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Original Article

Effect of buffer additives on solubilization and refolding of reteplase inclusion bodies

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Abstract

Reteplase is a non-glycosylated and recombinant form of tissue type plasminogen activator, which is produced in *Escherichia coli.* However, its overexpression usually leads to formation of inactive aggregates or inclusion bodies. In the present study, we report on the development of optimized processes for isolation, solubilization, and refolding of reteplase inclusion bodies to recover active protein. After protein overexpression in *E. coli* BL21 (DE3) inclusion bodies were isolated by cell disruption and repeated wash of pellet with buffer containing Triton X-100. To solubilize the inclusion bodies, different types, concentrations, pHs, and additives of denaturing agents were used. Rapid micro dilution method was applied for refolding of solubilized reteplase. Different chemical additives including sugars, alcohols, polymers, detergents, amino acids, kosmotropic, and chaotropic salts, reducing agents, and buffering agents were used in the refolding buffer. To evaluate the biological activity of refolded reteplase, an indirect chromogenic assay was performed. The best solubilizing agent for dissolving reteplase inclusion bodies was 6 M urea at pH 12. The optimized buffer for refolding of solubilized reteplase was found to be 1.15 M glucose, 9.16 mM imidazole, and 0.16 M sorbitol which resulted in high yield of biologically active protein. Our results indicate type, concentration, and pH of solvent and type, concentration, and combination of chemical additives can significantly influence the yield of inclusion bodies solubilization and refolding.

Keywords: Chemical chaperone; Inclusion bodies; Refolding additives; Reteplase.

INTRODUCTION

Nowadays, sedentary lifestyle and unhealthy diet have increased the incidence of cardiovascular diseases (CVD) in modern societies (1,2). Acute myocardial infarction (AMI) is one of the leading causes of mortality from CVD. AMI occurs when a clot is formed and blocks the coronary arteries. One of the most efficient therapeutic approaches for patients with AMI is thrombolysis therapy (3). Reteplase is one of the thrombolytic medicines, which has better kinetic, and safety profile compared with other thrombolytic agents (4).

Reteplase is a non-glycosylated and recombinant form of tissue type plasminogen activator (t-PA) which is produced in *Escherichia coli* (5). However, there are some obstacles in reteplase expression inside the prokaryotic cells. Overexpression of reteplase in *E. coli* leads to accumulation of insoluble and inactive aggregates called inclusion bodies (6). It is necessary to solubilize and refold inclusion bodies to recover biologically active form of the protein. There are different techniques for refolding of inclusion bodies including direct dilution (7), dialysis (8), diafiltration (9), and chromatographic methods (10) including size exclusion or gel filtration chromatography. In addition to refolding techniques, physical, and chemical parameters can also influence the refolding yields. Chemical additives (11) like amino acids (12) (*e.g.,* arginine and glycine) and thiol agents (13) (*e.g.*, dithiothreitol and beta-mercaptoethanol) can assist efficient refolding of proteins. Chemicals such as polyols (14) (*e.g.,* glycerol and sorbitol) increase hydrophobic interactions and stabilize protein conformations.

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Detergents (15) (*e.g.*, Triton X-100 and Tween 80) decrease non-covalent forces and suppress aggregation of protein during refolding process. Refolding and recovery of active form of proteins from inclusion bodies is a time-consuming and challenging task. There is no universal approach for obtaining native protein form of solubilized inclusion bodies and the optimum refolding conditions must be experimentally determined for each protein. Furthermore, refolding of proteins with a complex disulfide bridge structure like reteplase is considered more difficult (16). Reteplase molecule contains 18 cysteine residues (17) which make it more susceptible to formation of incorrect disulfide bonds during refolding process. In previous studies, we could successfully express reteplase in *E. coli* although almost all of overexpressed protein was aggregated as inclusion bodies (18,19). In the current study, we aimed to improve refolding yield of reteplase by optimization of solubilizing and refolding conditions.

