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1 # L-Argininamide improves the refolding more effectively than
2 L-arginine

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Abstract

L-Arginine (Arg) is a widely used additive for suppressing protein aggregation during refolding. Systematic screening of Arg analogs provides superior additives that enhance the refolding yield more effectively than Arg. The refolding yield of hen egg lysozyme in the presence of 500 mM L-argininamide (ArgAd) increases 1.7-fold higher than Arg. Thermal unfolding experiments indicate that ArgAd has a greater denaturing effect than Arg, which positively relates to the net charge of Arg analogs. Moreover ArgAd was also effective for the refolding of bovine carbonic anhydrase. High potency to increase the refolding yield of ArgAd compared to Arg results from high positive net charge and the denaturing property.

Keywords: L-argininamide; L-arginine; aggregation; lysozyme; refolding; renaturation

1. Introduction

Protein aggregation during refolding is a serious problem in biotechnology. The high-level expression of heterologous protein in prokaryotic and eukaryotic hosts frequently leads to the formation of insoluble aggregates, referred to as inclusion bodies (Marston 1986; Lilie et al. 1998; Villaverde and Carrió 2003). In order for the recovery of biological active protein, solubilization of the inclusion bodies with a denaturing agent such as urea or guanidine (Gdn) and subsequent *in vitro* refolding by dialysis or direct dilution are required (Fischer 1994; Rudolph and Lilie 1996). However, the refolding yield of reactivation is usually lowered because correct folding *in vitro* competes with unproductive side reactions, e.g., the formation of misfolded species and the aggregation of denatured protein (Zettlmeissl et al. 1979; Goldberg et al. 1991; Kiefhaber et al. 1991).

During the refolding reaction, the hydrophobic interaction drives the unfolded protein to sequester their hydrophobic patches from water when the denaturant is removed (Dill 1990). The difference between protein folding and aggregation is described as an intramolecular reaction (folding) or an intermolecular reaction (aggregation) by the following reactions:

$$U \rightarrow N \qquad (1a)$$

$$U + U \rightarrow A_2 \qquad (1b)$$

where U, N, and A_2 represent the unfolded protein, native protein, and a dimer, respectively (Baynes et al. 2005). The simple model suggests that the marginal balance from the unfolded state to the native structure or aggregates affects the refolding yields of unfolded proteins.

In order to improve the refolding yield, many types of additives have been used in the refolding buffer. There are two types of refolding additives, folding enhancers and aggregation suppressors (Tsumoto et al. 2003). The former is ammonium sulfate, polyols, sugars, and

certain amino acids such as glycine and proline (Maeda et al. 1996; Kumar et al. 1998; Samuel et al. 2000; Meng et al. 2001; Ou et al. 2002; Mishra et al. 2005). The folding enhancer stabilizes the native structure and enhances intermolecular interactions. The latter includes denaturants such as urea and Gdn (Orsini and Goldberg 1978), mild detergents (Zardeneta and Horowitz 1994; Wetlaufer and Xie 1995), polyethylene glycols (Cleland and Wang 1990; Cleland et al. 1992), and organic acids (Yang et al. 1996). Aggregation suppressors inhibit the folding reaction due to destabilization of the native structure. In most cases, these additives improve the refolding yield due to the deceleration of aggregation by weakening the intermolecular hydrophobic interactions.

L-Arginine (Arg) has the most basic side chain that possesses a similarity to Gdn. Arg has been found to increase the refolding yields of human tissue type plasminogen activator (Rudolph and Fischer 1990). Thereafter, Arg has been used for refolding various proteins, such as F_{ab} antibody fragments (Buchner and Rudolph 1991), single-chain immunotoxins (Buchner et al. 1992; Brinkmann et al. 1992), interleukin-6 receptor (Stoyan et al. 1993), interleukin-21 (Asano et al. 2002), human matrix metalloproteinase-7 (Oneda and Inouye 1999), and recombinant human neurotrophins (Suenaga et al. 1998; Rattenholl et al. 2001). Arg consists of four functional groups, i.e., guanidino-, amino-, carboxy-, and methylene-groups. Although Arg contains a guanidino group, it has only a minor effect on the thermodynamic stability of folded proteins (Taneja and Ahmad 1994; Lin and Timasheff 1996; Shiraki et al. 2002; Arakawa and Tsumoto 2003; Reddy et al. 2005). Arg does not accelerate the refolding kinetics, but increase the solubility of aggregate-prone molecules (Hevehan and De Bernardez Clark 1997; Reddy et al. 2005). Arg is the most effective suppressor for heat-induced aggregation among natural amino acids (Shiraki et al. 2002). In addition to the inexpensive and nontoxic

properties of Arg, it is expected to act as a solution additive in long term storage and affinity column chromatography (Arakawa et al. 2003, 2004; Ejima et al. 2005a,b). In spite of wide usage of Arg as an aggregation suppressor, a mechanistic explanation of the effects of Arg on protein refolding is rarely provided.

The aim of this study was to find a better additive among Arg analogs. We used hen egg white lysozyme as the model protein (Epstein and Goldberger 1963; Saxena and Wetlaufer 1970; Rudolph and Fischer 1990; Goldberg et al. 1991; Fischer et al. 1992, 1993). The results of this study show that L-argininamide (ArgAd) is more effective for oxidative refolding of lysozyme than Arg. Moreover ArgAd was effective for the refolding of bovine carbonic anhydrase (CA) which has lower pI than lysozyme. As discussed below, comparative analysis of additives provides information requiring superior refolding additives.

1 **2. Materials and methods**

2 *2.1. Materials*

3 Hen egg white lysozyme, Bovine carbonic anhydrase (CA),
4 ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), *p*-nitrophenyl acetate
5 (*p*-NPA), L-arginine hydrochloride (Arg), L-homoarginine hydrochloride (HArg),
6 L-2-amino-3-guanidinopropionic acid hydrochloride (AGPA), L-arginine ethylester
7 dihydrochloride (ArgEE), L-argininamide dihydrochloride (ArgAd), L-citrulline (Cit),
8 L-ornithine hydrochloride (Orn), guanidinopropionic acid (GPA), glycine (Gly), and
9 glycinamide hydrochloride (GlyAd) were purchased from Sigma (St. Louis, MO, USA).
10 Guanidine hydrochloride (Gdn), *Micrococcus lysodeikticus*, dithiothreitol (DTT), and oxidized
11 and reduced glutathione (GSSG and GSH) were purchased from Wako Pure Chemical
12 Industries (Osaka, Japan). Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) and
13 sodium phosphate were purchased from Nacalai Tesque (Kyoto, Japan). All the compounds
14 were of the highest grade available commercially.

