Optimizing refolding condition for recombinant tissue plasminogen activator

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Abstract
Low molecular size additives such as L-arginine and the redox compounds have been used both in the culture medium and *in vitro* refolding to increase recombinant proteins production. Additives increase protein refolding and yield of active proteins by suppressing aggregate formation or enhancing refolding process. In this work, a comparative study was performed on refolding of recombinant plasminogen activator (rPA) in the presence of different concentrations of denaturants and additives. *Escherichia coli*-expressed rPA inclusion bodies were solubilized in chaotropic denaturants and subjected to protein refolding by dilution method. The effects of various additives, the impact of pH, residual Guanidin Hydrochloride (Gn-HCl) and Dithiothreitol (DTT) on refolding process were investigated. The refolding process was assessed by determination of protein solubility and biological assay. The results of the study demonstrated that the best condition for solubilizing the rPA inclusion body was 6M guanidine hydrochloride at pH=10. In refolding step, L-arginine showed increasing effect on suppression of aggregation at concentrations of 200-1000 mM. Glutathione pairs (GSH-GssG) showed refolding enhancer effect in a range of 2-20 mM. The highest refolding yield was obtained in 500 mM L-arginine and reduced/oxidized glutathione 10:1 ratio in pH 10. In conclusion, the results show that L-arginine plays an important role in the refolding of human PA, preventing the aggregation of folding intermediate, and glutathione pair is essential for the correct refolding. The results also revealed that higher solubility in the presence of higher concentration of L-arginine (> 500 mM) or pH (>10) is not associated with higher activity.
Keywords**:** Refolding; Recombinant Plasminogen Activator; arginine; glutathione

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INTRODUCTION

Recombinant protein expression is used in both biotechnology and basic research, but most of the expressed proteins are in an insoluble form (Service, 2002; Christendat *et al*., 2000). Thus, soluble protein expression is one of the most important requirements in these fields. Different systems for expression of recombinant proteins have been developed, including mammalian cells, yeast, insect cells, plants and bacterial cells. *Escherichia coli* is a commonly used expression system which has several advantages to other systems, such as rapid growth, low cost cultivation, the availability of variable vectors and well known genetic structure (Carrio and Villaverde, 2001; Mukhopadhyay, 1997). However, the main disadvantage of this system is that the recombinant protein is often expressed as inclusion bodies (IBs).

Expression as inclusion bodies has some beneficial effect in some specific circumstances. Formations of IB can facilitate downstream processes such as purification of recombinant proteins (Flaschel, 1993). In the case of formation of IBs, *in vitro* refolding is a selective strategy for converting the inactive and insoluble inclusion bodies into soluble form, correctly folded and biologically active products. However, *in vitro* refolding is a time consuming process, based on trial-

error experiments and optimizing the refolding factors are highly complicated.

The *in vitro* refolding of IBS involves four main steps including, isolation of inclusion bodies from bacterial cells; solubilization of protein aggregates; refolding of solubilized protein and purification. To avoid aggregate formation during refolding process, refolding is usually performed at low protein concentration in a range of 10-100 µg/ml (Lilie *et al*., 1998). However, this leads to high costs of refolding process, thus refolding at high concentration of protein is of high interest. For this purpose various additives are used to improve refolding yield. Additives are low molecular size compounds that assist the refolding process by different mechanisms; some of them enhance the refolding process whereas the others work by suppression of aggregation. L-arginine is one of additives that has been used widely in refolding of recombinant proteins and acts by suppressing the aggregation (Marston, 1986). Aggregation suppression by arginine occurs on the partially unfolded proteins and intermediate structures. The other groups of additives are refolding enhancers and the most commonly used enhancers are reduced and oxidized glutathione (GSH/GSSG). In the case of proteins with disulfide-bonds, refolding buffer should assist disulfide bond-formation. Oxido shuffling systems consist of a mixture of reduced and oxidized low molecular weight thiol reagents. Commonly used oxido-shuffling reagents are reduced and oxidized glutathione (GSH/GSSG) but the pairs cysteine/cystine, cysteamine/cystamine have also been utilized (Clark, 2001). The required concentration of additives and shuffling system for refolding are protein specific and depend on nature and the number of disulfide bands. Thus, determination of optimized concentration of additives and shuffling system is a prerequisite for any given refolding process.