MATERIALS AND METHODS

Materials

Luria-Bertani (LB) broth was obtained from Himedia (India). Isopropyl β-D-1-thiogalactopyranoside (IPTG) was bought from Thermo Scientific (Italy). Benzonase and dithiothreitol (DTT) were purchased from Sigma (USA). All other buffer additives and solvents were obtained from Merck (Germany).

Expression of reteplase

Competent *E. coli* BL21 (DE3) cells were transformed with the expression plasmid (pDEST-reteplase) using heat shock method. A single positive colony was inoculated into 10 mL LB broth containing 100 µg/mL ampicillin and incubated overnight. Fifty mL of LB broth medium inoculated with this culture and incubated overnight was used as an inoculum culture for 500 mL LB broth supplemented with antibiotic. The culture was incubated at 37 °C until reached an OD_{600} of 0.4-0.6. Then expression of histidine-tagged reteplase was induced by addition of 1 mM IPTG. After 2 h incubation at 37 °C, the culture was centrifuged at 7,500 ×g for 10 min and the bacterial pellet was stored at -70 °C for further analysis.

Isolation of inclusion bodies

The pellet was resuspended in the buffer solution (50 mM Tris-HCl, 25% sucrose, 1 mM NaEDTA, 10 mM DTT, pH 8) and sonicated three times (70 % amplitude and 30 pulses) on ice. Next, lysozyme (1 mg/mL), benzonase (10 U/mL) and $MgCl_2$ (2 mM) were added to the sample and vortexed shortly. Then, Lysis Buffer (50 mM Tris-HCl, 1 % Triton X-100, 100 mM NaCl, 10 mM DTT, pH 8) was added and incubated at ambient temperature for 30-60 min after a short vortex. To the sample, NaEDTA (15 mM) and $MgCl_2$ (4 mM) were added and incubated at room temperature until its viscosity decreased. Then, the sample was centrifuged at 11,000 ×g for 20 min at 4 ºC. The supernatant was discarded and the pellet was resuspended in washing buffer (50 mM Tris-HCl, 0.5% Triton X-100, 1 mM DTT, 100 mM NaCl, 1 mM NaEDTA, pH 8.0) and the sonication process was repeated. The sample was centrifuged at 11000 × g for 20 min at 4 ºC and the pellet was resuspended in washing buffer not containing Triton X-100. The sonication process was repeated and the sample was centrifuged at 11000 × g for 20 min at 4 ºC. This step (resuspending in washing buffer without Triton, sonication and centrifugation) was repeated once more.

Solubilization of inclusion bodies

Two denaturing agents, urea and guanidine hydrochloride (GdnHCl), at different concentrations (2-6 M) were used to dissolve the inclusion bodies pellets. The combinations of two denaturing agents (6 M urea and 6 M GdnHCI at 1:3, 1:1, and 3:1 ratios) were also used to solubilize isolated inclusion bodies. The effect of different pH and presence of additives (DTT, n-propanol, and β-mercaptoethanol) in the best solubilizing agent were also evaluated. For each solubilizing condition, same amount of inclusion bodies was used. After centrifugation at 7,500 ×g for 10 min, the amount of protein in the supernatant (soluble fraction) was evaluated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Bradford method.

Purification of reteplase

Solubilized inclusion bodies were applied into a column containing nickel-nitrilotriacetic acid (Ni-NTA) agarose (Invitrogen®, USA) as described previously (19). Briefly, the column was washed twice with denaturing binding buffer (8 M urea, 20 mM NaH_2PO_4, 500 mM NaCl, pH 7.8). The column was washed twice with denaturing wash buffer (8 M urea, 20 mM NaH_2PO_4, 500 mM NaCl, pH 6). The column was washed twice with denaturing wash buffer (8 M urea, 20 mM NaH_2PO_4, 500 mM NaCl, pH 5.3). Next, the protein was eluted by denaturing elution buffer (8 M urea, 20 mM NaH_2PO_4, 500 mM NaCl, pH 4).