15

16 *2.2. Protein concentration*

17 Protein concentration was determined photometrically by measuring the absorbance
18 at 280 nm with an appropriate blank, using extinction coefficients of 2.63 mL mg^{-1} cm^{-1} for
19 native and 2.37 mL mg^{-1} cm^{-1} for denatured lysozyme (Saxena and Wetlaufer 1970) and 1.83
20 mL mg^{-1} cm^{-1} for CA (Pocker and Stone 1967). Absorbance was measured with a UV-Vis
21 spectrophotometer model ND-1000 (NanoDrop Technologies, Inc., Wilmington, DE, USA).

22

23 *2.3. Preparation of denatured protein*

1 Lysozyme at 16-132 mg mL^{-1} was reduced and denatured in a solution containing 6
2 M Gdn, 40 mM DTT, 0.1 M Tris-HCl (pH 8.0), and 1 mM EDTA. Bovine CA was denatured
3 in 6 M Gdn containing 0.1 M Tris-HCl (pH 8.0) at 44 mg mL^{-1}. The resulting protein solutions
4 were incubated for 2 h at 37°C. The denatured protein solution was stored at 4°C and used
5 within 1 week.
6
7 *2.4. Protein refolding*
8 The reduced and denatured lysozyme solution was diluted by 40-fold into the
9 refolding buffer containing 0.1 M Tris-HCl (pH 8.0), 1 mM EDTA, and refolding additives at
10 an appropriate concentration. The final refolding solution has a volume of 200 μL containing
11 0.1 M Tris-HCl (pH 8.0), 1 mM EDTA, 5 mM GSH, 5 mM GSSG, 150 mM Gdn, 1 mM DTT,
12 and 0.4-3.3 mg mL^{-1} lysozyme. The denatured CA was diluted by 40-fold into the refolding
13 buffer containing 0.1 M Tris-HCl (pH 8.0) and refolding additives at the concentration of 250
14 mM. The final CA concentration was 1.1 mg mL^{-1}. The diluted solution was mixed by a
15 vibrating mixer for 2 s and then incubated at 25°C for 12 h without shaking.
16
17 *2.5. Activity assay*
18 Prior to measuring the enzymatic activity, refolding solutions were centrifuged at
19 15,000 *g* for 20 min to remove precipitates. The activity of lysozyme was assayed by
20 bacteriolysis of *M. lysodeikticus*. Ten μL of the refolded lysozyme was added to 1490 μL of
21 0.5 mg mL^{-1} *M. lysodeikticus* solution containing 50 mM Na-phosphate buffer (pH 7.0). The
22 decrease in the light scattering intensity at 600 nm of the solution was monitored using a
23 UV-Vis spectropolarimeter model V-550 (Japan Spectroscopic Co., Tokyo, Japan) at room

temperature. The enzymatic activity was determined from the initial velocity of the substrate degradation. The activity of CA was determined by hydrolysis of *p*-NPA. A hundred μL of the refolded CA solution was diluted into the substrate solution containing 1 mM *p*-NPA, 50 mM Tris-HCl (pH 7.5) and 5 mM EDTA. The increase in absorbance at 348 nm was measured using the spectrophotometer. The refolding yield was determined as for lysozyme.

2.6. Alkylation of free sulfhydryl groups

In order to quench the formation of disulfide bonds, free sulfhydryl groups were alkylated with iodoacetic acid. The kinetics of the reactivation of lysozyme was monitored by removing 50 μL samples of refolding lysozyme at specific time intervals and quenching the reaction by addition 5 μL of 0.5 M iodoacetic acid dissolved in 1 M Tris-HCl (pH 7.0) and 1 M potassium hydroxide (Darby and Creighton 1995). The quenching procedure had no effect on the activity of native lysozyme.

2.7. Kinetic model

Figure 1 shows the refolding kinetic model as described previously (Hevehan and De Bernardez Clark 1997). Briefly, this model assumes the formation of the transient intermediate (I) from the unfolded protein (U) in the early phase of refolding. This is a rapid process with a millisecond time range, so that the rate constant of the formation of I (k_I) can be assumed to be infinite. Thus, this folding scheme is simplified to the parallel reaction, which consists of the formation of native structure (I -> N) and aggregates (I -> A). The folding is a unimolecular reaction, whereas aggregation is a higher order reaction. In the oxidative refolding of lysozyme, the aggregation can be best described by third-order kinetics (Hevehan and De Bernardez Clark

1997). The refolding yield of lysozyme (Y) over time can be described by the following equations:

$$Y = \phi\{\tan^{-1}[(1+\phi^2)\exp(2k_N t)-1]^{1/2}-\tan^{-1}\phi\} \quad (2a)$$

where ϕ is defined as

$$\phi = (k_N/k_A U_0^2)^{1/2} \quad (2b)$$

where U_0 represents the initial unfolded protein concentration, t the refolding time, k_N and k_A the folding and aggregation kinetic constants, respectively.

2.8. Circular dichroism spectroscopy

Circular dichroism (CD) spectroscopy was performed using a Jasco CD spectropolarimeter model J-720W with a Peltier cell holder with a temperature controller model PTC-348W (Japan Spectroscopic Co., Ltd., Tokyo, Japan). The near-UV CD spectra of lysozyme were monitored in the wavelength range of 265-310 nm. The heat-induced unfolding was monitored at 288.5 nm with a heating rate of 1℃ min^{-1} using a 1 cm path-length cell. All spectra representing the native lysozyme were solubilized into the buffer containing 0.1 M Tris-HCl (pH 8.0), 1 mM EDTA, and 500 mM additives. The final protein concentration was adjusted to 0.2 mg mL^{-1}.

2.9. pH titration

In order to determine the charged state of additives at pH 8.0, the pK_a values of the amino group of additives were determined by pH titration. A small quantity of 1.0 M NaOH was added to 3 mL of 0.1 M additive solution. The change of pH was monitored with pH meter model TPX-90i (Toko Chemical Laboratories Co., Ltd., Tokyo, Japan) and equivalent points

1 were calculated by derivation of titration curves.

3. Results

3.1. Screening of Arg analogs

In order to obtain superior additives to increase the refolding yields of protein, we investigated Arg analogs using oxidative refolding of lysozyme as a model. Figure 2 shows the 11 kinds of refolding additives tested in this paper. The compounds in class A are Arg and Gdn as well-known additives for refolding. The compounds in class B have no guanidino groups. Gly and GlyAd are selected to reveal the effect of the side chains and amidation of the carboxy group, respectively. In class C, ArgAd and ArgEE possess amide and carboxy ethylester groups substituted for the carboxy group of Arg, respectively. GPA has no amino group and two methylene groups shorter than Arg. In class D, HArg and AGPA have identical ionizable groups to Arg but different lengths of methylene groups.