Human tissue plasminogen activator is a 60-68 kD polypeptide composed of 527or 530 amino acid residues and containing 7-13 percent carbohydrate. rPA is a serine protease secreted from endothelial cells and converts plasminogen to plasmin and plasmin degrades fibrin. Clinically, rPA is used as a major thrombolytic agent in treatment of the stroke, myocardial infarction, and venous thromboembolisms. The molecule has 17 disulfide bands, made up of five distinct domain structures. A truncated version of tissue-type plasminogen activator, consisting of the kringle-2 and the protease domain known as rPA is produced by recombinant expression in *E. coli* (Zhang *et al*., 2005; Zhu, 2003). The nine disulfide bridges of rPA are essential for the native conformation and consequently the activity of the protease. This protein is expressed as insoluble IB and need in-vitro refolding for conversion into bioactive forms. The objective of this study was to evaluate the effects of arginine, glutathione and sucrose on refolding of rPA at different PH.

MATERIALS AND METHODS

Materials and equipments: Reagents for SDS-PAGE were purchased from Sigma Aldrich (Munich, German), protein molecular weight marker from Fermentas (Lithuania), *p*-nitrophenyl phosphate (pNPP) from ASSAYPRO, GnHCl, Urea, DTT, reduced glutathione (GSH), and oxidized glutathione (GSSG) from Applichem (Darmstadt, Germany), and BL21 (DE3) were from Novagen (Madison, WI).

Isolation of r-PA inclusion bodies: About 5 g of frozen biomass was homogenized in 10 ml of 0.1 M Tris-HCl buffer pH 6.8, containing 20 mM EDTA and the cells were sonicated for 10 cycles each 30 seconds with 30 seconds intervals on ice using a Sonicator (vibra cell). PMSF to a final concentration of 1 mM was added just before the sonication. Cell breakage was checked by light microscopy. The suspension of disrupted cells was centrifuged at 10,000 g for 10 min at 4°C. The collected pellets of IBs were resuspended in 50 ml of washing solution 1 (2.5% Triton X-100, 1 M NaCl, 20 mM EDTA in 0.1 M Tris-HCl buffer pH 6.8) and centrifuged at 10,000 g for 10 min at 4°C. Then, the washing step was repeated using the 0.5% Triton X-100 in the same buffer followed by 3 washing with buffer without Triton X-100. The purity of IB was analyzed by SDS-PAGE (Fig. 1).

Solubilization of inclusion body: For solubilizing the IBs, buffers (0.1 M Tris-HCl, 1mM EDTA) containing urea (2-8 M) and guanidine hydrochloride (Gn-HCl) (2-6 M), DTT (0-50 mM) at different pH (ranges 6-12) were used. IBs were solubilized in solubilization buffers and incubated for 2 h at 25ºC with gentle stirring. The amount of solubilized protein was determined by measurement of turbidity or transmittance at 600 nm (Bajorunaite *et al*., 2007).

Refolding: For refolding process, 50 µl of solubilized protein (the concentration of rPA was 20 mg/ml) was diluted in 1 ml of refolding buffer 0.1 M Tris-HCl, pH

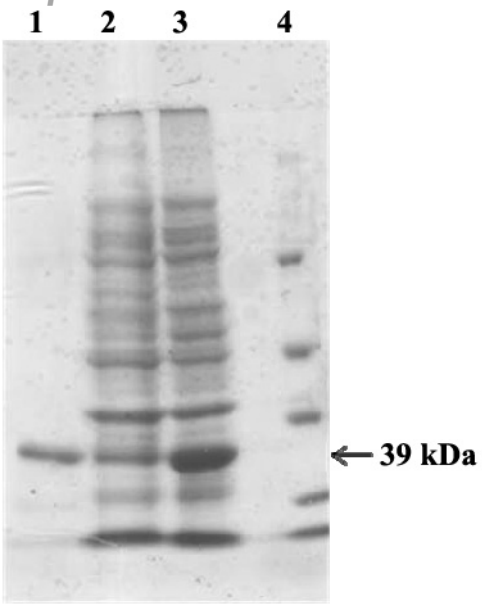

Figure 1. SDS PAGE analysis of rPA expression and purification. Lane 1, purified inclusion body of rPA; lane 2 , *E. coli* BL21 expressing rPA before induction; lane 3, *E. coli* BL21 expressing rPA after induction; lane 4, protein size marker.