Refolding of inclusion bodies

The effect of different buffer additives on refolding of solubilized and purified inclusion bodies was evaluated by rapid microdilution method using a 96-well plate. Solubilized inclusion bodies (20 µL) were diluted with 180 µL of each buffer additive and the plate was incubated at 4 ºC. On the next day, the protein aggregation in the samples was evaluated by measurement of turbidity at 600 nm. Soluble (refolded) and insoluble (misfolded) fraction of each sample was separated by centrifugation at 7,500 ×g for 5 min and the concentration of protein was measured using SDS-PAGE and Bradford method. A 2-level factorial design with 16 runs (Table 1) was used to evaluate the effects of 5 of the best buffer additives. After analysis of the results, the optimum refolding conditions predicted by Design Expert® (version 7.0.0, Stat-Ease, Inc., Minneapolis, USA) were used for large scale refolding.

SDS-PAGE analysis

For preparation of protein samples, 5 µL of 5× gel loading buffer (250 mM Tris-HCl, pH 6.8, 30% (v/v) glycerol, 5% M 2-mercaptoethanol, 10% SDS, and 0.02% bromophenol blue) was added to 20 µL of sample and incubated at 95 °C for 5 min to denature proteins. Samples were then electrophoresed on a 12% SDS-PAGE and stained with Coomassie Blue R-250. The amount of protein in each sample was estimated by densitometry analysis of corresponding band using TL120 software (nonlinear Inc, Durham nc, USA). The yield of refolding was calculated as the amount of protein after refolding to the amount of protein before refolding.

Table 1. 2-Level factorial design of five best buffer additives for refolding of reteplase.

Runs	Glucose (M)	Sucrose (M)	Sorbitol (M)	Ethanol (%)	Imidazole (mM)	Refolded protein concentration (µg/mL)	Refolding yield (%)
1	0	0	0.5	25	100	63.16	47.37
2	0	0.5	0	25	100	109.6	82.23
3	0.5	0.5	0.5	25	100	116.4	87.32
4	0	0.5	0	0	0	66.02	49.52
5	0.5	0.5	0	0	100	77.48	58.11
6	0	0	0	0	100	59.83	44.87
7	0	0.5	0.5	0	100	86.93	65.20
8	0	0	0	25	0	42.58	31.93
9	0.5	0	0.5	0	100	103.35	77.52
10	0.5	0	0	25	100	48.07	36.05
11	0	0	0.5	0	0	61.94	46.45
12	0.5	0	0.5	25	0	113.18	84.89
13	0.5	0.5	0.5	0	0	53.93	40.45
14	0	0.5	0.5	25%	0	67.83	50.87
15	0.5	0	0	0	0	51.93	38.94
16	0.5	0.5	0	25%	0	68.10	51.07
Effect	243.446	-247.451	392.893	156.785	603.804		
Sum of squared	395.371	800.896	426.118	1524.09	1703.04		
Contribution (%)	3.644	7.38159	3.92739	14.047	15.6963		

Bioactivity of refolded reteplase

Activation of plasminogen by reteplase was determined by AssaySense Human tPA Chromogenic Activity Kit (Assaypro, USA). To 20 µL of refolded samples, was added 80 µL of assay mix (50 µL assay diluent, 10 µL plasminogen, and 20 µL plasmin substrate). The commercial reteplase (Retelies®) was used as a positive control and water as the negative control. The samples were incubated at 37 ºC in a humid incubator and the absorbance was measured at 405 nm after 8 h.

RESULTS

Expression and isolation of reteplase inclusion bodies

E. coli BL21 (DE3) cells harboring pDEST reteplase was used for expression of reteplase by addition of 1 IPTG at 37 ºC for 2 h. After SDS-PAGE analysis, the overexpression of reteplase was confirmed and the protein appeared as a 39 KDa band (Fig 1a).

After large-scale expression of reteplase, bacterial cells were collected and disrupted by sonication. Reteplase inclusion bodies were isolated by washing with buffers containing detergent and high speed centrifugation. As shown in Fig. 1b, washing with buffers containing detergent and high speed centrifugation yielded in relatively pure inclusion bodies.