The reduced and denatured lysozyme was diluted into refolding buffer in the presence of additives and then the refolding yield was compared by enzymatic activity. Figure 3 shows the refolding yields of lysozyme in the presence of 500 mM additives. As the refolding was accomplished by 40-fold dilution, 150 mM Gdn and 1 mM DTT remained in the final solution. The refolding yield was only about 8% in the absence of additives. In the presence of Arg and Gdn, the refolding yields were 37% and 40%, respectively. Orn, Gly, and GlyAd rather decreased the refolding yields below 7%. In the presence of Cit, which has an ureido group substituted for the guanidino group of Arg, the refolding yield slightly increased up to half in the presence of Arg. This result should be due to the ureido group sharing a common structure of urea, which is apparently similar to the weak chaotropic effect of Gdn. The additives that showed higher refolding yields than Arg were ArgAd, ArgEE, GPA, HArg, and Gdn, though they have a guanidino group. AGPA did not affect the refolding yield of lysozyme, while

HArg showed 1.3-fold higher yield than Arg. ArgAd showed the best data for the refolding yield of lysozyme ~ 63% among 11 additives tested. The substitution of amide group for the carboxy group of Arg is favorable for the refolding of lysozyme.

3.2. Comparative data of Arg, Gdn, and ArgAd for refolding additives

We found that ArgAd increased the refolding yield of lysozyme more effectively than Arg. In order to clarify the superior property of ArgAd, the oxidative refolding of lysozyme was performed at various concentrations of additives and protein.

Figure 4A shows the refolding yield of lysozyme in the presence of various concentrations of Arg, Gdn, and ArgAd at a protein concentration of 1.0 mg mL^{-1}. The maximum refolding yields of lysozyme in the presence of ArgAd was 89% at around 0.8-1.0 M. The profile of Gdn was similar to that of ArgAd with a maximum yield of 79% at 1.0 M. However, the refolding yield in the presence of Arg was saturated above 1.3 M Arg. At 2.0 M Arg, the refolding yield was 72%. These data show that ArgAd is the best additive under a practical concentration for *in vitro* refolding below 1 M.

Figure 4B shows the refolding yields of lysozyme depended on the protein concentration in the absence and presence of Arg, Gdn, and ArgAd. Reduced and denatured lysozyme at various concentrations (16–114 mg mL^{-1}) was diluted by 40-fold into the refolding buffer containing 500 mM additive and then the residual activity was measured. In the absence of additives, the refolding yields steeply decreased and nearly all the lysozyme could not refold above 1.6 mg mL^{-1}. In the presence of 500 mM Arg, Gdn, and ArgAd, the profiles were clearly improved. At a high concentration of protein above 1.6 mg mL^{-1}, ArgAd increased the refolding yield by 8-fold compared to absence of additive, while Arg and Gdn increased

1 5-6-fold (Fig. 4B inset). It is interesting to note that at the low protein concentration, the
2 refolding yield is little improved by the additives, implying that preventing intermolecular
3 interaction plays a key role in increasing the refolding yield.
4

5 *3.3. Kinetics for refolding in the presence of ArgAd, Arg, and Gdn*

6 To further investigate the efficacy of ArgAd and its mechanism of action, refolding
7 kinetics were measured in the absence or presence of 500 mM Arg, Gdn, and ArgAd. The
8 refolding yields increased over time and reached plateau after 2-3 h, as shown in
9 Supplementary Figure S1. The experimental data were well fitted to Eqs. (2a) and (2b). The
10 deduced values of k_N, k_A, and ϕ are summarized in Table 1. As can be seen from the table, the
11 values of k_N, which corresponds to the kinetic parameter from unfolded state to native, were
12 slightly decreased when ArgAd, Arg, and Gdn were added at a concentration of 500 mM. In
13 the presence of 500 mM additives, the values of k_A, which correspond to the kinetic parameter
14 from aggregate-prone intermediate to aggregates, were 2 orders of magnitude lower than that in
15 the absence of additives. Interestingly, the value of k_A in the presence of ArgAd was about half
16 of that in the presence of Gdn. The ratio of k_N and k_A or the value of ϕ defined by Eq. (2b)
17 directly relates to the final refolding yield. As the ϕ value in the presence of ArgAd was higher
18 than that of Arg and Gdn. These data indicate that ArgAd preferentially suppresses the
19 aggregation process as compared to the folding process.
20

21 *3.4. Thermodynamic stability of lysozyme in the presence of ArgAd, Arg, and Gdn*

22 In order to clarify the denaturing effects of additives, thermal unfolding profiles of
23 lysozyme were measured by CD spectroscopy. Figure 5A shows the near-UV CD spectra of

1 non-reduced lysozyme at 25°C in the absence or presence of Arg, Gdn, and ArgAd. There were
2 no significant differences even in the presence of additives. Figure 5B shows the thermal
3 unfolding curves of native lysozyme monitored at 288.5 nm. No aggregation was observed
4 even at 98°C and thermal unfolding was irreversible even in the presence of additives. In the
5 absence of additives, the apparent midpoint temperature of unfolding (T_m) was 74.9°C. In the
6 presence of 500 mM Arg, the T_m value was 74.5°C, which is slightly lower than the absence of
7 additives. On the other hand, in the presence of 500 mM Gdn and ArgAd, the T_m values were
8 70.4°C and 71.2°C, respectively. This indicates that Gdn and ArgAd possess denaturing
9 properties. Interestingly, the denaturing effect of ArgAd was larger than that of Arg but smaller
10 than that of Gdn. Thus, the improvement of the refolding yield in the presence of ArgAd cannot
11 be explained by only the stabilization of aggregation-prone species relative to aggregates.
12
13 *3.5. pH titration of Arg, Gdn, and ArgAd*
14 The refolding process is generally dependent on the charged state of both the additive
15 and the protein. Lysozyme was positively charged under the present experimental conditions
16 due to the high isoelectric point (pI) from 9.3 (reduced form) to 10.8 (oxidized form). In order
17 to determine the charged state of additives, we performed pH titration experiments and
18 determined the pK_a value of the amino group responsible for these experimental conditions at
19 pH 8.0. The pH titration curves of the solution at a 0.1 M concentration are shown in
20 Supplementary Figure S2. The pK_a values of the amino group and the net charge of Arg,
21 ArgAd, HArg, and AGPA, are summarized in Table 2. The pK_a value of the amino group of
22 HArg was higher than that of Arg, while that of AGPA was lower. This would be due to the
23 inductive effect depending on the number of the methylene group between the guanidino group

1 and α–carbon. Although the pK_a of the amino group of ArgAd was the lowest value of all
2 additives tested, the net charge of ArgAd was highest due to the substitution of the amide group
3 for the carboxy group. The relationship between the net charge of Arg analogs and refolding
4 yields suggests that the high net charge may contribute to the effect of ArgAd as discussed in
5 the following section.
6
7 *3.6. The effect of ArgAd on CA refolding*
8 In order to confirm versatility of ArgAd as a refolding additive, we compared the
9 additives on the refolding yields of CA, which is a monomeric protein with the molecular mass
10 of 30 kDa with neutral pI. As shown in Figure 7, the refolding yield of CA in the absence of
11 additives was about 13%. In the presence of 250 mM Arg and Gdn, the refolding yields were
12 23% and 36%, respectively. On the other hand, the refolding yield in the presence of 250 mM
13 ArgAd was 1.4-fold higher than that in the presence of Arg. This result suggests the possibility
14 of ArgAd as a versatile additive for protein refolding.