10.5 containing 1 mM EDTA and various concentrations of additives, including arginine (0-1M), glutathione pair (1-50 mM), at 10:1 ratios of reduced to oxidized glutathione) and sucrose (0-1M) were added into the refolding buffer. After incubation for 24 hours at 25°C, the percentage of refolded protein was determined by biological activity assay.

SDS PAGE: SDS-PAGE was carried out for analysis of protein purity according to the method of Laemmli (23). The acrylamide concentration of the resolving gel was 15%. Proteins were stained with Coomassie Brilliant Blue R 250 dye (Lammli, 1970).

Determination of rPA activity: The activity of the enzyme was determined using chromogenic substrate S2288. The structure of choromogenic substrate (S-2288) was H-D-Ile-Pro-Arg-pNA.2HCl. The release of *p*-nitroaniline dihydrochloride, as the product, was monitored at 405 nm. The renaturation yield was determined by the measurement of activity of refolded protein using Human tPA Chromogenic Activity Kit (LOXO GMBH, Germany).

RESULTS

Effect of different concentration of denaturant on solubilization of rPA: 7 mg of rPA IBs was solubilized in 1 ml of solubilization buffers containing different concentration of urea or Gn-HCl and incubated for 2 hours at 25ºC. After centrifugation, the amount of solubilized rPA was evaluated by measurement of transmittance at 600 nm (Tables 1 and 2). The solubilization of rPA at alkaline pH in the presence of 6 M Gn-HCl was more efficient than 8 M urea. The highest solubilization was achieved with 6 M Gn-HCl. For determination of the optimum pH in this step, IBs were solubilized in presence of urea and guanidine at pH 8.0, 10 and 12. As shown in Table 1, urea at concentration of <4 M was not effective at any pH values whereas the solubilization of IBs were begun from higher concentration of guanidine. The highest solubilization

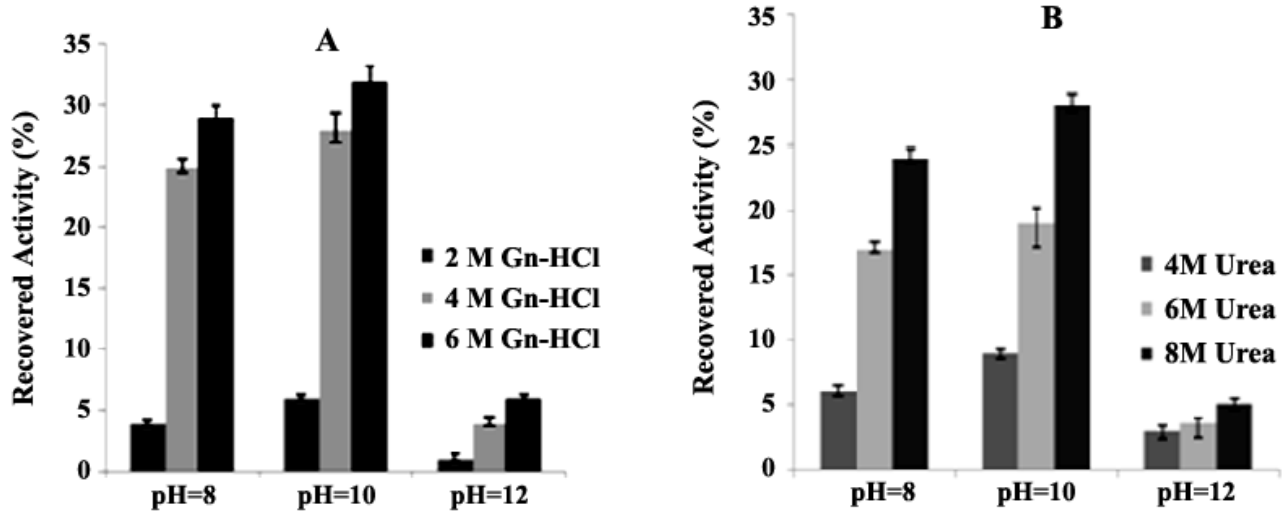

Figure 2. A: Effects of urea and B: Gn-HCl on refolding yield at various pHs.

Table 1. Effect of urea on solubility of rPA inclusion bodies.