Solubilization of inclusion bodies

Different concentrations of urea and GdnHCl (2-6 M) were used to dissolve the isolated reteplase inclusion bodies. As shown in Fig. 2a, urea 6 M dissolved most of the inclusion bodies. Additionally, different combinations of urea and GdnHCl (mixture of 6 M urea and 6 M GdnHCl at various ratios) were used for solubilization of inclusion bodies. It was found that enhancement of urea concentration in the mixture resulted in the improvement of inclusion body solubilization (Fig. 2b). The effect of pH on inclusion body solubilization was also evaluated. The pH of 6 M urea which used for inclusion bodies solubilizing was varied between 7 and 12.33. Fig. 2c revealed that pH 12.33 was the optimum pH for solubilization of reteplase inclusion bodies. Effect of additives on solubilizing of inclusion bodies was examined and as shown in Fig. 2d the presence of additives did not increase the inclusion body solubilization yield and even for n-propanol solubilization yield was decreased. Based on our results, 6M urea at pH 12.33 was found to be the best solubilizing agent.

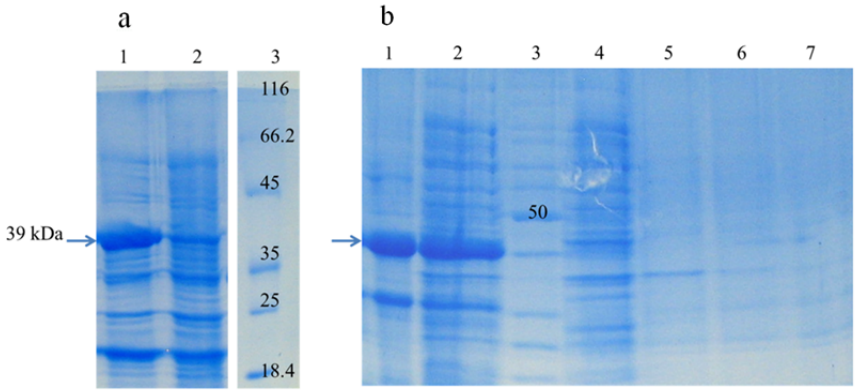

Fig. 1. (a), Expression of reteplase. Lane 1: induced total bacterial protein; lane 2: uninduced total bacterial protein; and lane 3: protein marker; (b), Isolation of reteplase inclusion bodies. Lane 1: inclusion bodies pellet; lane 2: total protein after induction; lane 3: protein marker; lane 4: supernatant after cell disruption; and lane 5-7: supernatants after 1-3 washing of inclusion bodies pellet.

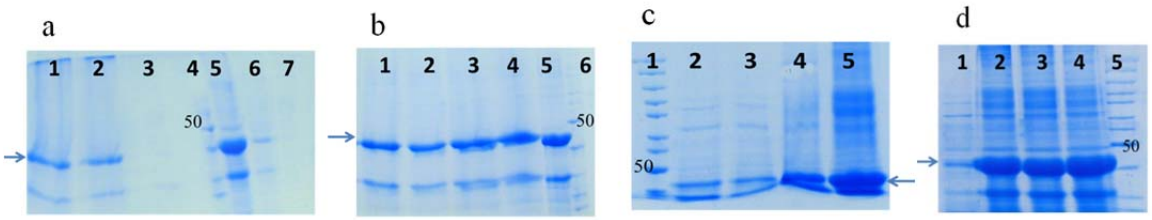

Fig. 2. Soluble fraction after solubilizing of reteplase inclusion bodies using (a), different concentrations of urea and guanidine hydrochloride (GdnHCl). Lane 1-3: 2, 4, and 6 M GdnHCl; lane 5-7: 2, 4, and 6 M urea. (b), different combination ratios of 6M GdnHCl to 6M urea. Lane 1-5: 4:0, 3:1, 2:2, 1:3, and 0:4 ratios. (c), 6 M urea at different pH. Lane 2-5: pH 7, pH 8.5, pH 10.1, and pH 12.33. (d), 6 M urea at pH 12 supplemented with different additives. Lane 1-3: n-propanol, DTT, and β-mercaptoethanol; lane 4: no additive. Lanes 4a, 6b, 1c, and 5d are protein marker.