1 **4. Discussion**

2 The systematic screening of Arg analogs for oxidative refolding of lysozyme revealed
3 that ArgAd is the most effective among the additives tested. We discuss why ArgAd is superior
4 as a refolding additive as follows.

5 The simple finding is that guanidino compounds improve the oxidative refolding of
6 lysozyme (Fig. 3). Gdn or the guanidino group binds to the aromatic side chains, as well as the
7 peptide backbone and negatively charged side chains, resulting in stabilization of
8 aggregation-prone species or folding intermediates to prevent aggregation (Nozaki and Tanford
9 1970; Arakawa and Timasheff 1984; Timasheff and Arakawa 1988; Lin and Timasheff 1996;
10 Tsumoto et al. 2004). These facts suggest that the guanidino group should be an indispensable
11 functional group in a refolding additive.

12 Early studies on refolding additives have shown that protein denaturants, typically
13 Gdn, at a nondenaturing concentration improve correct refolding (Orsini and Goldberg 1978).
14 The low concentration of the denaturant inhibits the intermolecular hydrophobic interactions,
15 which lead to the suppression of the aggregation and improvement in the correct folding. In
16 fact, Arg as well as Gdn decrease the melting temperature and perturb the spectroscopic
17 properties of a certain protein (Ishibashi et al. 2005). These data suggest that the denaturing
18 effect plays a key role in suppression of aggregation. In our data, ArgAd decreased the
19 refolding yield of lysozyme at high concentrations (above 1.3 M) as shown in the case of Gdn
20 (Fig. 4A). This phenomenon is consistent with the abilities of these chemicals to destabilize the
21 native state (Fig. 5). These facts prompted us to the hypothesis that the amide group of ArgAd
22 mainly contributed to the denaturing effect. Amide compounds, such as acetamide, are
23 effective additives on the refolding of lysozyme (Yasuda et al. 1998; Dong et al. 2004).

1 Moreover, acetamide is a weak protein denaturant (Gordon and Jencks 1963; Warren and
2 Gordon 1970). The amide group on ArgAd acts as both hydrogen bond donors and acceptors,
3 as ArgAd shows a higher denaturing effect than Arg. However, Gdn is also a stronger protein
4 denaturant than ArgAd. Although the protein-denaturing effect is key factor to understanding
5 the refolding additives, not all the properties of additives could be described.

6 One of the finding of our study is that ArgAd preferentially suppressed intermolecular
7 aggregation comparing to intramolecular folding (Table 1). This phenomenon is apparently
8 similar to the thermal unfolding of lysozyme under the acidic condition; i.e., lysozyme does not
9 aggregate but it reversibly refolds at acidic pH (Ueda et al. 2000). ArgAd has a higher positive
10 net charge than Arg due to the unionizable amide group (Table 2). ArgAd may tend to interact
11 with folding intermediates and neutralize local negative charges leading to enhanced
12 electrostatic repulsion between aggregation-prone species. This idea is supported by the data
13 that polyamine (Kudou et al. 2003) and ArgEE (Shiraki et al. 2004) suppress the heat-induced
14 aggregation of lysozyme. In the case of ArgEE, the hydrophobic end of the ethyl group
15 interacts with the hydrophobic patch on the unfolded polypeptide, leading to enhanced
16 electrostatic repulsion between aggregation-prone unfolded lysozyme. Binding of ArgAd on
17 the aggregation-prone species is related to the increase of net charge substituting the
18 unionizable amide group for the carboxy group leads to improved refolding yield.

19 There is a positive relation between the refolding yield and the positive charge of Arg
20 analogs (Fig. 6). The refolding yields in the presence of Arg, HArg, and AGPA were clearly
21 different although they have the same ionizable groups. This difference would be due to the
22 charged state of the C_α–amino group of additives (Table 2). Moreover, ArgAd has the highest
23 net charge of all additives tested. These results support the idea that positively charged additive

1 increases the refolding yield.

2 In summary, we showed that a new refolding additive ArgAd improves the refolding
3 yield due to the suppression of aggregation. The comparative analysis of ArgAd to similar
4 compounds reveals that the refolding additives require the guanidino group and some positive
5 net charges. These experimental facts provide information for developing new protein
6 aggregation suppressors and refolding additives. It should be noted that ArgAd is somehow
7 toxic and too expensive to use the bioprocess. These properties can be overcome by searching
8 the analogues of ArgAd.

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5

6 **Appendix A. Supplementary data**

7 Supplementary data associated with this article can be found, in the online version.

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1 Table 1

2 Kinetic constants of the folding (k_N), aggregation (k_A), and ϕ value calculated by Eqs. (2a) and

3 (2b)[a]

Additive	k_N (min^{-1})	k_A ($mL^2\ mg^{-2}\ min^{-1}$)	ϕ (-)
No Additive	0.0233±0.0019	23.4±2.1	0.03
Arg	0.0192±0.0006	0.498±0.026	0.20
Gdn	0.0150±0.0008	0.187±0.002	0.28
ArgAd	0.0140±0.0008	0.100±0.008	0.37

4 [a] Triplicate experiments were performed and average values of k_N and k_A and their standard

5 deviations are represented (Supplementary Figure S1).

1 Table 2

2 The pK_a values and net charges of Arg analogs

Additive	pKa	Net Charge[a]
Arg	9.04	+0.92
ArgAd	7.39	+1.20
HArg	9.33	+0.96
AGPA	7.66	+0.31

3 [a] The net charge of additives were measured at pH 8.0 (Supplementary Figure S2).

1 **Figure legends**

2 Figure 1. Simplified kinetic model represented by Hevehan and De Bernardez Clark. U,
3 unfolded protein; I, folding intermediate; A, aggregates; N, native protein. k_I, kinetic constant
4 of formation of intermediates; k_N, kinetic constant of folding; k_A, kinetic constant of
5 aggregation.

6

7 Figure 2. Chemical structure of additives. (A) Arginine and guanidine as standard additives.
8 Arginine derivatives, (B) substituted for guanidino group, (C) modified in carboxy group, and
9 (D) having shorter or longer methylene groups.

10

11 Figure 3. Refolding yield of lysozyme in the presence or absence of additives. Reduced and
12 denatured lysozyme at 40 mg mL^{-1} were diluted 40-fold with buffer containing final
13 concentration of 500 mM additives. Data represent the mean and error bars of three
14 independent experiments.