Urea concentration (M)	2	4	6	8
	Percentage (%)			
pH=8	1.1±0.03	9.1±0.25	9.7±0.26	32.5±0.51
pH=10	1.7±0.03	10.5±0.37	26.0±0.60	59.9±1.77
pH=12	3.2±0.11	38.8±0.92	56.0±1.21	63.8±1.74

Each value is the average of four independent runs of experiment.

in Gn-HCl was achieved in 6 M Gn-HCl (Table 2).

Effect of different concentration of denaturant on refolding yield: The solubilized inclusion bodies, in different concentrations of urea or Gn-HCl, were refolded by dilution in the same refolding buffer. The activity of samples was measured after 24 h. Although the maximum solubility was found at pH=12, the activity decreased by increase of the pH to values higher than 10 and the highest activity was observed at pH 10 by solubilization at 4 M Gn-HCl (Fig. 2A, 2B).

Table 2. Effect of Gn-HCl on solubility of rPA inclusion bodies.

Gn-HCl Concentration (M)	2	4	6
	Percentage (%)		
pH=8	6.2±0.14	45.7±0.81	65.1±1.15
pH=10	5.5±0.12	62.1±1.17	68.9±1.20
pH=12	6.1±0.11	57.8±1.11	73.0±1.22

Each value is the average of four independent runs of experiment.

Effect of residual Gn-HCl and DTT concentrations on the refolding of rPA: To demonstrate the effect of residual Gn-HCl and DTT on refolding process during dilution process, rPA was denatured in 2, 4 and 6 M Gn-HCl and subjected to refolding by dilution method in same refolding buffer. After incubation for 24 h in 25°C, the turbidity of samples was measured.

As shown in Figure 3A the refolding of rPA affected by residual concentration of Gn-HCl in the refolding buffer. Residual guanidine showed an increasing effect consequent to increase in the concentration of Gn-HCl.

The results also indicated that refolding of rPA is extremely sensitive to residual concentration of DTT in the refolding buffer (Fig. 3B) and refolding strongly inhibited in the absence of DTT. The highest refolding was seen at 5 mM DTT but it fell significantly at 20 mM. This is probably due to the change in the redox potential of the system. Since increasing of DTT concentration leads to a more reduced refolding, it results in the suppression of disulphoide bonds formation. Therefore, in the absence of DTT, the effect of PH was not significant, however, in the presence of DTT, pH 9 showed highest activity.

Effect of arginine on refolding of rPA: After solubilization stage, rPA was refolded by dilution in refolding buffer (0.1 M Tris-HCl, 1 mM EDTA, 10 mM GSH

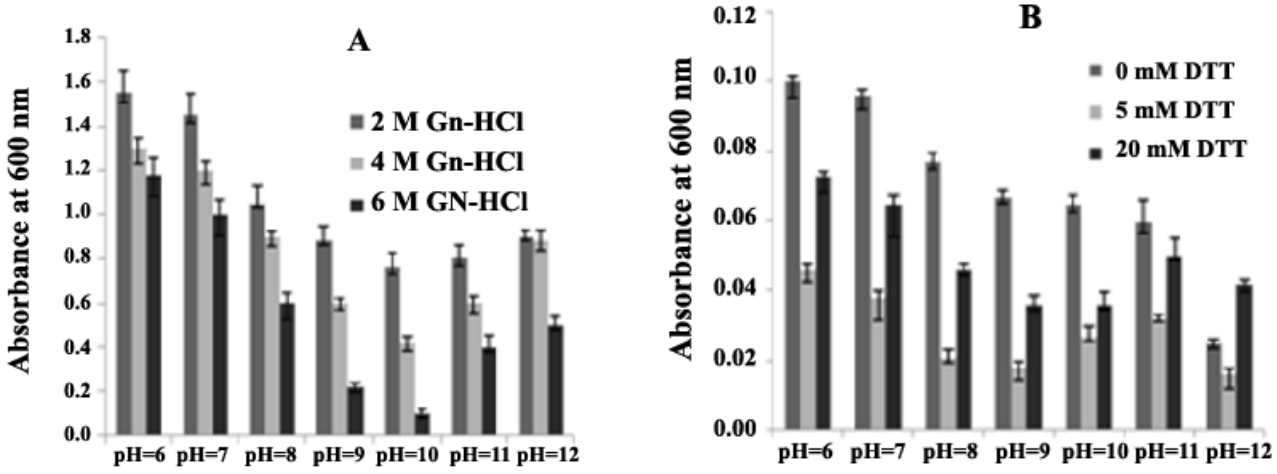

Figure 3. A: Effect of residual concentration of Gn-HCl and B: DTT in refolding buffer at various pHs on refolding of rPA.