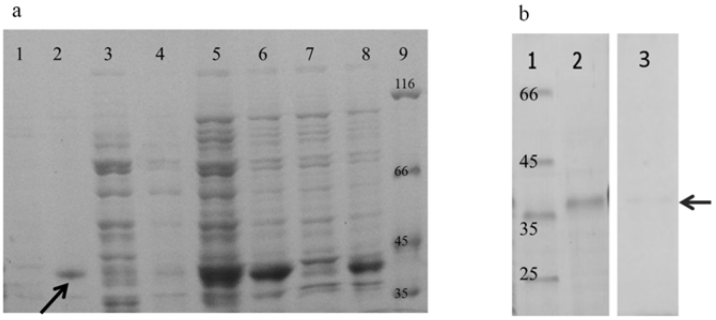

Fig. 3. (a), Purification of reteplase inclusion bodies. Lane 1: wash fraction by denaturing buffer at pH 5.3; lane 2: protein eluted at pH 4; lane 3: wash fraction by denaturing buffer at pH 6; lane 4: flow-through of column; lane 5: induced total bacterial protein; lane 6: pellet after cell disruption; lane 7: supernatant after cell disruption; lane 8: solubilized inclusion bodies before purification; and lane 9: protein marker. (b), Lane 1: protein marker; lane 2: protein refolded with the optimum buffer; and lane 3: protein refolded with water.

Purification of reteplase

Solubilized inclusion bodies were applied to a Ni-NTA affinity column and eluted under denaturing condition by decreasing the pH. As exhibited in Fig. 3a a highly pure reteplase was obtained after purification process.

Refolding of inclusion bodies

Rapid dilution method was used for refolding of reteplase inclusion bodies. Different additives were used to refold the protein and different refolding patterns were seen. Interestingly, high concentration (10%) of Tween 80 led to the formation of protein oligomers (Fig. 4). Among these buffer additives, imidazole, ethanol, sorbitol, glucose, and sucrose yielded more refolded protein (Fig. 4). A 2-level factorial design was applied to examine the effects of 5 buffer additives in combination. Imidazole, sorbitol, and glucose exhibited the highest positive effect on protein refolding (Table 1).

A wide range of refolding yield (Refolding yield (%) = concentration of refolded protein/concentration of solubilized protein × 100) from 31% to 87% (concentration of refolded protein from 43-116 µg/mL) were found for the 16 investigated runs, suggesting the importance of determining an optimal refolding condition. Based on software analysis of the results, the best refolding condition was predicted as 1.15 M glucose, 9.16 mM imidazole, and 0.16 M sorbitol which were used for large scale refolding of the protein. As shown in Fig. 3.b, high protein yield (130 µg/mL) was obtained using this refolding condition.

Bioactivity of refolded reteplase

Bioactivity of refolded reteplase was evaluated by an indirect chromogenic assay. The change in the absorbance of chromogenic substrate at 405 nm was directly proportional to the reteplase enzymatic activity. The enzymatic activity of refolded reteplase was determined as 0.75 IU/mL whose concentration was measured as 30 µg/mL (specific activity = 25 IU/mg). The concentration of commercial reteplase (positive standard) was 1 IU equivalent to 12 µg/mL (specific activity = 83 IU/mg).