15

16 Figure 4. Refolding yield of lysozyme in the presence of additives. (A) Reduced and denatured
17 lysozyme at 40 mg mL^{-1} were diluted 40-fold into buffer containing various final
18 concentrations of Arg (solid circles), Gdn (solid squares), and ArgAd (open circles). (B) The
19 effects of lysozyme concentration on the refolding yield in the absence (cross) and presence of
20 500 mM Arg (solid circles), Gdn (solid squares), and ArgAd (open circles). (B, Inset) The
21 relative refolding yield of lysozyme in the presence of additives. The vertical axis was divided
22 by the refolding yields of no additives at respective concentrations. The final protein
23 concentrations of 0.4-3.3 mg mL^{-1} were shown in the horizontal axis. Data represent the mean

1 and error bars of three independent experiments.

2

3 Figure 5. (A) Near-UV CD spectra of lysozyme in the presence of 500 mM additives, Arg
4 (solid line), Gdn (short-dashed line), and ArgAd (long-dashed line), or in the absence of
5 additives at 25°C (dotted line) and at 98°C (dash-dotted line). (B) Normalized near-UV
6 thermal transitions of lysozyme monitored by CD in the absence (cross) and presence of
7 additives, Arg (closed circle), Gdn (closed square), and ArgAd (open circle).

8

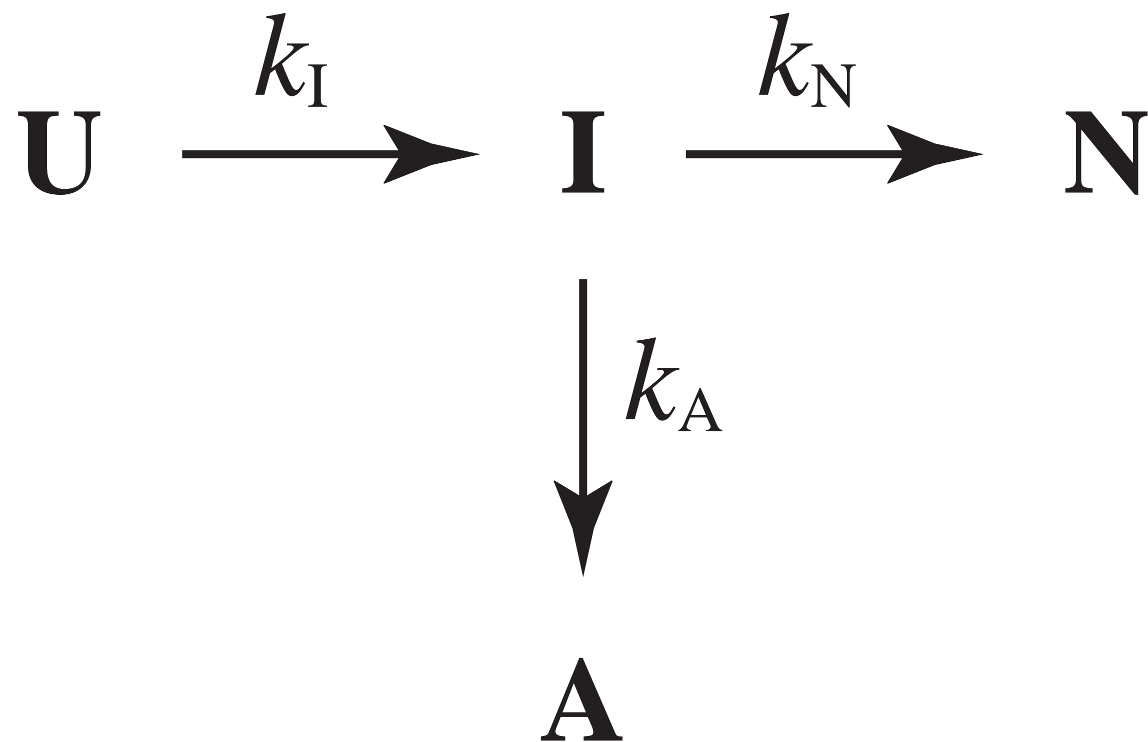
9 Figure 6. Relationship between refolding yield and net charge of additives. The additives are
10 Arg, ArgAd, HArg, and AGPA. The refolding yields were referred to Figure 2. The net charges
11 of additives were referred to Table 2. Data represent the mean and error bars of three
12 independent experiments.

13

14 Figure 7. Refolding yield of CA in the presence or absence of additives. Denatured CA at 44
15 mg mL^{-1} was diluted 40-fold with buffer containing final concentration of 250 mM additives.
16 Data represent the mean and error bars of three independent experiments.

Figure(s)

Hamada and Shiraki, Figure 1



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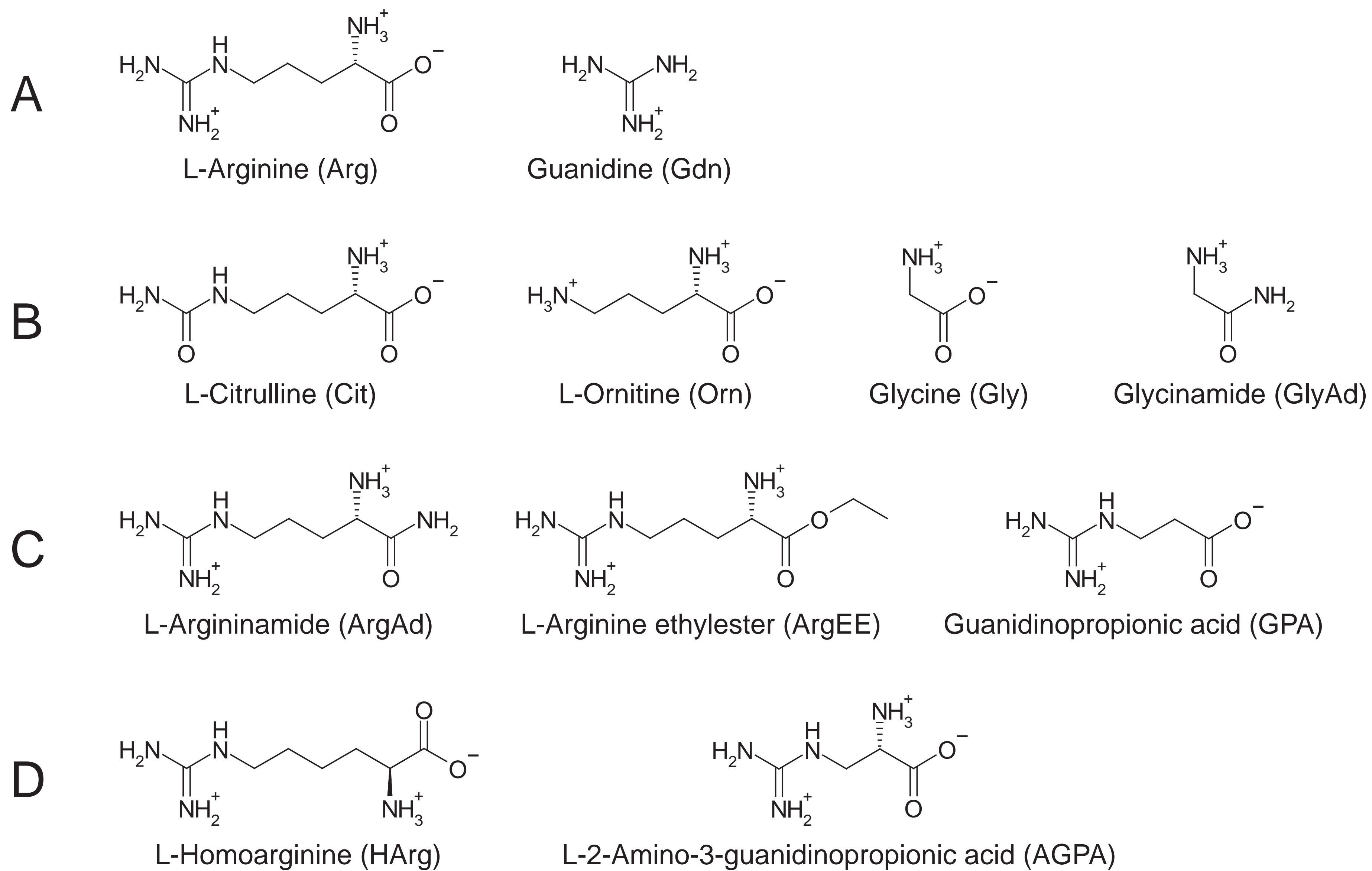