and 1 mM GSSG) containing different concentrations of arginine (0-1000) at different pH (8, 10, and 12). The amount of solubility and activity was determined after 24 h of renaturation at 25ºC by spectroscopy and bioassay, respectively. Analysis of solubility results revealed that arginine has a significant effect in decreasing the aggregation during refolding process. Subsequent to reduction of aggregation, the solubility increased. Arginine showed an increasing solubilizing effect in the range of 250-1000 mM with the highest solubility achieved at 1000 mM (Table 3); however the biological assay showed that the highest rPA activity is at 500 mM arginine. At higher concentration of arginine, the solubility was high but the activity reduced. Investigation of the effect of refolding buffer pH on refolding yield showed that the best pH for refolding was 10 (Table 4).

Effect of glutathione pair on refolding of rPA: For evaluation of the effect of GSH-GSSG on refolding, solubilized rPA was refolded by dilution in refolding buffer (0.1 M Tris-HCl,1 mM EDTA, 200 mM arginine) containing different concentrations of GSH (0, 1, 5, 20, 50) and GSSG (0, 0.1, 0.5, 1, 5) at different pH (8, 10, 12). The solubility and activity of protein was determined after 24 h of renaturation at 25ºC by spectroscopy and bioassay, respectively. As shown in Table 5, the highest refolding effect was seen in 10 mM/1 mM of GSH/GSSG pair at pH 10.

Effect of sucrose on refolding yield: Denatured and reduced rPA was prepared by incubating rPA (20 mg/ml) in 100 mM Tris-HCl buffer containing 6 M Gn-HCl and 50 mM DTT for 2 h and then diluted 1:10 with Tris-HCl buffer containing 200 mM arginine, 10 mM GSH and 1 mM GSSG and different concentration of sucrose. Then refolding yield was analyzed by enzymatic activity. The results showed that sucrose decreases the aggregation and increases the refolding yield. In the absence of arginine the sucrose effect is not significant, however, in the presence of arginine, it has an increasing effect in the concentration range of 100-200 mM with the highest effect in 200 mM (Fig. 4).

DISCUSSION

In vitro refolding is a commonly used method for large scale production of several commercially produced

Table 3. Effect of different concentration of arginine on suppression of aggregation.

Arginine Concentration (mM)	250	500	750	1000
Optical density (pH=8)	1.21±0.02	1.39±0.02	1.86±0.03	2.06±0. 03
Optical density (pH=10)	1.47±0.03	1.78±0.03	1.90±0.03	2.18±0.04
Optical density (pH=12)	1.32±0.02	1.6±0.03	1.82±0.03	2.02±0.03

Optical density was measured at 280 nm. Each value is the average of four independent runs of experiment.

Table 4. Effect of different concentrations of arginine on refolding yield.

Arginine Concentration (mM)	0	250	500	750	1000
Refolding yield (pH=8)	0.8±0.03	18.12±1.5	26.2±2.1	24.4± 2.8	22.6± 1.4
Refolding yield (pH=10)	1.7±0.08	26.68±1.2	39.98±3.4	38.14±3.0	30.58±2.3
Refolding yield (pH=12)	1.2±0.07	23.7± 1.7	37.4± 2.6	31.8± 3.3	29.5± 1.9

Each value is the average of four independent runs of experiment.

Table 5. Effect of different concentrations of glutathione pair on refolding yield.

GSH Concentration (mM)	0	1:0.1	5:0.5	20:2	50:5
Refolding Yield (pH=8)	9.18±0.8	15.18±1.4	20.35±1.7	30.61±2.3	26.85±2.2
Refolding Yield (pH=10)	19.02±1.5	27.15±1.9	33.15±2.4	33.93±2.5	28.2±2.3
Refolding Yield (pH=12)	20.55±1.6	30.39±2.1	33.9±2.9	29.70±2.1	24.6±1.9

Each value is the average of four independent runs of experiment.