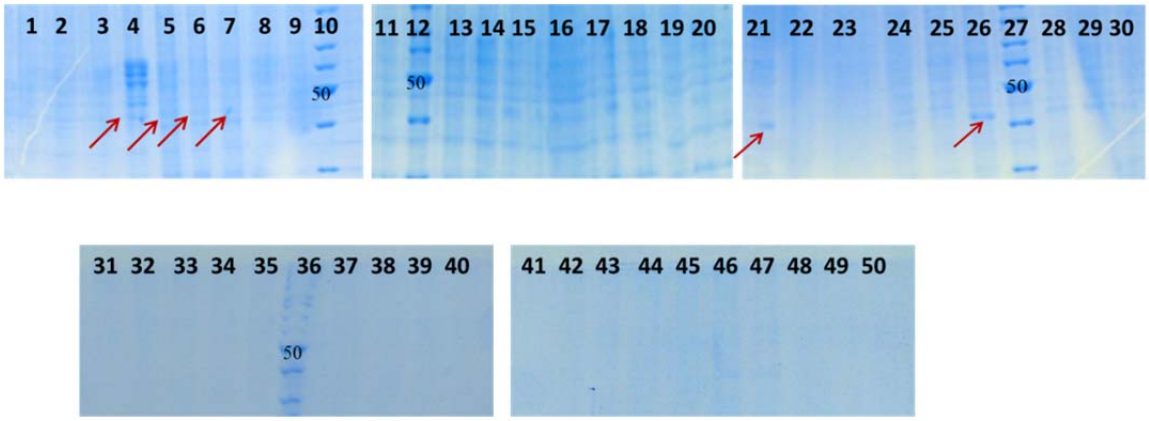

Fig. 4. Effect of different buffer additives on refolding of reteplase. Lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 28, 29, 30, 31, 32, 33, 34, 35, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, and 50 are 2 mM β-mercaptoethanol, 300 mM NaCl, 0.5 M urea, 10% Tween 80, 0.5 M sorbitol, 0.5 M sucrose, 0.5 M glucose, 6 M urea, water, 1% Tween 80, 1% Triton X-100, water, 6 M urea, 2 M guanidine hydrochloride, 1 M sucrose, 2 M urea, 5% polyethylene glycol 4000, 0.1% sodium dodecyl sulfate, 100 mM imidazole, 10 mM $FeCl_3$, 2 M glycine, 10 mM DTT, 1% Tween 20, 25% ethanol, 1 M sorbitol, 6 M urea, water, 50 mM citric acid, 10 mM $CoCl_2$, 10 mM $CuCl_2$, 10 mM $MnCl_2$, 10 mM $NiCl_2$, 40 mM $CaCl_2$, 40 mM $MgCl_2$, 200 mM KCl, 100 mM $MgSO_4$, 50 mM$(NH4)_2SO_4$, 50 mM Na_2SO_4 ,10% glycerol, 10 % ethylene glycol, 2% mannitol, 1 M betaine, 2 M arginine, 20 mM EDTA, 50 mM Tricine, and 1 M Tris, respectively. Lanes 10, 12, 27, and 36 are protein marker.

DISCUSSION

Reteplase, a non-glycosylated deletion type mutein of t-PA, is a thrombolytic agent widely used in management of AMI. As a non-glycosylated protein, reteplase can be easily overexpressed in prokaryotic systems like *E. coli*. However, it usually leads to insoluble expression of protein and formation of inactive aggregates or inclusion bodies. Recovering active protein needs development of optimized processes for isolation, solubilization, and refolding of inclusion bodies. In the current study, a protocol including cell lysis and pellet washing steps with low concentration of detergent (Trition X-100) and reducing agent (DTT) was used for inclusion body isolation which resulted in partially pure protein at high concentration. The choice of appropriate techniques for cell disruption is a critical issue in downstream processing (20). Here, bacteria cell lysis was performed using a combination of mechanical (*i.e.,* sonication) and non-mechanical (*i.e.,* lysozyme treatment) methods.