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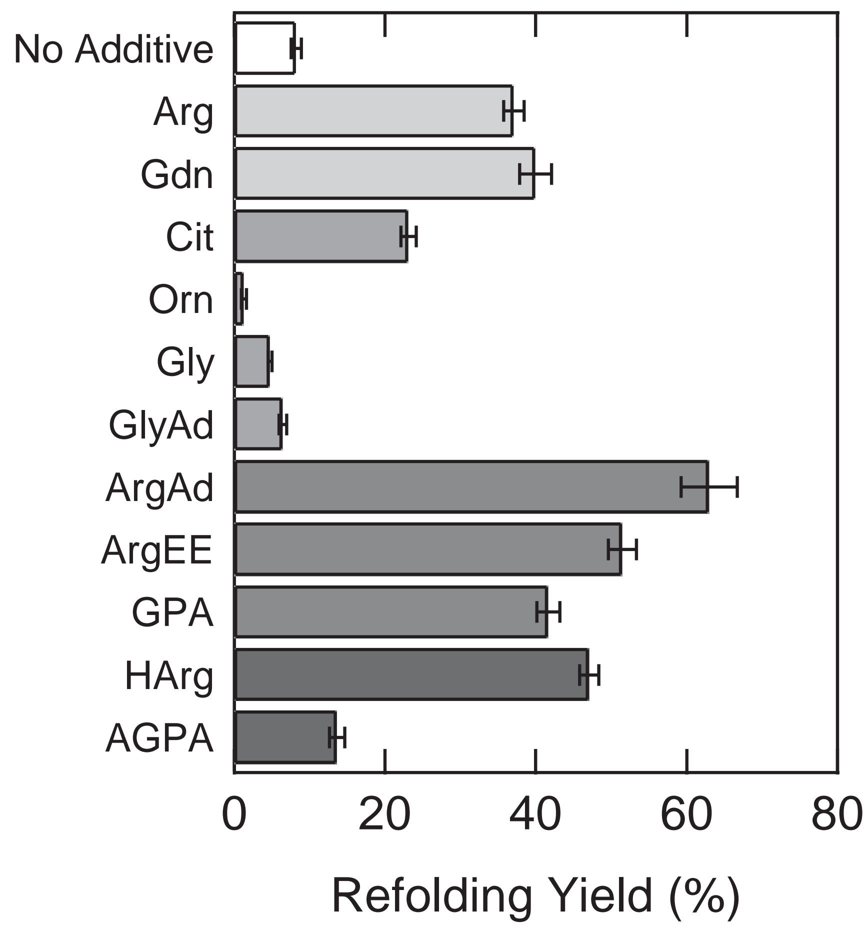

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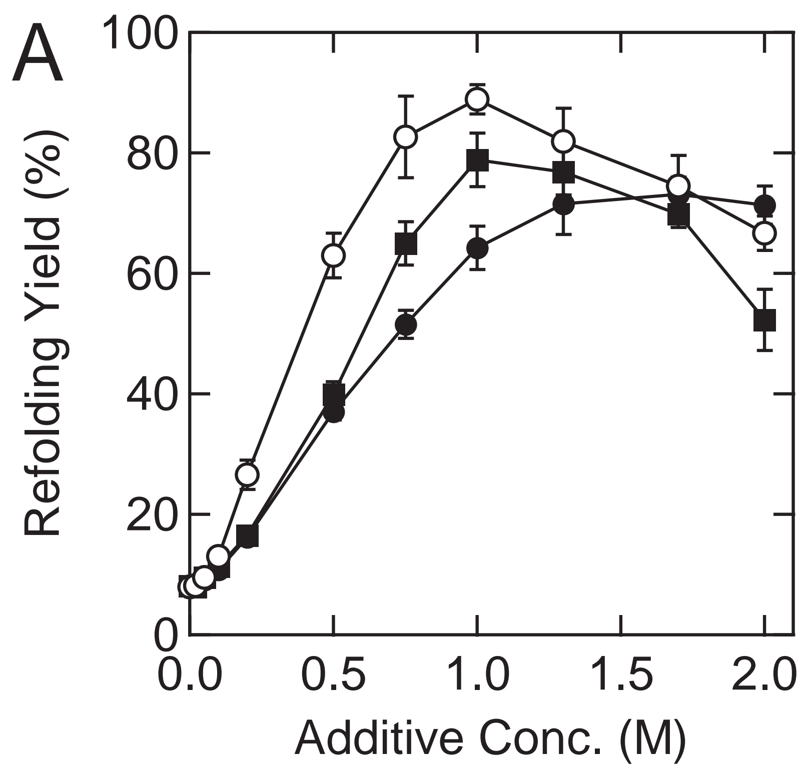

