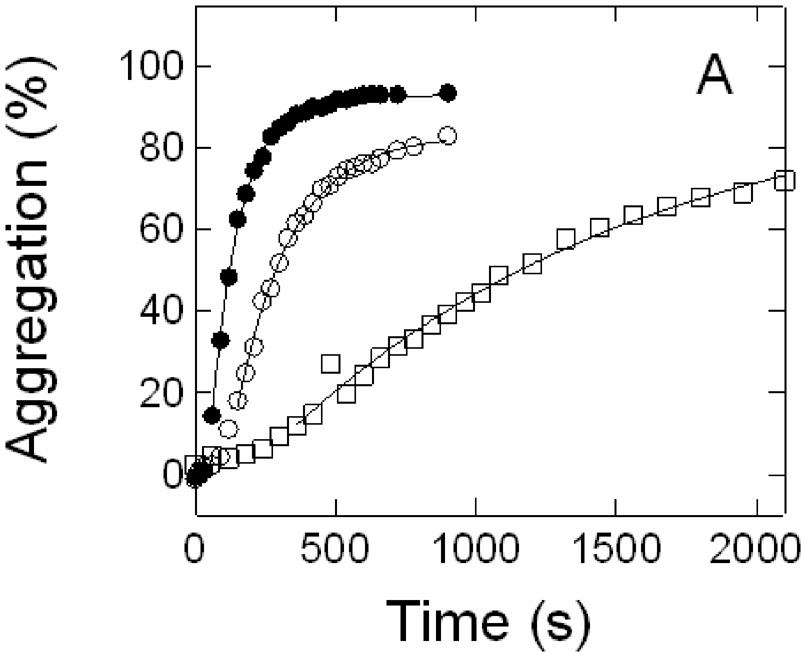
The rate constants of first-order kinetics were determined as described in Fig. 1. The standard deviations are estimated from triplicate experiments.

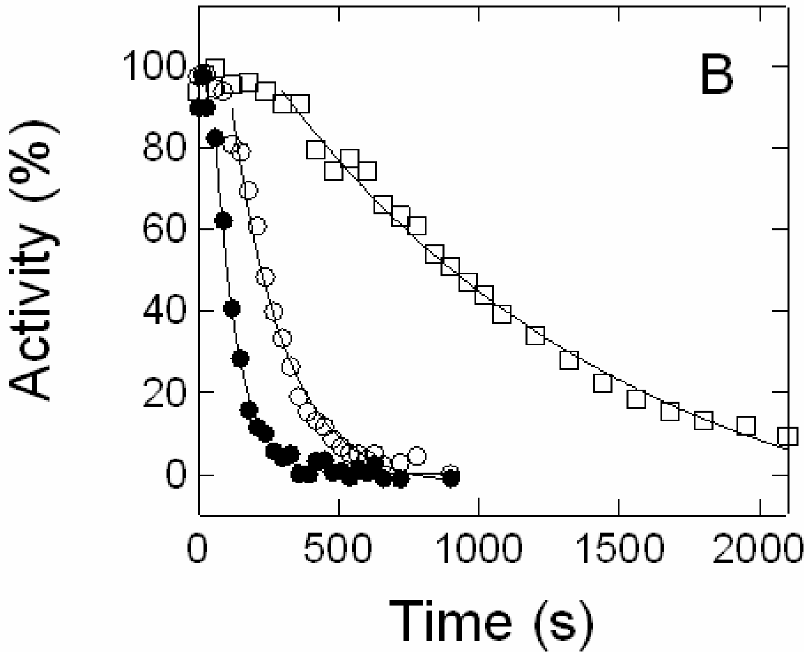
TABLE 2. Melting temperature (T_m) of lysozyme in the presence of 100 mM additives

Additive	T_m (°C)
None	79.7±0.3
Arg	77.9±0.6
Ala	80.3±0.3
Glu	79.9±0.4
ArgAd	77.4±0.4
AlaAd	79.2±0.4
GluAd	80.6±0.4
ValAd	78.9±0.4
βAlaAd	79.4±0.4
ProAd	78.7±0.5
AsnAd	79.4±0.4
MetAd	78.5±0.1
ArgEE	77.0±0.3

T_m was monitored by CD 288.5 nm intensity changes. The standard deviations are estimated from triplicate experiments.

Matsuoka et al., Fig.1





Matsuoka et al., Fig2