Rodríguez-Carmona, *et al.* evaluated the effectiveness of different cell lysis techniques such as treatment with lysozyme, the French Press, freeze-thawing, and combination of lysozyme treatment and sonication (21). In agreement with our approach, they reported that the most effective cell disruption method, which resulted in high yields of inclusion bodies, and less viable cells contamination was combination of mechanical and non-mechanical methods. After cell disruption, dense inclusion bodies could be collected by centrifugation. However, this pellet should be repeatedly washed with denaturants (*e.g.* urea and GdnHCl) or detergents (*e.g.* Tween, Triton, and SDS). This step can significantly reduce level of contaminants (22). It has been demonstrated that the existence of contaminants in the prepared inclusion bodies leads to significant reduction of protein refolding and purification yields (23). Addition of low concentrations of reducing agents (*e.g.* DTT and β-mercaptoethanol) to lysis and washing buffers especially for protein with many cysteine (*e.g.*, reteplase) keeps cysteine side chains in the reduced state and prevents formation of unwanted disulfide bonds (24). Solubilization of inclusion bodies can be influenced by pH and composition of solubilizing agent. Based on our results, effectiveness of inclusion bodies solubilization depends on type, pH and concentration of denaturant. It was found that high concentration of urea at high pH was the best solubilizing agent. It has been demonstrated

that alkaline pH of solubilizing agent promotes complete inclusion bodies solubilization upon reducing and breaking intermolecular disulfide bridges (25). High pH of denaturant can also increase efficiency of refolding for cysteine rich proteins, as it decreases formation of scrambled disulfide bonds and prevents misfolding of solubilized inclusion bodies (25). Additionally, Singh, *et al.* suggested that dissolving inclusion bodies at high pH could be an alternative approach to the use of high concentrations of denaturant (26). The composition of the refolding buffer is another important factor influencing the yield of refolding process. Chemical additive are usually applied to increase yield and speed of refolding, to suppress formation of aggregates and to stabilize refolded protein (27). In the present study, different buffer additives including sugars, alcohols, polymers, detergents, amino acids, kosmotropic and chaotropic salts, reducing agents and buffering agents were used in refolding buffer of reteplase. Imidazole, an amino acid derivative, can act as a chaperone molecule to accelerate refolding and inhibit aggregation. According to our results, 100 mM imidazole significantly improved the refolding of reteplase inclusion bodies. In agreement with our results, other group also reported improvement of *in vitro* refolding rate and yield for enhanced green fluorescent protein by addition of 0.1-1 M imidazole in the refolding buffer (28). Here, addition of ethanol to refolding buffer remarkably enhanced the yield of reteplase refolding. It could be explained based on the effect of organic solvents (*e.g.* ethanol, methanol, and trifluoroethanol) on secondary and tertiary conformation of protein by increasing hydrogen binding and breakage of hydrophobic interactions (29). However, alcohols can stabilize or destabilize the structure of refolded protein depending on the type and concentration of organic solvent and the type of target protein (30,31). Glucose and sucrose also increased the refolding efficiency of reteplase probably by their ability to stabilize native protein by preferential hydration. Similarly, Abe, *et al.* reported the refolding of unfolded 3Hmut Wil protein to its native conformation under a high sugar concentration condition (32). We also found that addition of sorbitol to the refolding buffer improved the yield of reteplase refolding. Sorbitol is a polyhydric alcohol which can promote the stability of refolded protein (33). Altogether, it can be concluded that hydrophobic interaction is one of the most important parameters influencing the formation of insoluble reteplase aggregates. Our study showed high concentration of Tween 80 led to the formation of soluble oligomeric species for reteplase. It may be due to the improvement of reteplase oligomer solubility with increase in surfactant concentration. Similarly, Wang, *et al.* reported that the addition of tween 80 at high concentration to formulation resulted in oligomerization of interlukin-2 during storage, which is mostly related to non-disulfide bind aggregates (34).

CONCLUSION

In this study, we evaluated the impact of different solubilizing agents on solubilization of reteplase inclusion bodies. Great amounts of inclusion bodies were dissolved when solubilization was performed at high concentration of alkaline urea. It was observed that chemical additives decreasing hydrophobic interactions could improve refolding of reteplase. The optimum refolding efficiency was observed for the buffer containing 1.15 M glucose, 9.16 mM imidazole, and 0.16 M sorbitol. The result of biological assay suggested that refolded reteplase with optimized buffer was functionally active. The methodology reported here may also be used to isolate, solubilize and refold other proteins with similar structural features.

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