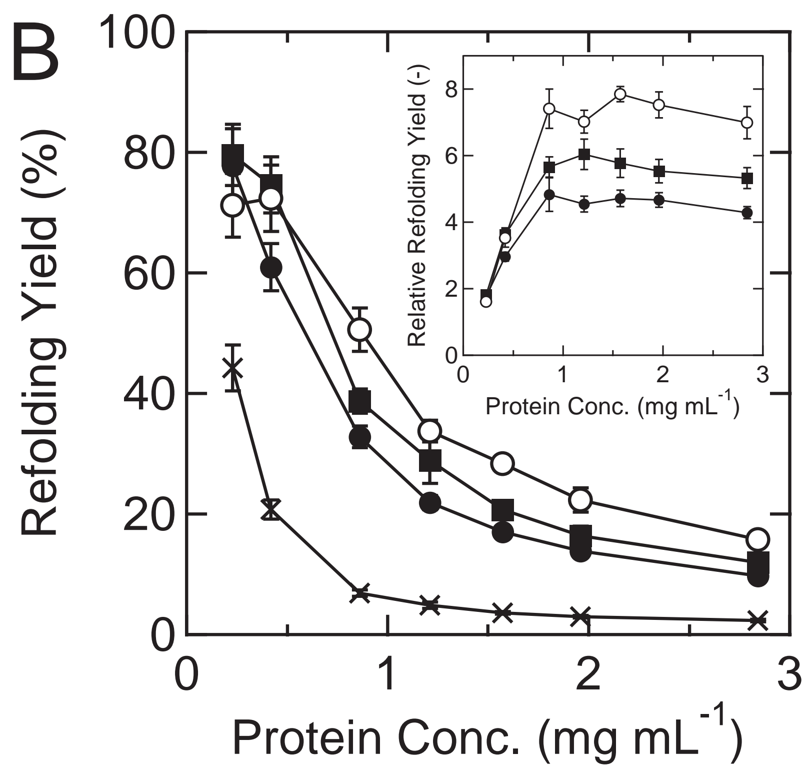

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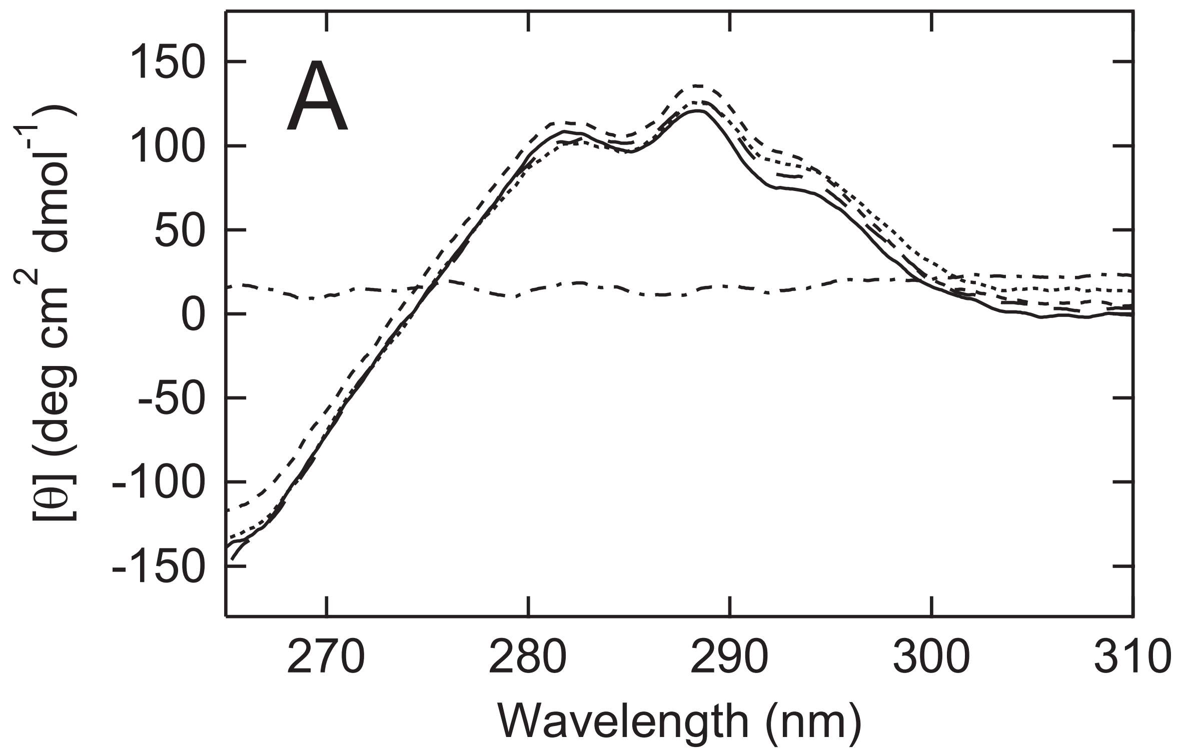