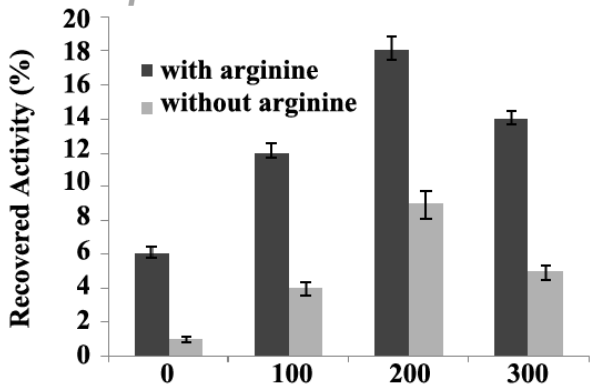

Figure 4. Effects of different concentrations of sucrose on refolding recovery yield in the presence and absence of arginine.

therapeutic proteins including EGF (Sharma *et al*., 2008), rhG-CSF, growth hormone (Bajorunaite *et al*., 2007) and etc. The main challenges in *in vitro* refolding process of IBs are optimizing renaturation condition by preventing the formation of inactive aggregates. Refolding is a dynamic process in which the misfolded proteins and aggregates compete with correct folded proteins. For prevention of aggregates formation during the refolding process, different refolding aids have been attempted including arginine, chaotropes detergents and oxidizingn/reducing agents. In most cases, improvement in the yield of correctly refolded protein requires the use of combination of different co-solvent systems depending on the nature of target protein.

In the present study, the effects of different concentrations of denaturants and DTT at various pH on refolding of rPA have been evaluated. In the first step, contaminant cellular proteins were removed by extensive washing of IBs and the purity of the IBs reached up to 90 percent. This is a required step for further refolding of IBs (Lilie *et al*., 1998). For solubilization, different concentration of urea and Gn-HCl were checked. Comparison of the effect of urea and Gn-HCl showed that Gn-HCl is more effective than urea in solubilization of IBs protein. This finding is in agreement with result of Chandra and colleagus that showed higher solubilization effect for Gn-HCl than urea (Chandra *et al*., 1985). pH of denaturation buffer can affect the solubility of inclusion bodies expressed in *E. coli*. Our result indicated that alkaline pH, especially pH higher than 8 can increase the amount of solubilization but decrease the active yield at pH values higher than 10. Although the pH higher than 12 has been used for solubilization of some proteins such as growth hormones (Tayyab *et al*., 2002; Patra *et al*., 2000) and proinsulin (Cowley and Mackin, 1997) however, the pH higher than 12 exerts irreversible chemical modifications on protein and leads to lesser activity. Thus, this high pH solubilization method may not be applicable for most pharmaceutical proteins.

In addition to the solubilizing agent, the presence of low molecular weight thiol reagents such as DTT or 2-mercaptoethanol (2 ME) is required especially for complete solubilization of disulfide bound rich proteins. Addition of these reducing agents denatures S-S bonds that are necessary for complete solubilization of IBs. Thiol groups prevent formation of non-native intra- and inter molecule disulfide bond in highly concentrated protein solutions (Lu and Liu, 2008; Chongjuan *et al*., 2000; Long and Wang, 1998; Darby *et al*., 1995). Furthermore, the residual concentration of DTT showed an effect on the refolding of rPA inclusion body. Although the effect of DTT on the refolding of proteins has not been reported in detail, the effect of other thiol agents such as reduced and oxidised glutathione (GSH and GSSG) has been studied. There are several reports about the use of gluthatione pair in refolding of other recombinant proteins such as recombinant prochymosin (Wei *et al*., 1999), recombinant growth hormone (Bajorunaite *et al*., 2007), NTA protein (a new thrombolytic agant) (Fang and Huang, 2001) and in refolding of recombinant alkaline phosphatase (Khodagholi and Yazdanparast, 2007). These studies showed that the optimal concentration of gluthatione pair differs significantly for each protein.

Several studies have examined the effect of additives especially arginine on refolding process. L-arginine was identified to be an efficient folding additive. Addition of arginine during refolding of rPA leads to aggregates suppression which leads to the increased yield of native protein (Arakawa *et al*., 2007; Bajorunaite *et al*., 2007). In our study, increasing arginine concentration to 1 M resulted in higher solubilization of rPA; however, the highest activity was achieved at 500 mM. Addition of sucrose as additive into refolding buffer apparently improved the refolding yield. The mechanism of sucrose effect on aggregation is not clear but its positive effect on refolding yield can not be ruled out.

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