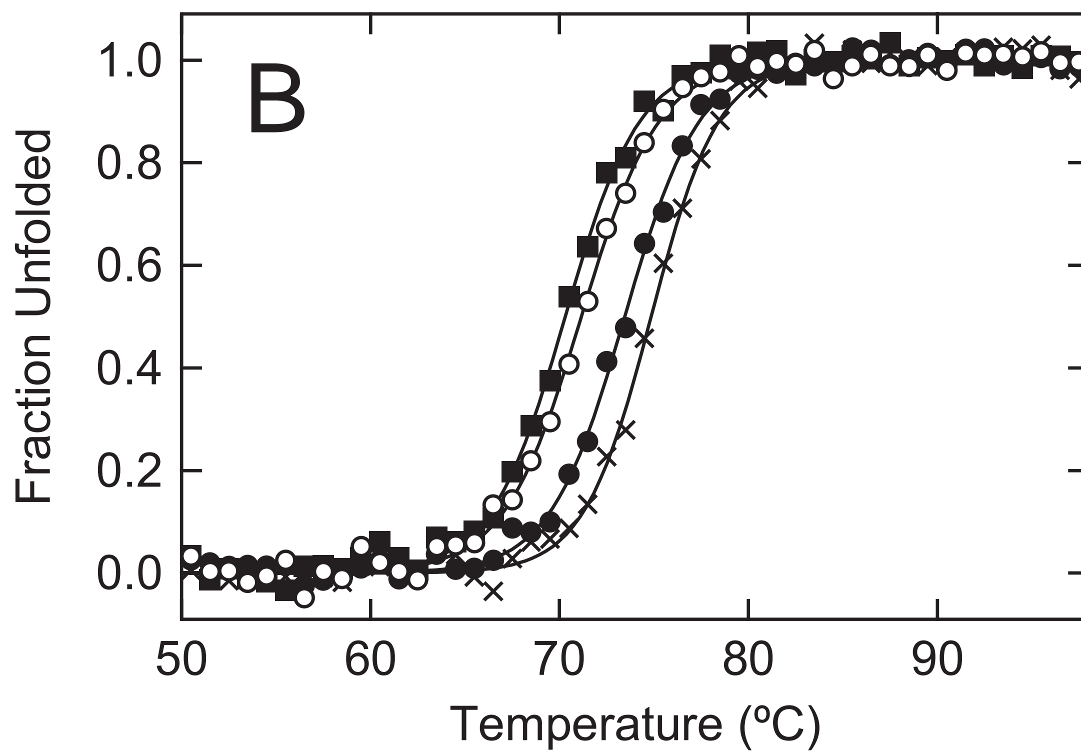

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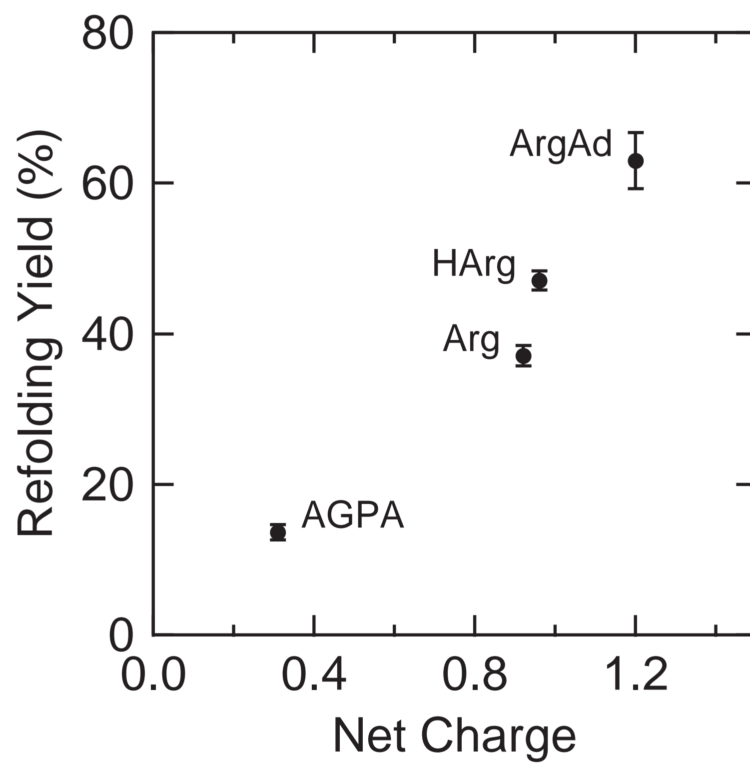
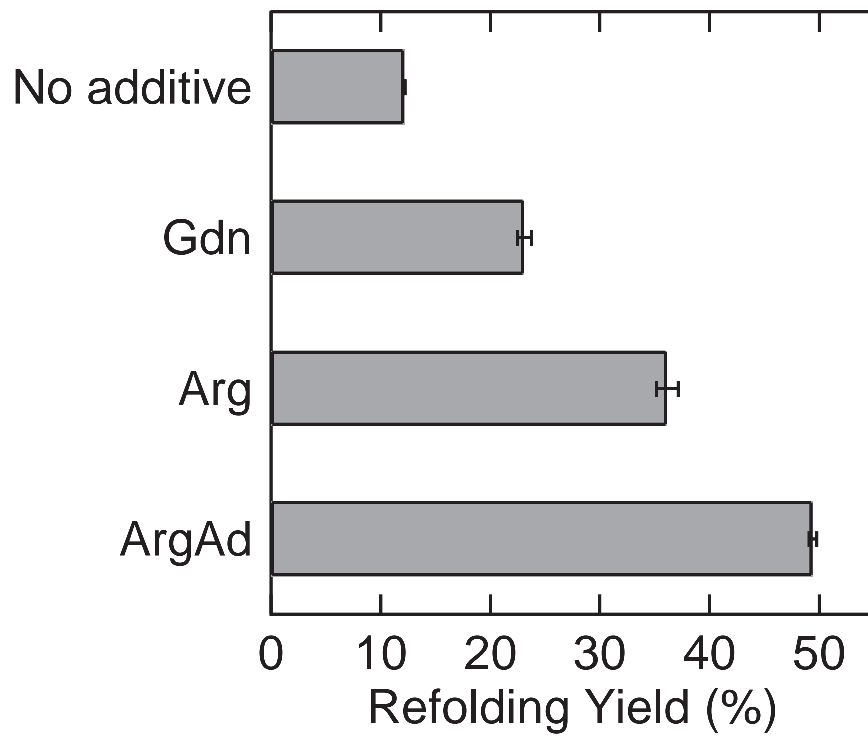
Hamada and Shiraki, Figure 6



Figure(s)

Hamada and Shiraki, Figure 7



Figure(s)

Electronic Supplementary Material for

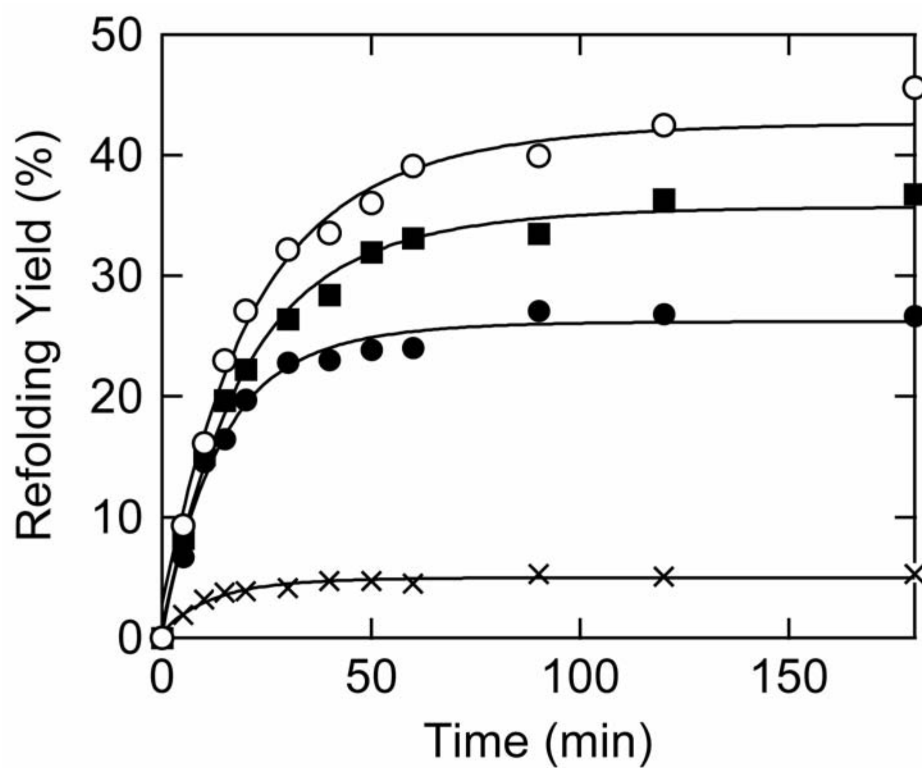
L-Argininamide improves the oxidative refolding more effectively than L-arginine

Hiroyuki Hamada and Kentaro Shiraki

Supplementary Figure S1

Kinetic profiles of lysozyme refolding.

The reduced and denatured lysozyme at 40 mg mL^{-1} were diluted 40-fold in the absence (cross) or presence of 500 mM Arg (solid circles), Gdn (solid squares), and ArgAd (open circles). After the incubation at respective periods, the residual activity was measured. Continuous curves show the nonlinear least-squares fitting to Eqs. (2a) and (2b).



Supplementary Figure S2

pH titration curves in the presence of 100 mM additives.

Determination of pK_a values of amino groups of Arg analogs. The additives are Arg (closed circle), HArg (closed square), AGPA (closed triangle), and ArgAd (open circle).